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May 2017

Factors Influencing Induction of Desiccation Tolerance in Bryophytes: Redefining Fundamental Aspects of the Organism's Relationship with the Environment in Xeric Habitats

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Repository Citation

FACTORS INFLUENCING INDUCTION OF DESICCATION TOLERANCE IN

BRYOPHYTES: REDEFINING FUNDAMENTAL ASPECTS OF THE

ORGANISM'S RELATIONSHIP WITH THE

ENVIRONMENT IN XERIC HABITATS

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A doctoral project submitted in partial fulfillment
of the requirements for the

Doctor of Philosophy – Biological Sciences

School of Life Sciences
College of Sciences
The Graduate College

University of Nevada, Las Vegas
May 2017

Dissertation Approval

The Graduate College
The University of Nevada, Las Vegas

February 28, 2017

This dissertation prepared by

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entitled

Factors Influencing Induction of Desiccation Tolerance in Bryophytes: Redefining Fundamental Aspects of the Organism's Relationship with the Environment in Xeric Habitats

is approved in partial fulfillment of the requirements for the degree of

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Abstract

If one wishes to understand the ecological role, range, habitat preferences, selective pressures, reproduction, response to climate change, and the potential for survival under climactic change, range expansion and range contraction for species and populations of bryophytes, an understanding of their survival of the desiccated state, is essential. In order to further our understanding of desiccation tolerance in mosses, three projects were undertaken. The first of these investigated the nature of desiccation tolerance in mosses, specifically if desiccation tolerance can be induced; thereby providing a desiccation tolerant phenotype in a moss species generally considered desiccation sensitive (*Physcomitrella patens*, the model organism for mosses). This hypothesis supposes the traditional dogmatic assumption that mosses can be cleanly divided into two categories (desiccation tolerant or desiccation sensitive) is wrong. The second project expanded upon the first by investigating if a hardened desiccation tolerant phenotype is retained for a time following the cessation of stimuli (using *P. patens* as a study system). The third project was designed to disentangle the interacting factors of ecotypic variation in *Bryum argenteum* (both in mean trait values as well as phenotypic plasticity), rate of desiccation (time allotted for the induction of desiccation tolerance), and life history phase (five categories considered).

Inducible desiccation tolerance exists in mosses and the examined "desiccation sensitive" species can survive desiccation if given proper stimuli, refuting the conventional dogma of desiccation tolerance existing as a binary state of tolerant or sensitive. Hardening toward desiccation tolerance was shown for *P. patens*, attenuating within eight days. All factors examined within the third study (rate of drying, phase, and ecotype) proved to significantly

impact desiccation tolerance. Adult shoots and bulbils (lateral dispersal agents across a local landscape, vegetative propagules) were found to display a desiccation tolerant phenotype with either rapid or no required induction for all ecotypes examined. Juvenile and intermediate shoots displayed an inducibly desiccation tolerant phenotype in response to the rate of drying applied (longer times resulting in greater health upon rehydration), with variation in response detected between ecotypes examined. For some juvenile and shoot ecotypes a rather low inducible capacity was shown requiring long rates of drying to achieve a high degree of desiccation tolerance, other ecotypes however displayed either a strong inducible response requiring only a short rate of drying for induction. The protonemal phase (responsible for lateral growth across the substrate, and giving rise to shoots) showed a pattern similar to juveniles, but more pronounced with more damage apparent with rapid dries. Some protonemal ecotypes did not appear to respond to slower dries, apparently lacking an inducibly desiccation tolerant phenotype, while some ecotypes (typically from the Southwestern United States of America; CA, NM, NV) showed a high potential for rapid induction of a desiccation tolerant phenotype. These results are interesting as they show a wider breadth of capacity for desiccation tolerance in regards to desiccation tolerance as well as a varying capacity for the phenotypic plasticity in response to slow drying rates for induction.

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Chapter One: Introduction and Background Information Concerning Desiccation Tolerance in Mosses

Organismal Information

The two species examined, *Physcomitrella patens* (Hedw.) and *Bryum argenteum* (Hedw.), serve as representative organisms near the terminal ends of a hypothesized gradient from desiccation sensitive to desiccation tolerant, and, as such, make excellent bookends (as study-species) for this project. *Physcomitrella patens* is a desiccation sensitive moss (Koster 2010) with a range restricted to mesic habitats. *Bryum argenteum* on the other hand has one of the widest distributions worldwide of any moss with specimens found on all continents and habitats ranging from mesic to xeric (Flora of North America Editorial Committee 2014).

Mechanistic Basis of Desiccation Tolerance

As a general rule when bryophytes are desiccated beyond their capacity to tolerate (i.e., <1 h), vital physiological recovery processes are negatively impacted, such as microtubule disassembly and reassembly (Pressel & Duckett 2010). In addition, when exposed to rapid drying (RD), plants exhibit chlorosis, delayed regeneration, reduced fitness, and damaged shoot apices (Schonbeck & Bewley1981a, b; Barker et al 2005; Stark et al. 2011). For some species however desiccation poses a much smaller threat, and individuals such as *Syntrichia ruralis* can survive multiple decades equilibrated to single digit humidities (Stark et al. 2016). The fact that mosses can survive such extreme physiological stresses and the mechanistic factors that underlie this survival are fascinating.

There is a large body of evidence suggesting that abcisic acid (ABA) is the signaling molecule most directly responsible for the control and regulation of the inducible aspect of a desiccation tolerant phenotype. Abcisic acid is known to generate a desiccation tolerant phenotype when exogenously added to a desiccation sensitive moss, and serves as a signaling molecule for many stress responses in vascular plants. Phenotypic desiccation tolerance induction

by ABA has been shown for the spp. *Funaria hygrometrica* at a conc. of 10 µM (Werner et al. 1991) and by for *P. patens* at a conc. of 100 µM (Oldenhof et al. 2006). Werner et al. (1991) furthermore showed that this induction of a desiccation tolerant phenotype required protein synthesis by applying ABA in conjunction with cyclohexamide (a protein synthesis inhibitor), which resulted in the loss of the desiccation tolerant phenotype. The timing of ABA activity in relation to a cycle of desiccation-rehydration was further refined by Beckett (2001). Becket gave samples of *Atrichum androgynum* ABA, but only provided cyclohexamide for a portion of the drying-rehydration cycle. Cyclohexamide was applied during the drying to desiccation (occurred over 3 days), during the desiccated state, and during rehydration, as well as stages in combination. He found no inhibition of the desiccation tolerant phenotype was detectable when cyclohexamide was added during desiccation or rehydration, showing that protein synthesis during these stages is not needed to achieve desiccation tolerance. Cyclohexamide added during the drydown however resulted in death, showing protein synthesis during the drying period is essential for desiccation tolerance, and most likely similar timing for ABA synthesis is required in nature. Furthermore these results show, in inducibly desiccation tolerant mosses at least, that protection plays the lion's share of responsibility for desiccation tolerance, with repair aspects not needed for desiccation tolerance (note this does not prove that repair aspects have no beneficial effects, just that they are not essential for desiccation tolerance.

These findings oppose the constitutive theory of desiccation tolerance in mosses and observations by other researchers in the field (Bewley 1973; Oliver & Bewley 1984). Both Beckett (2001), as well as Werner (1991) theorized that the different results that they observed are due to differences in experimental technique. Namely they believe that the source material used in most experiments on desiccation tolerance use field collected mosses, and therefore is already in

an induced state. Secondly they thought that the rates of drying applied for a "slow dry" are too rapid (usually 0.5-3 hours) for significant protein synthesis to occur.

Addition of ABA to mosses has been studied using both microarrays (Cuming et al. 2007), as well as proteomics (Wang et al. 2009; Cui et al. 2012; Cruz de Carvalho et al. 2014), and microscopic observation of cytoskeletal responses (Pressel et al. 2006; Proctor et al. 2007). For the most part these studies uncovered synonymous findings. A down regulation of many genes associated with both metabolic functions and photosynthesis was observed (a notable exception was upregulation of enzymes and pathways related to sucrose formation). Downregulation was seen for cytoskeletal proteins (confirmed by microscopic observation of cytoskeletal disassembly during desiccation). Upregulation was observed for late embryogenesis abundant proteins (LEA proteins), heat shock proteins (HSP), dehydrins, and compounds associated with the scavenging of free radicals.

The down regulation of metabolic compounds would function to lower the risk of high energy compounds (associated with mitochondria and chloroplasts) forming free radicals by lowering the total number of precursor compounds present in the cell during desiccation. Down regulation of cytoskeletal proteins and the associated dis-assembly of much of the cytoskeleton is though to aid in withstanding the shifts in cell volume (and plasmolysis) that occur with drying (Pressel 2006). Upregulation of sucrose metabolism and formation would aid in the vitrification of the cytoplasm leading to bioglass formation, which acts as a molecular shield preventing membrane-membrane and protein-protein interactions as well as filling hydrogen bonding requirements by locking a monolayer of water to organelles and proteins (Crowe et al. 1992, Smirnoff 1992). Late embryogenesis abundant proteins and dehydrins play numerous roles in preventing protein aggregation, and the stabilization of hydrophobic regions of membranes and

proteins (Tunnacliffe & Wise 2007). Heat shock proteins play roles both in the preventing of protein mis-folding and the protein aggregation this leads to, as well as tagging damaged proteins for destruction (Bakau & Horwich 1998).

In addition to exogenous ABA leading to an inducibly desiccation tolerant phenotype, the act of slow drying itself has been shown to lead to an increase in ABA levels in mosses. The formation of ABA in conjunction with the studies described earlier shows a link between a slow rate of water loss, the formation of a signaling molecule in response to water loss, and the formation of a desiccation tolerant phenotype in response to the signaling molecule generated by a slow dry.

Methodological Developments

The most commonly used method for drying mosses is to equilibrate them to an atmosphere of a specific relative humidity generated in the head space above a saturated salt solution. The use of a saturated salt solution to generate a controlled relative humidity is a well-established method that produces accurate results, somewhat modulated by the temperature at which the solution is stored (Greenspan 1976). This method however can be problematic, depending upon application, as it fundamentally links the rate of water loss to equilibrating relative humidity established by the salt solution, and the two cannot be independently manipulated. This leads to a situation where a saturated salt solution of potassium acetate (equilibrating relative humidity of 23%), for example, will always result in a more rapid drying rate than a solution of sodium chloride (equilibrating relative humidity of 75%).

To give us more flexibility for experimental design when working with equilibrating relative humidities, we implemented a system utilizing a hydrated artificial substrate in an enclosed space. The artificial substrate consisted of filter paper which is hydrated with a pre-

determined amount of water, and placed within a petri dish. This petri dish is placed within a relative humidity cabinet set to 50% relative humidity that is itself located within an environmental control room also set to 50% relative humidity for redundancy. As liquid evaporates from the filter paper the relative humidity within the Petri dish rapidly rises to 99%. Moist air in the petri dish slowly leaks out over time, being replenished with water from the filter paper until the source is exhausted at which point the Petri dish equilibrates to the relative humidity inside the chamber (usually 50% in this body of work). The volume of water added to the petri dish therefore controls the time a moss placed within the dish will spend in a sub-turgor state (at approx. 99% relative humidity), where we hypothesize physiological adaptations allowing survival of desiccating conditions occur.

Response Variables Used Across This Work

Chlorophyll Fluorescence

Chlorophyll fluorescence provides values directly related to the integrity of photosystem II and, by implication, provides information on downstream activities such as electron transport rate and the degree of non-photochemical quenching occurring (i.e., emission of energy as heat or fluorescence). This is achieved by the relativistic measurement of functional photosystem II molecules in relation to background fluorescent compounds. Fv/Fm represents the efficiency of excitation capture by open PSII reaction centers, indicating Φ_{PSII} and of photosynthetic performance (Genty et al. 1989; Maxwell & Johnson 2000). Fm represents the maximal fluorescence value achievable with all PSII reaction centers closed and all non-photochemical quenching parameters at minimum, and in stressed organisms shows a highly significant interaction with desiccation intensity (Kooten & Snel 1990; Pressel et al. 2009; Proctor 2012; Stark et al. 2013). Φ_{PSII} is a measure of the quantum yield of electron flow through PSII in vivo.

Φ_{PSII} therefore shows proportion of absorbed light used to drive photochemistry indicating overall photosynthetic rate and the relative rate of carbon fixation (Krall & Edwards 1992; Maxwell & Johnson 2000).

Regenerative Metrics

Regenerative metrics, shoot and protonemal regrowth, were used to determine if a stressed sample will be capable of producing new tissue within the observed timeframe (10 days). Secondly, if the organism is capable of regeneration, this will determine how many days it takes for regeneration to occur when the sample is re-hydrated. Regrowth makes an excellent response variable when feasible as it guarantees the tissue has survived desiccation.

Chlorophyllous Tissue Retention

Chlorophyllous tissue retention describes the amount of chlorophyll that is retained in shoots. Tissue retention was measured seven days post-rehydration from a desiccation event. Leaves were visually assessed and broken into three broad categories depending on color: green; semi-green; or dead (brown/white). Numbers of observed leaf categories per shoot were divided by the total number of leaves on the shoot and recorded as a percentage to account for shoots with different numbers of leaves.

Chapter Two: Rate of Drying Determines Extent of Desiccation Tolerance in *Physcomitrella patens*

Abstract

The effect of differential drying rates on desiccation tolerance in *Physcomitrella patens* is examined. In order to provide more evidence as to the status of desiccation tolerance in *P. patens* a system was designed which allowed control of the rate of water loss within a specific relative humidity. An artificial substrate consisting of layers of wetted filter paper to slow the drying process to as long as 284 hours, a significant increase over the commonly used method of exposure (saturated salt solution). By slowing the rate of drying, survival rates and chlorophyll fluorescence parameters improved, and tissue regeneration time was faster. These results indicate a trend where the capacity for desiccation tolerance increases with slower drying, and reveal a much stronger capacity for desiccation tolerance in *P. patens* than was previously known.

This first chapter, "*Rate of Drying Determines Extent of Desiccation Tolerance in Physcomitrella patens*", was published in the journal *Functional Plant Biology* in 2014.

Introduction

A plant may be considered desiccation tolerant (DT) if it can tolerate equilibrium at water potentials less than about -100 MPa (50% RH) (Koster et al. 2010). At present most bryophytes are considered constitutively desiccation tolerant (CDT) with a handful of inducibly desiccation tolerant (IDT) species reported (Cruz de Carvalho et al. 2011, 2012; Proctor et al. 2007a; Pressel & Duckett 2010). This study is concerned with the manifestation of IDT by which a desiccation sensitive (DS) species can exhibit a desiccation tolerant (DT) phenotype given appropriate stimuli, using *Physcomitrella patens* (the model organism for bryophytes) as our subject. The implication is that for IDT species, a slow drying rate (SD) is needed to induce protective mechanisms against desiccation and/or facilitate repair during subsequent rehydration.

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Status of *P. patens* is In Question

      Physcomitrella patens has traditionally been considered a DS, although drought tolerant moss (Koster et al. 2010). Recent reports however indicate *P. patens* may achieve a measure of DT if dried slowly (Wang et al. 2009). At present the status of the organism is unknown as methods employed by both camps failed to fully resolve the question. In the study supporting *P. patens* as DT, equilibrating humidity was not stated, confusing the degree to which drying was achieved (Wang et al. 2009). Equilibrating to an unspecified relative humidity (RH) adds a great deal of variability into the study, dependent upon local atmospheric conditions. The second and much larger concern is the method used to determine water loss, namely fresh weight divided by equilibrated weight. This technique is problematic in studies on mosses which retain a significant portion of water external to the organism between its leaves (Koster et al. 2010). This extra mass, when counted as part of the fresh weight, will artificially inflate water loss calculations.

      The most important concern in papers supporting *P. patens* as a DS organism is the use of saturated salt solutions to control drying rate. Contained atmospheric RH (via saturated salt solutions) has long been used as a surrogate for desiccation rate, with faster rates obtained at lower RHs and slower rates at higher RHs (Proctor et al. 2007b). Most studies enlisting salt solutions achieved a RD on the order of 30 minutes to two hours, and a SD ranging three to six hours (Bewley 1995). Desiccation rates, however, can be much slower in nature than the SD generated by salt solutions, dependent upon colony size, shoot architecture, and local atmospheric conditions (Rice et al. 2011). Furthermore salt solutions render impossible a SD and RD at the same RH. One study which assessed the influence of rate of desiccation at a single RH (55%) upon recovery found significant differences in the 3 rates employed (6.0, 1.5, & 0.6 h) (Penny & Bayfield 1982). As rate of desiccation can be critical to generate an IDT response, drying times

experienced in the wild for a species should be taken into account when designing an experiment to determine DT status. We therefore set out to control for both the rate of desiccation and the final equilibrating relative humidity (RH) within one experimental technique.

Advantages of An Inducible Desiccation Tolerance System

Induction of DT in *P. patens* provides a new opportunity to study the response to desiccation using the model bryophyte organism as a study system. To successfully uncover a transcriptomic or proteomic response to environmental stimuli requires variation in response between experimental and control samples, which was previously thought to be impossible, as *P. patens* was viewed as not possessing a desiccation tolerant phenotype. Desiccation sensitive species are problematic in that they apparently lack a capacity to survive desiccation and would lack a detectable response for mitigating damage, allowing survival. Constitutively desiccation tolerant species are also problematic, as they are always in a state of readiness and therefore may not have a detectable response to desiccation beyond baseline levels. With an ability to manipulate an IDT system in *P. patens,* it should be possible to tease apart differences in the induced and hydrated state, thus aiding in novel gene discovery related to DT.

Hypothesis

Our experiment addresses the following hypothesis: *P. patens* possesses an IDT response, and damage upon rehydration is related to the prior rate of desiccation, with longer drying times mitigating chlorophyllous tissue loss, increasing photosynthetic capacity (due to reduced photosystem II damage), and improving the ability to initiate development of new protonema and shoots upon replanting.

Methods

Cultivation Methods

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The Gransden strain of *P. patens* was provided by Scott Schutte (formerly of Southern Illinois Univ., Dept. of Biology). Plants were cultured using the technique of Horsley et al. (2011), and watered every 48 h with a 30% Hoaglands nutrient solution. After 4-6 weeks of cultivation shoots were extracted for experimentation (similar size and age shoots were used throughout). Plants were grown in a plant growth chamber (Percival model E30B, Boone, Iowa, USA) set to a 12 h photoperiod (20°C lighted, 8°C darkened), ~65% RH, 55μmol m^{-2} s^{-1}photosynthetically active radiation (PAR).

Method of Drying

All shoot manipulations were carried out in a walk-in environmental control chamber (R.W. Smith & Co.) set to constant temperature, relative humidity (RH) and light (20°C, 50% RH, 2-4 μmol γ m^{-2} sec^{-1}). Sheets of #1 Whatman filter paper (Whatman international LTD, Maidstone, England) were fitted to the inner dimensions of a 35 mm Petri dish (to serve as an artificial substrate) and equilibrated to 50% RH. One sheet of filter paper was added for every 100 μL of water used per treatment (two sheets for 200 μL, four for 400 μL, etc.). One hour before adding shoots to the petri dishes a 1000uL Gilson pipette (Gilson Inc., Wisconsin, USA) was used to add water to the filter paper. This provided a range of drying times dependent upon the initial amount of water added to the filter paper, 0 μL (less than 6 h to dry), 200 μL (65 h to dry), 400 μL (118 h to dry), and 1200 μL (284 h to dry). Measured drying times were validated and calibrated by including an ibutton inside a number of petri dishes and tracking the loss of humidity over time across a range of water volumes added to filter paper (Fig. 1).

Five replicates of 15 shoot clusters were prepared for each treatment. Surface free water was removed by blotting shoot clusters on Whatman No. 1 filter paper, while under observation with a dissecting microscope to allow visual confirmation of free water removal while also

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ensuring shoots retained full turgor (otherwise stress will be imposed by the blotting process itself). Shoot clusters were transferred to lidded Petri dishes, and placed in a benchtop RH chamber (Totech Super Dry) set to 50% RH which itself was inside a walk-in environmental control room set to 50% RH, 20°C, thus reducing potential humidity fluctuations (which would alter the rate of water loss) during the experiment.

Petri dishes were weighed (Sartorius BP 211D balance, sensitivity of 0.00001g) at intervals ≤ 8 h over a period of 12 d to determine when water loss had ceased for each replicate (indicating equilibration to environmental RH). When no further water loss was detected, we allowed an additional 24 h of exposure to ensure equilibration before rehydration. Half the samples were further equilibrated to 30% RH inside a sealed container above a CaCl saturated salt solution for an additional 24 h, to examine the influence of a more intense desiccating event. Samples were re-hydrated immediately at the end of their final 24 h drying event (all treatments spent the same amount of time equilibrated to 50% RH but took increasing lengths of time to reach that point). Rehydration was conducted upon a saturated chemical wipe to allow slower rehydration than direct immersion in water.

Methodology Validation

Validation of methodology was tested by determining water content for samples treated in the same manner as our experimental samples. Sample weight was recorded after equilibration to both 50 (Totech Super Dry chamber) and 30%RH (CaCl salt solution chamber), followed by drying in an oven at 70 °C for 48 h to remove residual water. Samples were then re-weighed to determine the dry weight, and water content ([equilibrated weight-dry weight]/dry weight). At 50% RH our samples reached 12.52% water content with a standard deviation of ±3.3% and a

standard error of ±0.6%. At 30% RH our samples reached 7.94% water content with a standard deviation of ±0.49% and a standard error of ±0.16%.

In a second experiment the RH inside lidded (400, 800, 1200 µL) and unlidded (RD, 0 µL) Petri dishes was measured using iButtons (Maxim, San Jose, California, USA). RH was measured every 10 min, n = four) on separate sets of non-experimental shoots carried out in the same desiccation cabinet used in experimental samples, set to 50% RH which ranged from 45–55% RH during the observation period.

Chlorophyll Fluorescence

Five replicates were prepared just prior to beginning fluorescence measurements to serve as an un-dried control. The moss shoots and their associated chemical wipes were placed inside of a Hansatech fluorescence clip (Norfolk, England) to dark adapt the shoots. Terminal ends of the chemical wipe were in contact with water to provide hydration at all times via capillary action. Measurements of Fv/Fm, Fm, and quantum efficiency of photosystem II (Φ PS II yield) were determined multiple times over a 24 h period with a Hansatech fluorescence monitoring system ($FMS2$, Norfolk, England). Fv/Fm was measured for dark adapted samples using the saturation pulse method (Bilger et al. 1995). Quantum efficiency of PSII ($[Fm'/Fs]/Fm'$) was determined as described in Genty et al. (1989).

Regeneration

Following fluorescence measurements shoots were planted in 95 Petri dishes (4 shoots per dish) for estimation of tissue survival and to determine time required for shoot and protonemal emergence (regeneration). After planting shoots were inspected daily with a dissecting microscope (60×) for 21 d, recording new protonema or shoots. Seven days post planting the number of undamaged, partially damaged, and dead leaves on each shoot was visually assessed

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with a dissecting microscope. Similar methods have been used in our laboratory to judge

organismal health in mosses exposed to thermal, and desiccation stress as well as age-dependent

effects upon regeneration (Stark et al. 2007, 2009, 2013).

Statistics

Values recorded at the final time-point of experimental treatments and controls were

compared by analysis of variance (ANOVA) and means were compared by a post-hoc pairwise

Tukey analysis in SPSS 20 for Windows (IBM, Armonk, NY). One way ANOVAs were

performed individually upon our measured variables $F\text{v}/F\text{m}$, $F\text{m}$, Φ PS II, Leaf health, and

regrowth times for all treatments and controls (Table 2).

Results

Relative Humidity Inside Petri Dish

Shoots experienced high humidity levels (~95% RH) over periods of time increasing with

the amount of water used (<3 h for 0 μL H_2O, ~125 h for 400 μL H_2O, ~175 h for 800 μL H_2O,

and ~200 h for 1200 μL H_2O). Once the RH inside the Petri dish dropped below 90–95%, shoots

desiccated within 20 h. The declining slope of RH within the Petri dish was similar across

treatments (although with shifted initiation time for the descent portion of the slope). Although

the ambient RH outside of the Petri dishes was 45–55%, the RH inside the dishes declined to 51-

54% in all treatments (Fig. 1).

Leaf Damage and Survival

As drying time is extended, leaf damage attenuates (Tables 1, 2; Fig. 2), with leaf damage

ranging from 100% for a rapid dry to 47% (± 4.1%) at 284 h (slowest dry) when equilibrated to

50% RH. Over 50% of leaves at the longest drying time appeared healthy one week after planting.

Increasing length of drying was positively associated with a greater percentage of chlorophyllous

tissue following desiccation and negatively associated with dead/brown tissue (Tables 1, 2; Fig. 2). No shoots rapidly dried to either 30 or 50% RH were capable of surviving desiccation. Intermediate drying times showed partial survival dependent upon length of drying time, with tissue health increasing in conjunction with drying time. All plants allowed to slowly dry to equilibrium were shown to have some degree of tissue survival (Table 1).

Regeneration of Protonema and Shoots

The number of days required for emergence of protonema and shoots was inversely related to the rate of drying in all experimental treatments (Tables 1, 2; Fig. 2). Shorter drying times increased time to emergence as well as leading to a lower percentage of plants producing protonema and shoots. Undried controls, however, required longer for the production of protonema and shoots than experimental samples, most likely due to apical inhibition of bud formation as the plant was essentially undamaged in most cases. No shoots or protonema were produced by any rapidly dried samples over the course of the experiment.

Chlorophyll Fluorescence

Initial fluorescence values (30 m) were similar among treatments; however, samples rapidly diverged during a 24-h period (Table 2; Fig. 3). Rapidly dried samples showed a steep decline in values, while slower drying rates showed increased performance over the next 24 hours.

F_v/F_m values (Table 2; Fig. 3 A, B) for all treatments showed recovery of photosynthetic capacity following rehydration, with a positive correlation between time to desiccation and higher F_v/F_m values, indicating greater health. Rapidly dried samples suffered extensive damage and showed no recovery over the 24-h period. Plants subjected to a 284 h drying time performed better while 65 h and 116 h samples showed intermediate performance upon rehydration. F_m

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values (Table 2; Fig. 3 C, D) also showed a strong correlation with drying time. Similar to our Fv/Fm results the rapidly dried treatments dropped sharply after rehydration while the longest drying times showed a significant improvement compared to rapidly dried samples. Φ_{PSII} values again showed a pattern similar to that of Fv/Fm and Fm, with longer drying times correlating with higher values (Table 2; Fig. 3 E, F).

Discussion

Measurements of Tissue Damage, Protonemal Emergence and Shoot Emergence

Chlorophyllous tissue damage, time until protonemal emergence, and time until shoot emergence all showed patterns indicating that slower rates of drying positively influenced organismal health (Table 1). Samples dried more slowly retained more chlorphyllous leaves and required shortened times until development of new tissue. These observations support our hypothesis that *P. patens* possesses an IDT response to drying, and that longer drying time positively influences organismal health following rehydration.

Chlorophyll Fluorescence

Measuring chlorophyll fluorescence of stressed photosynthetic organisms gives insight into the state of Photosystem II (PSII). Reduced fluorescence values manifest quickly following stress, and indicate the extent to which PSII is using the energy absorbed by chlorophyll (Maxwell & Johnson 2000; Proctor 2012). In all measures of chlorophyll fluorescence, slower drying times were positively associated with values reflecting greater health. Fv/Fm and Φ_{PSII} values (Table 2; Fig. 3) showed a pattern of increasing values over the 24-h observation period. When provided longer drying times, Fm values (Table 2; Fig. 3) showed higher values upon rehydration, as well as an ability to maintain higher Fm values over the 24-h observation period.

Observations of chlorophyll fluorescence therefore support *P. patens* as an IDT organism with its survival positively related to rate of drying.

Fv/Fm represents the efficiency of excitation capture by open PSII reaction centers, indicating Φ_{PSII} and of photosynthetic performance with lower Fv/Fm values observed in response to stress (Genty et al. 1989; Maxwell & Johnson 2000). The close correlation between *Fv/F*m, length of drying time, and ultimately tissue survival represents a strong argument that longer drying times lead to protection of PSII in a desiccated state.

Fm represents the maximal fluorescence value achievable with all PSII reaction centers closed and all non-photochemical quenching parameters at minimum, and in stressed organisms shows a highly significant interaction with desiccation intensity (Kooten & Snel 1990; Pressel et al. 2009; Proctor 2012; Stark et al. 2013). The value of Fm can vary between experiments and laboratories as it is an arbitrary value influenced by differences in tissue mass and fluorometer configuration. However Fm has been a reliable indicator of tissue survival in our laboratory and others, and may be more reliable than *Fv/F*m when a large portion of the organism is undergoing cellular death, as a high Fv/Fm value can be observed when little photosynthetic tissue remains (Cruz de Carvalho et al. 2011; Pressel et al. 2009; Proctor 2012). Fm values of slowly dried samples in our experiment suggest that in addition to having a higher percentage of functional PSII reaction centers (Fv/Fm), a greater amount of the PSII structures are undamaged in gross terms.

Φ_{PSII} is a measure of the quantum yield of electron flow through PSII in vivo. Φ_{PSII} therefore shows proportion of absorbed light used to drive photochemistry indicating overall photosynthetic rate and the relative rate of carbon fixation (Krall & Edwards 1992; Maxwell & Johnson 2000). The Φ_{PSII} of the samples in our experiment showed retention of near normal rates

in *P. patens* when desiccated slowly, supporting our hypothesis that slow drying allows for protection of the photosynthetic apparatus.

Conclusions

For over a decade *P. patens* has been a focus of non-vascular plant research, resulting from the ease with which homologous recombination can be performed, in conjunction with sequencing of the *P. patens* genome (Quatrano et al. 2007; Schaefer & Zyrd 1997). Until recently it was thought DT could not be achieved in *P. patens* without abscisic acid supplementation; reports indicated the organism perished if exposed to desiccating conditions (Frank et al. 2005; Oldenhof et al. 2006; Saavedra et al. 2006; Cumming et al. 2007). Our findings suggest that rate of drying is an extremely important variable in the degree of damage *P. patens* experiences. These findings likely carry ecological importance, most obvious of which is that estimations of habitat range for IDT mosses may be much wider than previously thought, as our recent findings suggest (Cruz de Carvalho et al. 2011; Stark et al. 2013).

Whether the manifestation of DT presented in this paper is due to the induction of gene expression in *P. patens* during a SD event; or if *P. patens* is simply more capable of handling a SD than a RD due to the SD being a more forgiving stress allowing constitutively expressed repair mechanisms to confer survival is in need of further investigation. Essentially either method would be illuminating as one would allow for novel gene discovery opportunities and the other would suggest that *P. patens* is using a similar survival strategy as the desert adapted moss, *Syntrichia ruralis*, only calibrated for the SD it would experience in its more mesic environment (Oliver & Bewley 1984). Recent discoveries however suggest gene induction is the likely explanation: firstly, exogenous ABA can induce a DT pathway in *P. patens* and secondly,

concentrations of ABA and LEA proteins increase in *P. patens* in response to dehydration (Cui et al. 2012; Shinde et al. 2012; Yotsui et al. 2013).

Chapter Three: Hardening and De-hardening to Desiccation Tolerance in *Physcomitrella patens*

Abstract

Until recently, the moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. was thought to be desiccation sensitive. Recent research suggests that *P. patens* can survive and recover from slowly applied desiccation. In this study, we examine induction of physiological hardening by a slow-dry pre-treatment of *P. patens* leading to increased survival of a subsequent rapidly applied desiccation. We also examined the length of time hardening is retained when *P. patens* is rehydrated, i.e., how long until de-hardening occurs. By applying a slow-dry pre-treatment before a rapid-dry treatment, survival rates, chlorophyll fluorescence values, chlorophyllous leaf retention ratios, and protonemal and shoot emergence rates all improved compared to non-hardened samples (given the same treatment otherwise). Hardening was retained for up to 8 d depending upon the variable used to assess health. These results indicate that the capacity for desiccation tolerance (DT) in *P. patens* increases in response to bouts of low water potential.

Introduction

Our understanding of water relationships and desiccation tolerance (DT) in the moss clade is currently under re-evaluation, shifting from considering mosses as either strictly tolerant or sensitive, to a gradient, partially modulated by environmental stimuli. This environmental stimulation leads to hardening as seen in seasonal hardening (influenced by weather and climate conditions), increasing from a minimum in spring to a maximum in summer (Richardson 1981; Dilks & Proctor 1976a). A plant is considered DT if it survives equilibrium at water potentials of < -100 MPa (50% relative humidity, RH). For the moss *Physcomitrella patens*, this equates to 12.5% water content on a dry weight basis (Koster et al. 2010; Greenwood & Stark 2014). *Physcomitrella patens* was thought to be desiccation-sensitive (Frank et al. 2005; Cui et al. 2010), however, recent research has shown it to survive and recover from slowly applied desiccation

(Greenwood & Stark 2014). These findings suggest that *P. patens* has the capacity to be induced to exhibit DT by a slow-dry event. We examined retention of DT in *P. patens* after cessation of hardening stimuli. Although *P. patens* is often used as a model organism for research on plant evolution, development, and physiology (Quatrano 2007), until recently exogenously applied abcisic acid (ABA) prior to desiccation was thought essential to survive desiccation (Khandelwal et al. 2010).

We address three predictions centered on *P. patens* possessing a capacity to harden to DT, and when hardened will retain the ability to tolerate a rapid drying event (<30 min). First, a slow-dry pre-treatment will protect against a subsequent rapid-dry (by mitigating chlorophyll loss, improving chlorophyll fluorescence values, and reducing time until protonemal and shoot emergence), compared to specimens that do not experience a hardening slow-dry pre-treatment. Second, hardening will diminish as the hydration interval increases (de-hardening). Third, the length of time the hardening stimulus is applied will influence the strength of hardening and the time required to de-harden. By testing a slow-dry pre-treatment followed by a rapid-dry treatment compared with un-hardened samples given a rapid dry, we determine if *P. patens* displays a DT response.

Methods

Cultivation

If moss samples are directly collected from the field to test DT, the experiences accrued by each plant under field conditions can result in differential health, as well as differential hardening (i.e., reflect differential histories of water stress; Stark et al. 2014). The genetic makeup of field collected plants also varies, whereas laboratory-cultivated samples are genetically identical due to clonal propagation. For these reasons, we chose to use the Gransden strain of *P. patens* (provided

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by Scott Schutte, Southern Illinois University, Dept. of Biology), cultured in the laboratory to remove field effects (Bopp & Werner 1993; Stark et al. 2014).

We cultivated plants using techniques from Greenwood & Stark (2014, also second chapter of this dissertation). Cultures were watered every 48 h with 30% Hoaglands nutrient solution (Hoagland & Arnon 1950). After 8-12 weeks of cultivation, we extracted shoots for experimentation.

Drying

We controlled rate of drying using techniques from Greenwood & Stark (2014), i.e., using wetted filter paper as an artificial substrate where volume of water pipetted is proportional to drying time; plants were equilibrated to 50% RH inside a desiccation cabinet at 20°C. Treatments consisted of an un-dried control, a rapid-dry control (<30 min), and a 4 d slow-dry (time from full turgor to equilibrium with 50% RH) or an 8 d slow-dry. Initial treatments were followed by a 1, 4, 8, or 12 d rehydration period (de-hardening) and ending with a rapid-dry treatment. As an additional control, un-dried plants were subjected to hydration times (1, 4, 8, 12 d) followed by a rapid-dry or no drying. Ten shoots were used for each of five replicates per treatment for a total of 100 sample units.

After the slow-dry pre-treatment, we transferred shoots to new Petri dishes containing autoclaved field-collected hydrated sand for rehydration. Prior to rapid-dry treatments, we removed any shoots or protonemata generated during the slow-dry pre-treatment or rehydrated de-hardening period to avoid conflation of results. For the subsequent rapid-dry, we again rinsed shoots, and placed them in un-lidded Petri dishes containing filter paper but with no water added, allowing a rapid-dry. We used unstressed hydrated controls (i.e., sample units not receiving a slow-dry) from our stock cultures. Controls were rinsed and blotted as described above, and

immediately transferred to Petri dishes containing sand. We used these unstressed shoots to

control for the effects of transferring materials between different containers and substrates.

Chlorophyll Fluorescence

Prior to measurement, we transferred shoots to new dishes containing sand saturated with

water for 24 h to rehydrate. All samples were transferred into a leaf clip and dark-adapted for 30

m before taking measurements. Reduced fluorescence values manifest quickly after stress events

and indicate the extent to which photosystem II (PSII) is using energy absorbed by chlorophyll,

with low utilization efficiency indicating damage (Maxwell & Johnson 2000; Proctor 2012). We

took measurements (following Genty et al. 1989) of dark-adapted fluorescence (F_v/F_m), non-

photochemical quenching (NPQ), and the quantum efficiency of PSII (efficiency of PSII electron

transport; ϕPSII). F_v/F_m represents the efficiency of excitation capture by open PSII reaction

centers, indicating Φ_{PSII} and of photosynthetic performance (Genty et al. 1989; Maxwell &

Johnson 2000). Φ_{PSII} is a measure of the quantum yield of electron flow through PSII in vivo.

Φ_{PSII} therefore shows proportion of absorbed light used to drive photochemistry indicating overall

photosynthetic rate and the relative rate of carbon fixation (Krall & Edwards 1992; Maxwell &

Johnson 2000).

Regeneration

Immediately following fluorescence measurements, we selected three shoots at random

and placed them on hydrated sand in Petri dishes to determine chlorophyllous tissue retention, and

to determine time required for shoot and protonemal emergence. We inspected these shoots over

ten days using a stereomicroscope, recording emergence of protonema and shoots and noting how

many days were required in that particular sample for emergence. At seven days after placement

on sand, we counted the number of undamaged (green tissue), partially damaged (partially green

tissue turning brown), and dead (brown, no obvious green tissue) leaves on each shoot to determine chlorophyllous leaf retention. Leaf counts were converted to a percentage to account for different numbers of leaves on individual samples.

Data Analysis

We compared experimental treatments to controls by analysis of variance (ANOVA), and compared means by a post-hoc pairwise Tukey test. We used one way ANOVAs individually to assess measured variables: F_v/F_m, ΦPSII, NPQ, leaf health, and regrowth times for all treatments and controls. Homogeneous subsets established by a Tukey test signified statistically similar response variables with an α of 0.05.

We conducted a Kaplan-Meier analysis at a 95% confidence interval upon the protonemal and shoot regeneration rates to predict emergence rates. We censored (recorded as 10 days) samples that did not produce shoots or protonema over the course of observation, resulting in a maximum predicted estimation of ten days. All statistical analyses were executed in SPSS 20 (IBM, Armonk, NY).

<center>Results</center>

Chlorophyll Fluorescence

Fluorescence parameters were significantly closer in value to unstressed controls when a slow-dry pre-treatment preceded a rapid-dry treatment (Fig. 4, 5, 6; homogeneous subsets established at an α of 0.05); however, with increasing time spent rehydrated (de-hardening period) after a slow-dry pre-treatment, fluorescence values decreased. At the longest de-hardening time, there was no significant difference among samples that spent 12 d hydrated (de-hardening) before a rapid-dry across any of the pre-treatment exposures, indicating the protective effects of the pre-treatment had been lost by 12 days. In all other cases, unhardened samples given a rapid-dry led

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to fluorescence values significantly lower than hardened treatments. In all cases but one, the unstressed controls had higher fluorescence values (Fig. 4, 5, 6; homogeneous subsets α of 0.05) and these values tended to be significantly higher compared to other treatments. NPQ showed some differences in trends, with detectable hardening lasting shorter for NPQ compared to other fluorescence values. The 4 d slow-dry pre-treatment appeared to assist with retention of higher NPQ values when accompanied by a de-hardening period <8 d (Fig. 6; homogeneous subsets α of 0.05).

Chlorophyllous Tissue Retention

Chlorophyllous tissue retention significantly increased when given a slow-dry pre-treatment prior to a rapid-dry (Fig. 7; homogeneous subsets established at an α of 0.05), with both the 4 d and 8 d slow-dry pre-treatments resulting in more green and semi-green leaf tissue (Fig. 7; A homogeneous subsets α of 0.05). A rapid-dry treatment without a slow-dry pre-treatment led to significant loss of green leaves (Fig. 7; B-D homogeneous subsets α of 0.05). As de-hardening periods increased there was a significant reduction of green leaves as well as semi-green leaves compared to samples with shorter de-hardening periods. Beyond an 8 d de-hardening period a rapid-dry resulted in nearly all leaves dying.

Time Until Shoot and Protonemal Emergence

In general, we observed that the slow-dry pre-treatment increased the likelihood of shoot and protonemal emergence when applied before a rapid-dry treatment (Fig. 8, 9; homogeneous subsets α of 0.05). As the rehydrated (de-hardening) period lengthened, shoot and protonemal emergence decreased to near zero following a rapid-dry treatment, regardless of the prior slow-dry pre-treatment. For example, after an 8 d de-hardening period, slow-dry pre-treatment did not result in new shoots or protonema production of samples that received a rapid-dry treatment.

Discussion

Does *P. patens* Exhibit Inducible Desiccation Tolerance?

Revisiting our original three hypotheses: first hardening exists in *P. patens* reducing damage caused by a rapid-dry, second hardening is a transient event, and third a longer slow-dry pre-treatment will result in more intense and longer lasting hardening). First, the evidence strongly supports the ability of *P. patens* to harden to DT given the wide disparity in performance between samples hardened by a slow-dry before exposure to a rapid-dry and those not hardened before a rapid-dry. Secondly the data support a transient nature for hardening with all metrics used to assess health returning to control values after 12 d spent in a hydrated state. Our third hypothesis, however, was not supported, as our 8 d slow-dry performed worse than or equal to a 4 d slow-dry in terms of hardening intensity and duration.

Physcomitrella patens exhibits patterns indicating inducible hardening to DT (by a prior slow-dry pre-treatment); fluorescence, chlorophyllous tissue retention, and new tissue emergence were improved by a slow-dry pre-treatment prior to a subsequent rapid-dry. With increased time spent hydrated (de-hardening) after slow-dry pre-treatment, response values decreased, displaying reduced health and a transient hardened phenotype. Fluorescence parameters were largely similar between the 4 d and 8 d slow-dry pre-treatment across hardening periods. However the 4 d slow-dry pre-treatments showed slightly higher green leaf percentages compared to the 8 d slow-dry pre-treatments. As for shoot and protonemal emergence, both slow-dry pre-treatments resulted in greater emergence after a rapid-dry treatment compared to after a rapid-dry treatment with no prior slow-dry pre-treatment.

Response to Treatments

Time spent hydrated (de-hardening) after the initial slow-dry pre-treatments led to attenuated DT. A slow-dry pre-treatment did protect against a future rapid-dry treatment. The protection was strongest after a 1 d hydration period, declining at 4 d, and by 8 d of a hydrated de-hardening period there was little discernable difference between slow-dry pre-treated and the samples not pre-treated with a slow-dry. The protection was essentially eliminated after a 12 d hydration for all parameters measured; experimental samples were statistically indistinguishable from rapid-dry control samples.

We found a strong relationship between slow-dry pre-treatment and fluorescence parameters that indicated hardening. Fluorescence parameters, such as ΦPSII, for slow-dry pre-treated samples exposed to rapid-dry treatment presented higher values compared to control samples, indicating a DT response. We also observed a decline in fluorescence parameters with lengthened hydration (de-hardening) after the slow dry pre-treatment, indicating decreased protection.

As expected, chlorophyllous tissue retention decreased as de-hardening interval increased. Hardened shoots retain a high degree of chlorophyllous tissue when subjected to a rapid-dry after a 1 d de-hardening period (with longer de-hardenings resulting in progressively worse performance). These results suggest that when severely damaged, *P. patens* will prioritize new shoot and protonemal development instead of chlorophyllous tissue retention.

Time until emergence of protonema and shoots was strongly improved by a slow-dry pre-treatment; indicative of hardening (Fig. 5, 6). Un-hardened control samples showed increased damage when given a rapid-dry treatment, providing evidence that the slow-dry pre-treatment contributed to DT. Our control rapid-dry samples lacked protonemal emergence. The heightened regenerative ability following a rapid-dry that a slow-dry pre-treatment imparts may be the most

important aspect for survival. Without hardening *P. patens* shows virtually no shoot or

protonemal regrowth, with only one shoot producing protonema out of 225 examined (samples

not pre-treated with a slow-dry before a rapid-dry Fig. 5, 6). This is in sharp contrast to hardened

samples given 4 d or fewer to de-harden which displayed protonemal emergence rates between

20-80% of the time depending on treatment combinations (Fig. 5, 6). When viewed in the context

of a colony where shoots exist as clones in clumps of hundreds or thousands, such patterns would

improve survival odds, as shoots in the center experience longer slow-dry periods.

Addressing the 4 d Slow-dry Outperforming the 8 d Slow-dry

Surprisingly, our 4 d slow-dry treatment performed better than or equal to the 8 d slow-dry

period in terms of hardening intensity and duration. Further investigation is necessary to elucidate

why this occurred, but one explanation may be the exhaustion of carbohydrate reserves over the 8

d slow-dry period. Mosses likely expend a large amount of energy preparing for the desiccated

state, and more during the respiratory burst to fuel damage repair that occurs during rehydration

(Krochko et al. 1979). Carbohydrate exhaustion is a leading hypothesis explaining why repeated

short intensity rainfalls can kill desert-adapted mosses, because the short photosynthetic window

before the organism again desiccates would not allow attainment of a positive carbon balance

(Coe et al. 2012). As *P. patens* is considerably more desiccation sensitive then desert-adapted

mosses, we would expect it to have lower carbohydrate reserves, and therefore be more

vulnerable to carbohydrate exhaustion. An alternative explanation is that by 4 d of slow drying

the maximal capacity for hardening (In response to a single exposure) had been reached.

Conclusions

Ecological Implications

Our findings indicate that hardening is an important variable for survival of a rapid-dry event in *P. patens*. Interestingly, the de-hardening time we observed (~8 d) in the laboratory for *P. patens* closely matches the de-hardening time frame of the field-collected desert moss *Crossidium crassinerve* (7 d; Stark et al. 2014), and may be a common length for de-hardening in mosses. These patterns further support a gradient of desiccation tolerance in mosses, as postulated by Pressel et al. (2006) & Wood (2007). This gradient spans from very sensitive species (requiring a long time frame for induction, such as *P. patens*), to extremely hardy species which can only be damaged with very rapid drying events to near 0% RH (e.g., *Syntrichia ruralis*).

Chapter Four: Effects of Rate of Drying, Life History Phase, and Ecotype on the Ability of the Moss *Bryum argenteum* to Survive Desiccation Events

Abstract

Desiccation stress is frequently experienced by the moss *Bryum argenteum* and can influence survival, propagation and niche selection. We investigated effects of rate of drying (RoD), life history phase, and ecotype on desiccation tolerance (DT). Using chlorophyll fluorescence as a survival index, we determined how our parameters influence desiccation tolerance for the following factors; five life history stages, 13 sampling locations or ecotypes, and five levels of drying time. We observed significant main effects from both factors and interactions. Rate of drying and phase significantly affected DT. The reaction norms of DT displayed by the 13 ecotypes showed a substantial degree of variation in phenotypic plasticity, particularly in protonemal and juvenile phases. We observed differences in survival response and chlorophyll fluorescence between rapid and slow drying events in juveniles. These same drying applications did not produce as large of a response for adult shoots (which consistently displayed already high values). However, we did observe differences in the magnitude of phenotypic plasticity in response to RoD treatments between ecotypes, particularly in juveniles, and protonema. Some juvenile and protonemal ecotypes, such as those from the southwest United States, possessed higher innate tolerance to rapid drying, and greater resilience compared to ecotypes sourced from mesic localities in the United States (particularly samples CA1, NM, CIMA, and VF). Taken as a whole these results show a complex nuanced response to desiccation with ecotypes displaying a range of responses to desiccation reflecting both inherently different capacities for desiccation as well as variation in capacity for phenotypic plasticity.

Introduction

There are two factors required for understanding and predicting survival of an organism in response to desiccation, its capacity for desiccation tolerance and the capacity to improve its

desiccation tolerance in response to environmental cues or past experience. The first of these is the inherent capacity of the organism to express a level of desiccation tolerance that is unaffected by external conditions or previous environmental exposure to drying. The second factor, phenotypic plasticity, is the degree to which an organism can increase DT in response to environmental cues or previous exposure to drying. The capacity for phenotypic plasticity itself is known to undergo ontogenic shifts as plants develop such that the plasticty may only be observable for specific periods (Mediavilla & Escudero, 2004).

Although phenotypic plasticity is the major means by which plants cope with environmental pressures a phenotypically plastic response for the trait of desiccation tolerance would not be free of cost (Valladares 2007). Generation of desiccation tolerant phenotypes would require the associated maintenance and production costs associated with the protein and sucrose formation believed necessary to develop a desiccation tolerant phenotype (DeWitt *et al*. 1998). In addition it is likely that the sensory apparatus to receive the information about a drying environment is not cost free, and should the organism fail to produce a desiccation tolerant phenotype within the necessary timeframe of a desiccating event mortal injury would most likely result (DeWitt *et al*. 1998). Phenotypic plasticity should be favored in response to either shifting environmental pressures at a locality, or in organisms which may be deposited into a variable location (such as seen for spore rain). In variable environments therefore a plastic response should be favored as the costs associated with producing the phenotype should be proportional to the rate at which the appropriate environments are encountered (Pigliucci 2005).

 Bryophytes possess a remarkable capacity to survive extremely low water potentials, an ability known as desiccation tolerance (DT). Desiccation tolerance is the ability of an organism to equilibrate to 50% relative humidity (RH, i.e., < —100 megapascals) and resume normal function

when rehydrated (Wood 2007). Mosses occur in all ecosystems of the world (except marine), from equatorial jungles to temperate forests to deserts and even Antarctica. Mosses can withstand extreme environmental stress, surviving temperatures and water content levels lethal to most eukaryotic organisms. All mosses are poikilohydric and their water content changes with relative humidity. Some species can survive up to 120°C for 30 minutes (Stark & McLetchie 2009), thousands of years beneath glacial ice sheets (La Farge et al 2013; Roads et al. 2014), fully submerged in water (the genus *Fontinalis*; de Carvalaho et al. 2011) and decades of continuous desiccation at room temperature (Stark et al. 2016).

Mosses are important for the many beneficial roles they play in ecosystems such as functioning as ecosystem engineers, nutrient cycling, colonizing disturbed habitats, increasing soil stability, and aiding establishment of seed plants (Belnap 2006; Chaudhary et al. 2009). Given these remarkable abilities, we may learn much about how organisms survive stressful conditions by studying mosses and also why mosses occur where they do in the environment.

The altered rainfall and temperature patterns forecast in models of anthropogenic climate change could have disastrous effects for mosses and destabilize ecosystems dependent upon mosses (Intergovernmental Panel on Climate Change 2014). Understanding the degree of DT possessed by both xeric and mesic ecotypes could be critical for predicting future survival of individual populations as well as the future of ecosystems reliant upon their presence (i.e., effects on ecosystem function; nutrient cycling, soil stability). In general, SD results in a greater survival after a desiccation-rehydration cycle (Bewley 1995; de Carvallho et al. 2011), while RD leads to chlorosis and damaged photosynthetic machinery (Schonbeck & Bewley 1981a; Stark et al. 2013). An understanding of how mosses (an essential component of many mature biological soil crusts) respond to and tolerate desiccation will help guide rehabilitation and restoration practices

in advance of predicted climate change. Understanding these dynamics will assist land managers and restoration practitioners in developing appropriate survey techniques for identifying vulnerable populations and developing strategies for conserving vulnerable populations, as well as matching at risk populations to appropriate habitats. With this study we illuminate aspects of the relationships between ecotypic variation, life history phase, and rate of drying (RoD) upon DT in the species *Bryum argenteum*. The methods and design displayed in this paper will also provide a roadmap for determining the physiological response to desiccation for populations of other mosses as well.

As non-climate related anthropogenic effects (e.g., habitat destruction and fragmentation) are already threatening many populations, predicting how mosses will respond to global climate change may be essential for conservation efforts to effectively predict and protect at risk populations and species (Tuba et al. 2011). One could imagine two scenarios with different outcomes with respect to the effect changing climate will have on moss distributions, and species survival. In the first, a species is "pre-adapted" (phenotypically plastic for desiccation tolerance). In this case one expects a relatively minor effect on moss cover, with many populations surviving climate change. A second scenario where ecotypes are highly adapted to their local environment and possess low phenotypic plasticity, in which case mesic ecotypes will be more sensitive to desiccation and many populations could perish if their locality sees reduced rainfall.

Phenotypic plasticity has been demonstrated to exist in *B. argenteum* previously for thermal tolerance across ecotypes and may reasonably expected for other physiological traits (He et al. 2016). Inducible phenotypic plasticity for desiccation tolerance (if present) may be key for survival and evolutionary adaption therefore to the new habitats and climactic regimes associated with the effects of anthropogenic climate change. A capacity for plasticity in response to

environmental signals resulting in increased desiccation tolerance would allow a wider range of genotypes to survive the altered habitats, preserving genetic variation (Matesanz et al. 2010). This persistence would permit time for either a return to climactic norms (if they are relatively short lasting), or for the development of mutations and selection of more desiccation resistant forms (if effects are longer lasting). The buffered protection from environmental selection (preservation of genetic diversity) would allow for genetic assimilation of traits related to desiccation tolerance over the long term if conditions persist (and or gene flow from more mesic habitats is relatively low) resulting in an overall more constitutive nature to desiccation tolerance in xeric mosses (Ghalambor et al. 2007; Matesanz et al. 2010; Pigliucci 2005; Shaw & Etterson 2012).

Recent research has demonstrated that DT in mosses is a variable and plastic trait for at least some species. For example, *Physcomitrella patens*, which is generally recognized as desiccation sensitive (DS), can withstand desiccation if a slow RoD is applied (Greenwood & Stark 2014). In many mosses, DT likely functions as an inducible trait, using environmental cues and water loss to drive the production of the stress hormone abscisic acid (ABA). Exogenous ABA induces DT in DS species, and ABA is produced during osmotic stress, suggesting it could have a role in DT within natural systems (Beckett 2001; Cruz de Carvalho et al. 2014; Wang et al. 2009; Werner 1991).

As spores of mosses have high dispersal capacity yet lack a method to control the location of deposition it has long been suggested that mosses act in accordance with the Baas Becking hypothesis *"Everything is everywhere, but the environment selects"* (Baas-Becking 1934). This agrees with studies of the genetic structure of moss populations, which have shown high genetic diversity (Shotnicki et al. 1998). An alternative explanation is that high levels of phenotypic plasticity formed by a long history of variable selective pressures in diverse environments over

multiple generations, rendering environmental selection and ecotypic variation to a specific

habitat a moot point (Pigliucci 2001). Aspects supporting high phenotypic plasticity include the

wide range of habitats used by *B. argenteum* and its cosmopolitan distribution (Shaw 1989).

During development, mosses may transition from an inducible form of DT, requiring a

slow dry (SD) to achieve protection to a constitutive form (experiences a RD with minimal to no

damage) or reducing the time required for inducible DT (Stark et al. 2016; Stark & Brinda 2015).

Evolution selects for phenotypic traits in a population that improve the odds of survival and

reproduction across life phases while minimizing expenditures. DT likely has energy costs to

maintain, therefore, it is expected that DT is regulated over development to reduce energy

expenditure while maximizing fitness (Stearns 1989). Factors that guide the development of traits

are efficient utilization of limited energy resources, habitat or ecosystem conditions, genetic

inheritance from ancestors, ability to deal with stress at various life history phases, or a

combination of these factors. We expect that organisms invest greater energy toward DT in

habitats subjected to desiccation (e.g., higher DT ability in organisms from xeric habitats

compared to mesic habitats). However if rates of gene flow with organisms from mesic habitats is

high this may not hold true.

Over the life cycle of an organism, the relative importance of abiotic (e.g., desiccation,

intense light, heat etc.) and biotic (e.g., intra and interspecific competition) factors can shift. For

example, juvenile shoots likely prioritize vertical growth to maximize energy gathering ability

(e.g., shade competition), similar to vascular plants allocating energy between defense,

reproduction, and growth over development (Bazzaz et al. 1987). Resource allocation shifts occur

in sporophytes of *Aloina ambigua* which switch from inducibly DT to constitutively DT as they

develop (Stark & Brinda 2015). *Bryum argenteum* shoots may therefore transition as well, with established adults prioritizing DT.

For this study, we examined possible relationships between five different life history phases (similar to vegetation phenotypic phases) with five rates of drying (rate of drying influences length of time that is required for an organism to reach a desiccated state, or low internal water content) on 13 ecotypes of *B. argenteum*. *Bryum argenteum* makes an excellent study system due to its cosmopolitan distribution across arid, mesic, and urban settings. Life phases included bulbils, protonema and juvenile, intermediate and adult shoots. Rate of drying included five rates of drying to an equilibrating relative humidity of 50%. By examining a range of drying times (i.e., time spent at sub-turgor prior to desiccation), we could determine not just if a tissue is DT or not, but the time required for inducible DT to manifest.

This study asks if DT in *B. argenteum*, is completely constitutive or if DT in this species falls along an inducible gradient of DT. We hypothesized that all life phases (protonema, juvenile shoots, adult shoots, and propaguliferous bulbils) types would perform best with longer drying periods, and that the effect of a slow vs. rapid dry will be more prominent in juvenile tissues and protonemata. We applied these factors to specimens obtained from thirteen different source populations (ecotypes), and sought to address to what degree ecotypes deviate in their capacity to tolerate desiccation and how this may influences survival in different environments. We hypothesized that evolutionary history influenced ecotypic variation, so that samples from arid regions (U.S. Southwest) will display greater DT and a shorter time required to induce DT compared to populations from mesic habitats (U.S. Northwest and Mid-Atlantic states). We also hypothesized that protonema and juvenile forms would be most susceptible to desiccation, requiring longer timeframes for effective inducible DT compared to mature forms, which would

indicate these phases are more vulnerable and less resilient to changes in environmental conditions.

Methods

Bryum argenteum has one of the widest distributions of any plant and is found on all continents and in diverse habitats: hot and cold deserts, temperate and polar climates, and urban settings (Flora of North America Editorial Committee 2014). Sexually dioecious, it is capable of short or long distance dispersal via sexually generated spores, as well as clonal dispersal over short distances via bulbils.

Sample Collection

Specimens were gathered from herbarium collections (collected between 1999 and 2009) from a range of North American (U.S.) habitats showing varied seasonal and yearly temperature and precipitation. Localities include Arizona (2 locations; male and female, ♂/♀), California (3 locations; ♂/♀), Kentucky (2 locations; ♂/♀), Nevada (1 location; ♀ only), New Mexico (1 location; ♀ only), Massachusetts (2 locations; (♂/♀), and Oregon (2 locations; ♂/♀) (appendix 1). Specimens were originally collected and stored dry in herbarium cabinets at low humidity and low light.

Selection of Material and Cultivation

Wet-dry cycles experienced under field conditions combined with differential levels of resources and pre-existing injury and disease make assessment of DT or inducibly-DT status difficult due to hardening (Stark et al. 2014). Induction of a hardening response has been observed in gametophytes of *Crossidium crassinerve*, protonema of *Funaria hygrometrica* (Werner et al. 1991), and even in gametophytic tissue of some aquatic mosses (Cruz de Carvalho 2014). For

these reasons, we used lab cultivated samples grown under common growth conditions to assess DT and to avoid variation due to unknown field hardening effects.

Moss shoots were isolated by subculturing and cultivated following Greenwood & Stark (2014, also second chapter of this dissertation) to remove habitat-acquired acclimation. In this study we tested for DT capacity in three major phenological classes: 1) bulbils, propagules specialized for roles as agents of dispersal; 2) protonemata, which provide lateral expansion of moss colonies through and on surface soil; and 3) shoots, the major photosynthetic structures and bulk of gametophytic tissue. Shoots were additionally divided into three developmental classes based on height (<3 mm, juvenile; 3 —5 mm, intermediate; and >5 mm, adult).

Protonemata required unique cultivation as they adhere to substrate, making isolation difficult without destroying tissue. Protonemata, therefore, were cultivated in a liquid media of 30% Hoagland's solution (Hoagland & Arnon 1950). To develop stock cultures, test tubes (16 × 100 mm) were inoculated with a single leaf from stock cultures. Protonemata were grown at 24°C with constant light (59 µmol m^{-2} s^{-1} PAR, photosynthetically active radiation). Tubes were sealed with parafilm and inverted twice daily to increase gas exchange. Bulbils were collected from shoots of stock cultures growing in sand substrate.

Sample Material Preparation

For each ecotype, from randomly selected stock cultures grown on sterile sand, shoots were selected when they reached target lengths (juvenile, <3 mm; intermediate, 3-5 mm; and mature, >5 mm), with five shoots used per sample unit. For protonema, material was collected from liquid stock to produce all sample units. Due to the small size of individual bulbils, 50 bulbils collected from sand substrate stock cultures were used per sample unit. All tissue material was blotted between two sheets of Whatman #1 filter paper to remove excess surface water before

placing the tissue on top of an artificial substrate. These were placed within a 35-mm diameter Petri dishes pre-treated with RoD manipulation treatments.

Stress Application

Rate of drying (RoD) was controlled for by adding specific volumes of sterilized water to an artificial substrate following Greenwood & Stark (2014, also second chapter of this dissertation) before the addition of tissue to the substrate. Specific volumes of water were dispensed with a 100 µL pipette. Volumes for RoD included: 0 µL with no lid (< 20 m), 0 µL lidded (< 30 m), 12.5 µL lidded (1 h), 25 µL lidded (4 h), and 50 µL lidded (11 h), times listed indicate time until leaf curling (~86% RH equilibration) when metabolic activity ceases. Controlled RoD were carried out in a benchtop relative humidity chamber (Totech Super Dry, Totech, Tokyo, Japan) set to 50% relative humidity, The chamber was located inside of an environmental control room (R.W. Smith and Co., San Diego, CA, USA) set to 20°C and 50% RH. Samples were stored for 24 h at 50% RH after equilibration to ensure all material was dried to equilibrium. Verification of dry material follows procedures in Greenwood & Stark (2014). Unstressed shoots with no prior drying events, obtained from the existing stock cultures served as controls. All treatment combinations, ecotype $\times$ life phase $\times$ RoD, were replicated five times.

Chlorophyll Fluorescence as a Measure of Stress

After samples were dry (and equilibrated) for 24 h, they were rehydrated for 24 h, transferred to leaf clips, and dark-adapted for 30 m. For shoot phases, all five shoots within the sample unit were used. For protonemata, enough material from within the sample unit was used to cover the base of the leaf clip. For bulbils, 50 were used per sample unit. Since chlorophyll fluorescence is a ratio, the amount of tissue does not necessarily affect readings but enough material is required to reach a minimum initial fluorescence signal.

Status of dark-adapted (F_v/F_m) fluorescence, non-photochemical quenching (NPQ) and quantum efficiency of photosystem II (ϕPSII) were determined with a fluorescence monitoring system (FMS2, Hansatech) using the saturation pulse method (Schreiber et al. 1995). F_v/F_m is the maximum quantum efficiency of PSII under dark-adapted conditions. Non-photochemical quenching is a method to dissipate excess excitation energy as heat, and a decrease in NPQ can indicate tissue damage. ϕPSII is the quantum efficiency of PSII electron transport in the light (Genty et al. 1989), with lower levels resulting from stress or damage.

Data Analysis

All 13 ecotypes, five life phases, and six rates of drying including controls resulted in a 13 $\times$ 5 $\times$ 6 treatment design, or 390 total potential treatment combinations, N = five per treatment, totaling 1,950 individual sample units. All fluorescence parameters were analyzed for significance of main treatments and all two-way and three-way treatment interactions using analysis of variance (ANOVA) in SPSS v.20 (IBM corp., Armonk, NY, USA), with life history phase, ecotype, and RoD set as fixed factors. For significant effects, post hoc Tukey tests were applied to determine homogeneous subsets within main treatments and all significant two-way interactions.

Results

All three main treatments, as well as all two-way and three-way interactions between treatments were significant for all fluorescence parameters. Significance of interactions varied within and between RoD treatments but results and trends were relatively consistent between fluorescence parameters. RoD appeared to have the most effect on fluorescence parameters, followed by life phase and ecotype (Fig. 10 A - D). For specific results per fluorescence parameter, see supplemental figures 10 – 12, and reaction norms on figures 13-17.

Rate of drying contributed strongly to the variability of fluorescence parameters within phases. There was a clear trend (Fig. 10 A, B), and across ecotypes (Fig 13-17; Supp. Fig. 10 - 12), that lengthened RoD increased fluorescence. Mostly, this increase was between a rapid dry and a 1 or 4 h drying time, indicating that after a 1 or 4 h drying time for most ecotypes, increasing drying time does not necessarily continue to significantly increase fluorescence, although this was variable within ecotype (Fig. 13-17; Supp. Fig.1-3). With increased drying times there was less difference across life phases (Fig. 10 A; reduced slope angle). The youngest shoots required 11 h to reach control values with but intermediate and mature shoots for many ecotypes demonstrated a strong response over a shorter drying time (Fig 10-17; Supp. Fig. 1-3). However, within the time frames examined by this study, protonemal values did not reach controls values and only achieved low mean values after an 11 h drying time. For example, F_v/F_m reached mean 0.368 ($\pm$0.025 s.e.) after 11 h. Overall, there was a strong trend toward greater recovery after rehydration with a longer RoD for most ecotypes within life phases.

Generally for all life phases, fluorescence parameters improved with slower drying rates (Fig 10 A). Significance between life phases varied within and between ecotype, although most of the variability appears to be contained within the protonema and juvenile shoots across RoD treatments (Fig. 10 -14; Supp. Fig. 1-3). Protonema performed the poorest in response to desiccation stress , i.e., the lowest F_v/F_m performance (mean $\pm$ standard error; 0.355 $\pm$0.01), compared to shoots and bulbils (Fig. 10 C; Supp. Fig. 1). Protonema tended to remain vulnerable to damage across the RoD treatments (Fig. 10 A), except for three instances with ecotypes from the Southwest US (NM, CIMA, CA1, VF; Fig. 13; Supp. Fig. 1). As shoots increase in age, mean fluorescence across all treatments and ecotypes increased: juveniles (0.635 $\pm$0.009); intermediates (0.697 $\pm$.007); adults (0.702 $\pm$.006). Bulbils display the greatest healthy retention regardless of

RoD (0.728 ±.003) (Fig. 10 C, Supp. Fig. 1) and tended to display the highest resistance to an extreme RD (Fig. 10 A, Fig. 17, Supp. Fig. 1). Mean values of all phases responded positively to longer drying times showing increased values approaching or equal to control values.

Ecotype, grouped into four significantly homogeneous subsets (Fig. 10 D), with most of the signal driving these groups derived from the protonemal and juvenile shoot phases (Fig. 13, 14; Supp. Fig 1). Variation diminished with development in shoot tissues with mature shoots from all ecotypes displaying a high tolerance for desiccation (Fig. 16). Likewise bulbils also showed very low ecotypic variation and an extreme capacity for desiccation tolerance across not showing variation for phenotypic plasticity in response to RoD (Fig. 17).

Reaction norms (ecotype by environment) for tissues showed both some overall similarities, as well as capacities unique to a subsets of ecotypes. First for all ecotypes examined bulbils displayed a remarkably flat response to the RoD applied, displaying the high importance of desiccation tolerance to this phase (Fig. 17). For adult shoots many ecotypes performed well at all drying rates; however AZ1, MA2, KY2, when dried more rapidly than 1h performed far below most other ecotypes, if given an induction of 1h or more they performed near the other ecotypes (Fig. 16). With intermediate shoots most ecotypes required 1h for induction of a DT phenotype. Intermediate shoots from ecotypes of NM, CA2 had high values after only 30 minutes of induction, while conversely AZ1 was worse than all other ecotypes with lower values at most RoD and requiring a 4h induction time before intermediate shoots showed values close to other ecotypes given a similar drying regime (Fig. 15). Juvenile shoots required a longer RoD to induce DT than other shoots on average with most ecotypes requiring 1 or more hours. Juvenile shoots of the AZ1 ecotype were again much less tolerant of desiccation than other ecotypes requiring 4 hours to induce a DT phenotype and displaying values consistently lower than all other ecotypes

at times below this (Fig. 14). Protonemal forms were the least likely to show a DT reponse of any kind in reponse to RoD, with only a handful showing signs of induction. Protonemal ecotypes of CIMA, VF, NM and CA1 were the exceptions with all showing induction after 11h of drying, with NM and VF having reached control values. Protonema from NM showed a more rapid response than other ecotypes with strong induction achieved with only a 1 hour slow dry, and control values reached by 4h (Fig. 13).

Discussion

The constitutive nature of DT in *B. argenteum* can and should be doubted if the organism is considered across all life phases. Although adults and bulbils would appear constitutively DT (CDT), as they show little damage from RD, juveniles and protonema display an inducible DT phenotype. Gao et al. (2015) found that the rehydration transcriptomes from adults of *B. argenteum* exposed to a RD to 20% relative humidity resulted in significant differences between the rehydration and pre-desiccation transcriptomes, suggesting similar conclusions. Up-regulation of stress response genes in response to drying provides a mechanism of DT involving transcript up-regulation in addition to the constitutive description of desiccation tolerance in *Syntrichia ruralis,* which produces and sequesters messenger ribonucleoprotein particles at all times regardless of stress conditions (Oliver & Bewley 1984). Although adults of *B. argenteum* may be considered constitutively DT, at least a part of their physiological response should be considered inducibly DT based upon transcriptomics (Gao et al. 2016) and fluorescence data presented here.

Among the ecotypes examined, significant differences were observed in DT response. However, this effect was mostly restricted to protonemal and juvenile phases. This variation in DT could be, in fact, an adaptive response to high dispersal capacity. With spore production ranging from 100 million to tens of billions per square meter of moss cover annually and any

spore potentially traversing hundreds to thousands of kilometers before settling (Miles & Longton 1992), it would be beneficial to possess a highly plastic capacity for inducible DT or DT across vulnerable life history phases, assuming costs for maintaining a rapid inducible DT response are low, and or only expressed in the appropriate environments (Pigliucci 2005).

The tissues examined showed a wider degree of responses than expected, but all tissues responded in a manner logically consistent with their roles in the organism. Protonema are most sensitive, but this may be mitigated in nature as the intrinsic properties of soil results in slower drying rates than tissues above ground can expect, providing a longer drying period, and thus more time for induction. Bulbils will lack the protective functions of colonial patch dynamics once separated (Zotz et al. 2000), which acts to slow the water loss across cushions of moss. It is logical that bulbils would display a constitutive DT response, as any dispersal agent functioning in a xeric habitat should be under strong selection for DT. Patch establishment and expanding regions of growth are likely sensitive to environmental conditions so that establishment and expansion only occur in wet seasons or favorable years. During other periods, the colony may experience high losses of protonemal and juvenile tissue. Taken together, these observations suggest that new growth under xeric conditions is limited by the ability of protonema and juvenile shoots to survive drying.

The reaction norms displayed by the 13 ecotypes showed a substantial degree of variation in phenotypic plasticity, centered around protonemal and juvenile phases. The variation at protonemal and juvenile shoot stages likely reflects increased competition during colony initiation, and along the expanding edge of established colonies. In mesic habitats energy expenditure toward a desiccation tolerant phenotype would provide little advantage, but the more rapid establishment of a colony, and more rapid transition from a juvenile to an adult phase due to

rapid growth would prove more advantageous. In habitats more likely to experience rapid and extreme drying events any advantage gained from rapid growth at the cost of desiccation tolerance would be easily offset by the occurrence of a single unexpected rapid drying event, favoring a rapidly inducible phenotypic plasticity as seen in some ecotypes.

Our results suggest that adults and established colonies are resistant to the effects of altered rainfall pattern that are expected in many areas due to global climate change due to their high capacity for DT with short drying times. However, over longer time frames and under current climate change estimates, the establishment rates of new colonies in xeric habitats are expected to decrease in locations becoming hotter and or drier due to climate change. Shifts to more xeric habitats would negatively impact protonema and juveniles, resulting in higher mortality rates (IPCC 2014). If the rate of successful colony establishment falls beneath the rate at which mature colonies are lost, the cumulative effects would result in either expatriation from localities transitioning to more xeric habitats or a strong selective pressure resulting in increased tolerance across many populations. Genetic diversity in response to desiccation tolerance we have shown for this species, as well as diversity in the capacity for plasticity of reponse suggest *B. argenteum* may have the variation needed for selective pressures to result in more tolerant phenotypes and or a more quickly initiated and stronger plastic response (Carlson et al. 2014).

The findings also provide an experimental outline for land managers to locate and assess vulnerable moss communities by defining methods to determine the susceptibility of ecotypes to altered rainfall patterns across life phases. Non-climate related anthropogenic effects (e.g., habitat destruction and fragmentation) are already damaging many moss populations furthering their need for protection. Predicting how mosses will respond to global climate change by assessing vulnerability of communities is an important step in estimating overall ecosystem health and

function and assist land managers in identifying and reducing negative consequences of disturbance and climate change, by choosing ecotypes for cultivation and transplantation that show a high capacity for desiccation tolerance in protonema and juvenile shoots when raised in culture.

Other direct applications of this research could be used in rehabilitation of disturbed dryland soils or restoring biological soil crusts in xeric habitats. Mosses are cultivated easily in the lab (e.g., Greenwood & Stark 2014; Antoninka et al. 2015), although field studies in the US are limited. Several studies from dryland systems in China suggest mosses can be used as a component for soil stabilization and possibly restoration (e.g., Bu et al. 2013). Few studies have used moss in restoration applications in the US (e.g., Chiquoine et al. 2016) and no studies to the authors' knowledge as of the writing of this article have incorporated laboratory cultivated mosses in field studies or restoration efforts. To increase successful incorporation of mosses into field applications, we suggest the following three strategies to improve outplanting attempts. First, we recommend cultivation to continue until shoots and colonies are fully mature before outplanting; second, harden samples with a slow drying event before outplanting to increase DT; and third, limit outplanting to cooler, wetter seasons, as this allows an entire season for new growth and adaptation to the local climate before desiccation stresses are encountered.

Salvaged biocrust has been effective in restoring habitats suffering from anthropogenic damage, but is limited to localities where there was foresight to collect and store material before construction (Chiquioine et al. 2016). Greenhouse cultivation has produced a six-fold increase in four months of biocrust containing moss (Antoninka et al. 2015) providing a valuable tool for outplanting. However, restoration efforts whenever possible should collect specimens from the local area for greenhouse cultivation to maximize the odds of choosing locally adaptive forms

(assuming the habitat for transplantation into is stable). Local specimens we believe will have decreased protonemal and juvenile mortality upon reintroduction to the wild. If using local materials is not possible, then the use of ecotypes from similar habitats should be considered when available.

Further we would recommend that future studies upon the use of mosses toward habitat restoration of drylands consider the desiccation tolerance capacity of individual ecotypes used for cultivation and later re-introduction. If a restoration effort simply chooses the most rapidly growing ecotype it may find that this advantage in growth rates comes at an unacceptably high cost when field mortality rates are measured. Instead a simple pilot study before the main effort of cultivation is begun should be employed to select for a balance of growth rate and desiccation tolerance leading to more successful re-introduction rates over the long term.

Conclusion

This study presents a pattern of ecotypic reaction norms to desiccation along a developmental trajectory. The use of two tissues with specialized adaptive roles (i.e., bulbils, protonema), as well as shoots during multiple developmental phases, presents a look at how an entire life cycle responds to desiccation. By applying five rates of drying, we examined not just whether the organism is desiccation tolerant, but also how different life history phases and tissue types prioritize rapid to slow induction times to achieve DT. We observed a gradient of responses, thereby allowing predictions of how organisms may respond to future desiccation challenges across life history phases under a variety of scenarios. An extrapolation of these findings would suggest that we should expect few short-term effects (due to high DT of adult shoots), but significant adverse long-term effects on colony establishment (due to low tolerance of protonema and juvenile shoots). If these trends hold true for mosses in generatl species with reduced

dispersal capacity in stressed habitats it could prove disastrous. In the future, we hope results from this study will contribute to other disciplines, including ecological restoration and land management.

The overall plasticity shown for *B. argentum* is encouraging and displays a greater deal of variation than expected (especially given only 13 ecotypes were examined). The plastic variation in response, current large species distribution (both geographic as well as habitat variety), ease of long distance dispersal (via spores) and short distance dispersal (spores and bulbils) suggests that the species may be better poised to respond to climate change than most (Kopp & Matuszewski 2014). This does however also cause this author to suspect that these benefits will also give the species a strong advantage against other species in colonizing new habitats formed by climate change, potentially allowing it to outcompete more narrowly restricted (both in terms of geographic range and suitable habitats) species of mosses. A quickly dispersing species such as *B. argenteum* could rapidly fill new niches as they appear acting as a highly invasive organism and excluding more slowly dispersing species by occupying these niches, further accelerating species loss of more vulnerable species as they are outcompeted.

Chapter Five: Dissertation Conclusions

Dissertation Conclusions

For *Physcomitrella patens*, time spent at sub-turgor led to an increased capacity for desiccation tolerance, displaying increased health by maintaining improved values for fluorescence and regrowth (see Tables 1-2; Figs. 2-3). This evidence strongly supports the conceptual position of desiccation tolerance existing as a gradient, and that the time spent at sub-turgor has a strong influence on the capacity for desiccation tolerance. It also suggests that desiccation tolerance may be more widespread than believed. If a species that is considered to be among the most desiccation sensitive can survive desiccation, it is likely that other "desiccation sensitive" species may display a pattern of desiccation tolerance if provided a slower drying time.

The ability to harden to desiccation stress was shown in *P. patens* when a slow-dry pre-treatment resulted in significantly improved survival following a subsequent rapid-dry compared to a rapid-dry without a slow-dry pre-treatment. The slow-dry pre-treatment improved values for chlorophyll fluorescence (Figs. 4-6), chlorophyll retention (Fig. 7), emergence rates for protonema (Fig. 9) and shoots (Fig. 8), as well as reduced emergence time (Figs. 8, 9). When hardened samples were given a hydration period (de-hardening) before rapid drying, desiccation tolerance diminished (Figs. 8, 9). As the de-hardening period extended, values indicative of health decreased, and by day eight samples were indistinguishable from rapid-dry (no pre-treatment) control samples (Figs. 8, 9).

The effects of rate of drying, life history phase, and ecotype upon *Byrum argenteum's* ability to survive desiccation, were all found to significantly influence desiccation tolerance. However not all factors equally affected desiccation and survival (Figs. 10-12). Life phase and rate of drying produced much stronger effects than were elicited by ecotype (Figs. 10-12). The variation observed in ecotypes was mostly restricted to the protonemal and juvenile phases (Figs.

10-17; Supp. Figs. 1-3). In intermediate and mature shoots as well as bulbils there were little differences across ecotypes. As expected lengthened rate of drying resulted in greater health (Figs. 10-17). The effect seen across life phases is logically consistent with the life phase roles in the organism (Figs10-17). Protonema are responsible for lateral expansion through the soil and should be somewhat temporally buffered against rapid humidity fluctuations. Experimentally, protonema are the most sensitive life phase examined. Juvenile shoots are likewise very sensitive, and I expect samples are prioritizing growth over desiccation tolerance. Intermediate and mature shoots are both hardy as would be expected of nearly fully developed samples. Bulbils, which act as vegetative dispersal structures, are extremely hardy, displaying a constitutive strategy of desiccation tolerance which would be extremely valuable for a structure required to traverse a harsh xeric environment.

The results presented over these three chapters strongly support the hypothesis that an inducible physiological response to low osmotic pressure is capable of increasing survival during desiccation showing desiccation tolerance in some species to be a phenotypically plastic response. This capacity for desiccation tolerance is modulated by the rate of drying, ecotype, life history phase, and tissue type. Desiccation tolerance can be enhanced across the organism in response to previous environmental exposure to low water content. Ecological environmental interactions of future experiments involving bryophytes will be easier to interpret and explain using these results.

I do however fear that some species (those similar to *B. argentum* in population size, dispersal capacity and a wide habitat preference), with a high capacity for dispersal are poised to act as invasive species in the event of climate change. A world-wide spore rain combined with an ability to succeed in many habitat types, combined with rapid environmental shifts could prove

disastrous. Without a concerted effort to identify at risk populations and transport them to newly forming habitats many species and a large portion of biodiversity in ecotypes will be lost.

Implications and Significance of This Work

Our understanding of how water relationships impact desiccation tolerance in the moss clade is undergoing re-evaluation, and this work helps to re-define the properties of desiccation tolerance within the mosses. The traditional view considers mosses as existing in one of two discrete non-overlapping categories: desiccation tolerant, and, therefore, able to survive desiccation with minimal damage; or desiccation sensitive, for which desiccating conditions are lethal. The alternative view, which this research supports, considers desiccation tolerant and desiccation sensitive status not as discrete categories but descriptors of two ends of a gradient with many levels of desiccation hardiness between these two extremes. This work has shown species to have different inherent capabilities for desiccation tolerance that is modulated by environmental factors, with time spent at sub-turgor, ecotypic variation, current life history phase, tissue type, and (we expect) a host of other environmental factors (temperature, rain frequency, solar irradiation, etc.) interacting to determine desiccation tolerance.

Previous work has established that desiccation tolerance could be achieved in desiccation sensitive mosses through the addition of abscisic acid addition, and that slowly dried mosses produce abscisic acid. With this work a link has been made between the act of slow drying leading directly to a phenotype that can survive desiccation, the implicit assumption being that abscisic acid is produced during this slow dry in sufficient quantities to generate a desiccation tolerant phenotype (chapter 2). This work also gives empirical evidence showing that the dogma of desiccation tolerance or desiccation sensitive is not conclusive and there is a grey transition zone between these two concepts.

In chapter three it was shown that hardening from a desiccation sensitive to a desiccation tolerant phenotype can be induced by a slow dry. This allows a temporal separation between exposure and phenotypic induction and allows for the possibility that periodic drying or partial drying will result in hardier mosses under field conditions and a wider habitat range. This finding also re-enforces the importance of hardening for environmental restoration projects, suggesting that mosses when given a hardening exposure will have better survival odds than un-hardened when transplanted back into the field.

As chapters two and three showed errors in the dogmatic representation of the desiccation sensitive or desiccation tolerant dogma was flawed by showing the generation of a desiccation tolerant phenotype in a "desiccation sensitive" species chapter four does by highlighting the desiccation sensitive aspects of a moss considered to be constitutively desiccation tolerant. Both protonemal and juvenile shoots were shown to be desiccation sensitive or inducible desiccation tolerant, but not desiccation tolerant at all rates of drying. The degree of desiccation sensitivity displayed was found to be ameliorated by slower drying rates and therefore displayed a phenotypically plastic inducible response to a slow drying rate that allowed survival.

In addition to further challenging the accepted dogma of desiccation tolerance chapter four serves to provide a look in to desiccation tolerance over the course of an organisms life cycle (protonema, three shoot phases, and bulbils). We uncovered significant differences in response in every stage examined showing a considerable (and unexpected) ontogenetic plasticity for desiccation tolerance with some forms being very resistant to induction (at the time frames examined), protonema and other, while bulbils displayed a near perfect constitutive desiccation tolerance response. Ecotypic variation was also examined in chapter four, across 13 ecotypes

from the continental United States of America. A surprisingly variable capacity in phenotypic plasticity was found (especially for the protonemal and juvenile shoot phases).

Taken together the factors uncovered over the body of this dissertation stands as a strong reason to question much of what we currently believe in the nature of desiccation tolerance within mosses and the methodology commonly used to asses desiccation tolerance of mosses. With this in mind the work not only offers critiques of the current methods to study desiccation tolerance in bryophytes, but also offers guidelines by which future efforts may be improved.

The major criticisms of the current common methods used to study desiccation tolerance can be summarized in three statements. First, the drying regimes used are for the most part performed far too quickly, especially for species currently categorized as desiccation sensitive. Second, the near exclusive use of adult shoots in determining the desiccation tolerance status for a species ignores the complexity present across an organism's life cycle. Third, the use of field collected specimens for determination of desiccation tolerance ignores the effects of field hardening. Fourth, using collections from a single location (one ecotype) to determine the desiccation tolerance capacity for an entire species ignores the variation found within individuals of the species.

Solving these concerns should be a relatively straight forward prospect, but will require overcoming significant institutional inertia. First, to solve the concerns over drying rate two parts of the traditional method should be amended. Salt solutions have long been used to control equilibrating relative humidity, however this intrinsically links drying rate to humidity. The solution would be either to adopt a saturated artificial substrate as used across chapters 2-4 of this work, or using a series of saturated salt solutions in a step-down method to control drying rate. The second part of amending drying rates will consist of choosing drying rates that reflect what

the particular species under study can be expected to receive in its normal range and act as natural upper and lower bounds to define the slow and rapid drying rates chosen.

Second to uncover the importance of life cycle stage in determination of desiccation tolerance status one should cultivate materials under laboratory conditions to gather enough material at all life cycle stages for examination. This suggestion would have the added side benefit of controlling for our third concern that of field hardened specimens used in testing. Cultivation adds considerable time input for studies to measure and test mosses, however the accurate determination of results should be valued as more important than expediency.

Fourth, the testing of multiple ecotypes for any species considered is necessary to confidently begin to quantify the capacity of a species. The importance of this is highlighted in the fourth chapter of this work. In only 13 ecotypes examined in chapter four we observed a wide display of phenotypic plasticity for the trait. If for this study we had restricted ourselves to only a single ecotype we could have concluded that the species possessed either a nearly constitutively desiccation tolerant phenotype, or in choosing another ecotype concluded that the species lacked a desiccation tolerant phenotype whatsoever in the protonemal or juvenile shoot phases. Obviously either of these conclusions would have been erroneous, and serve to highlight the potential pitfalls from making species descriptions from single ecotype observations.

Global climate change can be expected to lead to dynamic shifts in population structures as regions of earth transition to alternative climate regimes. This work increases our understanding of many factors (inducible desiccation tolerance, hardening, ecotypic and tissue variation in desiccation tolerance), enhancing our ability to make reasonable predictions for how organisms will respond to climate change. Another essential aspect for predicting of survival in a changing climate depends upon knowing if a species has sufficient genetic variation for selection

to act upon (Ghalambor et al. 2007; Matesanz et al. 2010; Shaw & Etterson 2012). Highly

variable species will possess a greater range of traits for selection to act upon, increasing the

likelihood that some will be sufficiently adaptive in the new climates allowing survival. If current

variation is not present at high levels an alternative method to overcome selective pressures and

survive climate change would be high levels of phenotypic plasticity for desiccation tolerance,

effectively acting as a buffer against selection and allowing a wider range of the existing

genotypes to persist until sexual recombination can provide a more competitive combinations of

alleles and chromosomes (Ghalambor et al. 2007; Matesanz et al. 2010; Shaw & Etterson 2012).

Although this work has uncovered a great deal of variation in desiccation tolerance and

phenotypic plasticity for this trait (for some ecotypes) this could be counter balanced by the low

rates of sexual reproduction and low occurrence of male sex expression in xeric habitats. The

effects of clonal propagation combined with a strong selective pressure for desiccation tolerant

phenotypes will likely lead to a loss of genetic variability over the short term in xeric habitats as

hardier forms expand into locations previously occupied by less desiccation tolerant forms. The

wide cosmopolitan distribution of *Bryum argentum*, as well as its high capacity for long distance

spore dispersal, should, over longer time frames re-introduce genetic variation to xeric habitats.

However many other xeric species with restricted and or patchy distribution combined with

potentially lower capacity for spore dispersal would have a less optimistic long term outlook as

they would not have a large source of genetic material preserved in other habitats to re-introduce

into the xeric localities.

Future Goals

The most obvious extension of this work that I feel is most ripe for future research

concerns the inherent differential capacity for desiccation tolerance of male and female mosses

and would in effect be an extension of the third chapter. Females are known to greatly outnumber males in xeric habitats, although the reasons have never been fully explained. One pattern I noticed (although statistically untenable due to the design parameters of the third chapter) was that males tended to have more desiccation sensitive protonemal tissue than females. At the time of this writing I am currently (as part of another project) gaining access to a large number of ecotypes (of *B. argenteum*) from across the continental united states. With this increased sample size I may be able to determine if the patterns I saw in my work hold true across a larger sample set.

Appendix

Bryum argenteum Ecotypes

AZ Male and Female

 Pima County, Santa Rita Mts, Madera Canyon, Arizona. near Santa Rita Lodge, Stark and Castetter 17 March 2008.

CA Male and Female

 Fresno County, Sierra Nevada Mts, San Joaquin River Gorge, California, Stark s.n., 2008.

KY Male and Female

 Fayette County, Lexington, Kentucky, campus of University of Kentucky, asphalt between greenhouses, Stark and McLetchie s.n., 12 October 2007.

MA Male and Female

 Suffolk County, Urban parking lot in Boston, Massachusetts, Duncan Souza s.n., 2008.

NM Female

 Sandoval County, Sandia Mts, Las Huertas Campground, New Mexico, near latrine, Stark and Castetter s.n., 7 April 2009, elev. 7700 ft, 35° 14.127 N 106° 24.779 W.

OR Male and Female

 Multnomah County, Portland, OR, sidewalk along the Willamette River, Oregon, Stark and Eppley s.n., 15 May 2009.

CA Female

 San Bernardino County, Cima Dome, California, Stark s.n., 8 January 2009.

NV Female

 Clark County, Valley of Fire State Park, Nevada, Stark and Bonine NV-3100, 1999.

s.n. = sans number (no collection number)

Tables

Table 1. Leaf Damage and Regeneration of *Physcomitrella patens* Exposed to Four Rates of Drying and two Relative Humidities.

Leaf damage and regeneration time in shoots of *Physcomitrella patens* exposed to four rates of drying and two equilibrating relative humidities (RH). N.A = not applicable as no regeneration occurred.

Treatment	Day 7 Green Leaves (%)	Day 7 Partially Green Leaves (%)	Day 7 Dead/Brown Leaves (%)	Days to first Protonema	Days to first Shoot
Unstressed Control	83.1 ± 2.4	11.3 ± 1.2	5.6 ± 0.9	7.5 ± 0.3	9.2 ± 0.5
284 h to 50% RH	52.9 ± 4.1	18.2 ± 3.5	28.9 ± 6.6	4.2 ± 0.2	4.8 ± 0.3
116 h to 50% RH	22.1 ± 7.6	22.7 ± 5.4	55.2 ±4.1	4.8 ± 0.4	6 ± 0.44
65 h to 50% RH	18.2 ± 8.6	13 ± 2.8	68.8 ± 3.3	5.7 ± 0.5	7.4 ± 0.5
RD to 50% RH	0	0	100	N.A.	N.A.
284 h to 30% RH	41.1 ± 4.3	30.0 ± 2.1	29.0 ± 3.3	4.6 ± 0.2	5.9 ± 0.4
116 h to 30% RH	13.9 ± 2.7	24.1 ± 2.8	62.0 ±3.7	5.1± 0.4	6.0 ± 0.5
65 h to 30% RH	2.3 ± 0.9	11.5 ± 1.9	86.2 ± 3.2	7.2 ± 0.5	10.2 ± 0.7
RD to 30% RH	0	0	100	N.A.	N.A.

Table 2. Single Factor ANOVA for Fluorescence and Re-growth of *Physcomitrella patens.*

Single factor ANOVA with a post hoc Tukey test for experimental treatments. N value = 5 for fluorescence comparisons (*Fv/F*m, *F*m, Φ_{PSII}), and N = 74 for regrowth comparisons (green Leaves, partially green leaves, dead/brown leaves).

	50% RH 24 h post rehydration *F*v/*F*m				30% RH 24 h post rehydration *F*v/*F*m			
	Rapid Dry				Rapid Dry			
Control	1.5×10^{-11}	Control			1×10^{-12}	Control		
65 h	9.5×10^{-8}	0.00005	65 h		3×10^{-7}	2×10^{-9}	65 h	
116 h	1.2×10^{-9}	0.022	0.087	116 h	2×10^{-10}	0.0000005	0.001	116 h
284 h	3.8×10^{-10}	0.116	0.016	0.923	8×10^{-12}	0.0018	0.0000005	0.1
	50% RH 24 h post rehydration PSII				30% RH 24 h post rehydration PSII			
	Rapid Dry				Rapid Dry			
Control	1×10^{-12}	Control			1×10^{-12}	Control		
65 h	3×10^{-8}	3×10^{-8}	65 h		.0004	3×10^{-11}	65 h	
116 h	2×10^{-10}	0.00001	0.24	116 h	1×10^{-8}	8×10^{-8}	0.0002	116 h
284 h	2×10^{-11}	0.001	.0004	0.4399	1×10^{-9}	0.0000001	0.00001	0.641
	50% RH 24 h post rehydration *F*m				30% RH 24 h post rehydration *F*m			
	Rapid Dry				Rapid Dry			
Control	4.8×10^{-12}	Control			4×10^{-12}	Control		
65 h	0.658	2×10^{-11}	65 h		0.954	8×10^{-12}	65 h	
116 h	0.011	4.2×10^{-10}	0.168	116 h	0.18	5×10^{-11}	0.477	116 h
284 h	.00005	1.4×10^{-8}	0.001	0.178	0.004	7×10^{-10}	0.015	0.355
	50% RH 7 d post planting green leaves				30% RH 7 d post planting green leaves			
	Rapid Dry				Rapid Dry			

Control	4.9×10^{-13}	Control			4.9×10^{-13}	Control		
65 h	0.01025	4.9×10^{-13}	65 h		0.986	4.9×10^{-13}	65 h	
116 h	0.00104	4.9×10^{-13}	0.9688	116 h	0.021	4.9×10^{-13}	0.0864	116 h
284 h	4.9×10^{-13}	4.9×10^{-13}	4.9×10^{-13}	4.9×10^{-13}	4.9×10^{-13}	4.9×10^{-13}	4.9×10^{-13}	4.9×10^{-13}

50% RH 7 d post planting partially green leaves					30% RH 7 d post planting partially green leaves			
	Rapid Dry				Rapid Dry			
Control	1.7×10^{-7}	Control			0.0000028	Control		
65 h	0.000072	0.73895	65 h		0.0009398	0.6972	65 h	
116 h	9.6×10^{-13}	0.23038	0.00924	116 h	6×10^{-13}	0.04364	0.00052	116 h
284 h	5×10^{-13}	0.03538	0.00051	0.93493	4.9×10^{-13}	5×10^{-13}	4.9×10^{-13}	1.8×10^{-5}

50% RH 7 d post planting dead/brown leaves					30% RH 7 d post planting dead/brown leaves			
	Rapid Dry				Rapid Dry			
Control	4.9×10^{-13}	Control			4.9×10^{-13}	Control		
65 h	5.5×10^{-9}	4.9×10^{-13}	65 h		0.98275	4.9×10^{-13}	65 h	
116 h	4.9×10^{-13}	4.9×10^{-13}	0.03111	116 h	6.2×10^{-9}	4.9×10^{-13}	1.3×10^{-7}	116 h
284 h	4.9×10^{-13}	8.7×10^{-9}	3.9×10^{-8}	0.01659	4.9×10^{-13}	4.6×10^{-10}	4.9×10^{-13}	0.000004

50% RH days until protonemal emergence				30% RH days until protonemal emergence		
	Control				Control	
65 h	0.00041	65 h		0.92025	65 h	
116 h	4.1×10^{-7}	0.48131	116 h	0.0000056	0.00031	116 h
284 h	2.4×10^{-11}	0.021335	0.492066	1.3×10^{-8}	0.0000026	0.72621

50% RH 7 d post planting dead/brown leaves			30% RH 7 d post planting dead/brown leaves	
	Control			Control

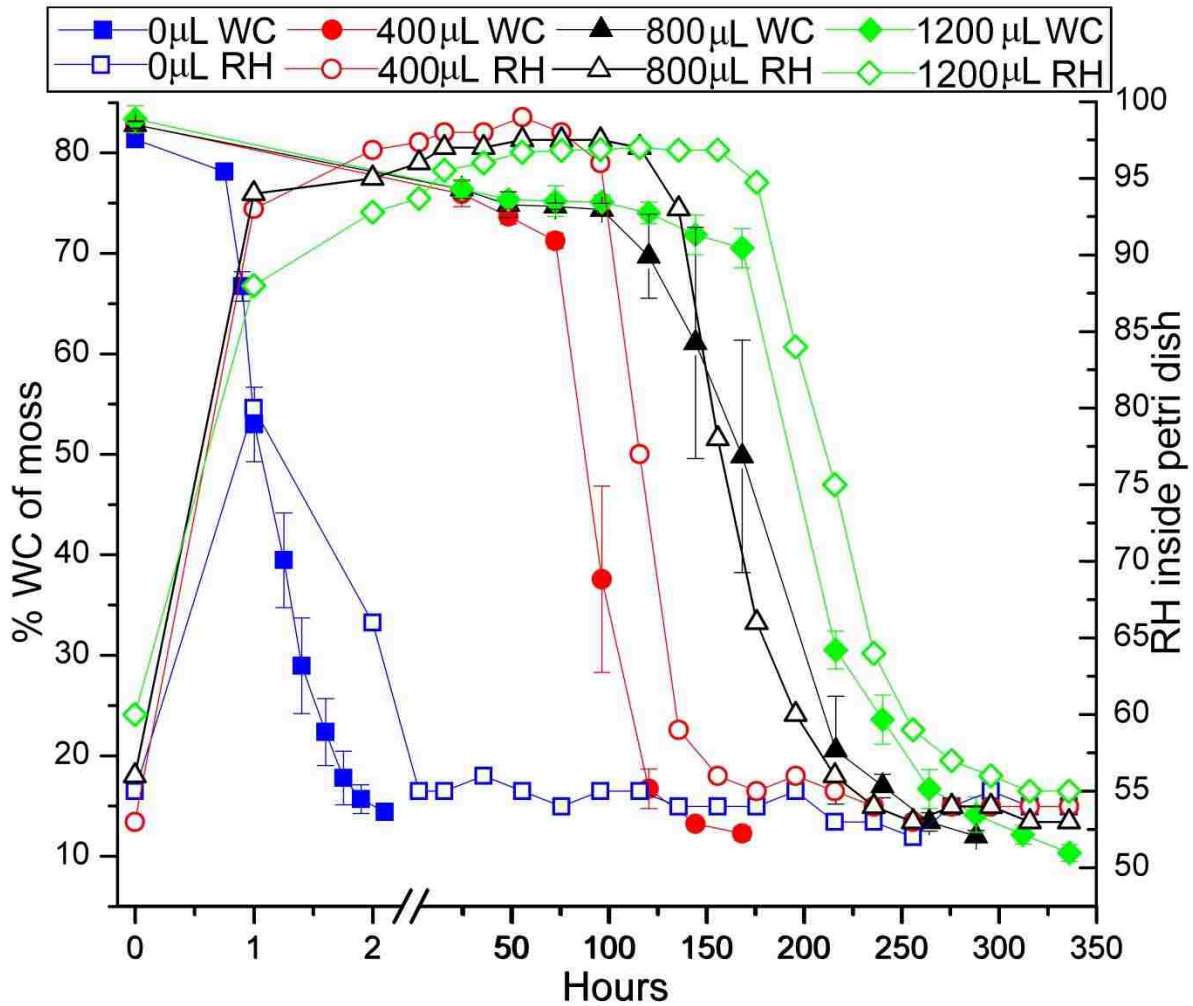|						
65 h	0.00584	65 h		0.51	65 h	
116 h	0.0000023	0.29062	116 h	0.000044	0.0000018	116 h
284 h	1.8x10-12	0.00062	.16079	0.0000054	$2.5x10^{-7}$	0.99844

Figures

Figure 1. Drying Curve Plotting Water Content Against Relative Humidity and Time.

Drying curve showing the relationship between water content of the mosses and relative humidity inside the petri dish and how both of these factors were dependent upon the amount of water initially added to the filter paper with the petri dishes.
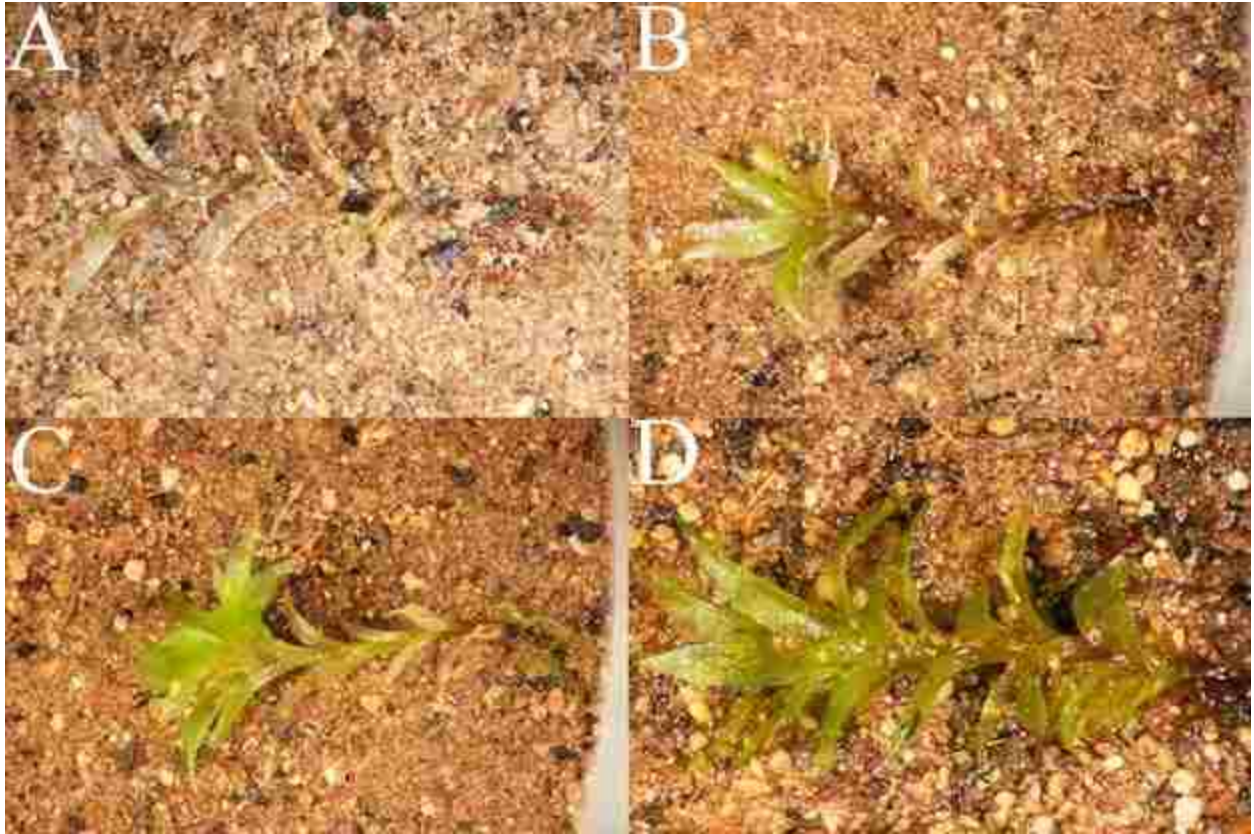
Figure 2. Representative Shoots of Physcomitrella patens Seven days Post Rehydration.

Representative shoots of *Physcomitrella patens* Seven days post rehydration for the 4 treatments; A = rapid dry, B= 65 h dry, C = 116 h dry, D = 284 h dry.
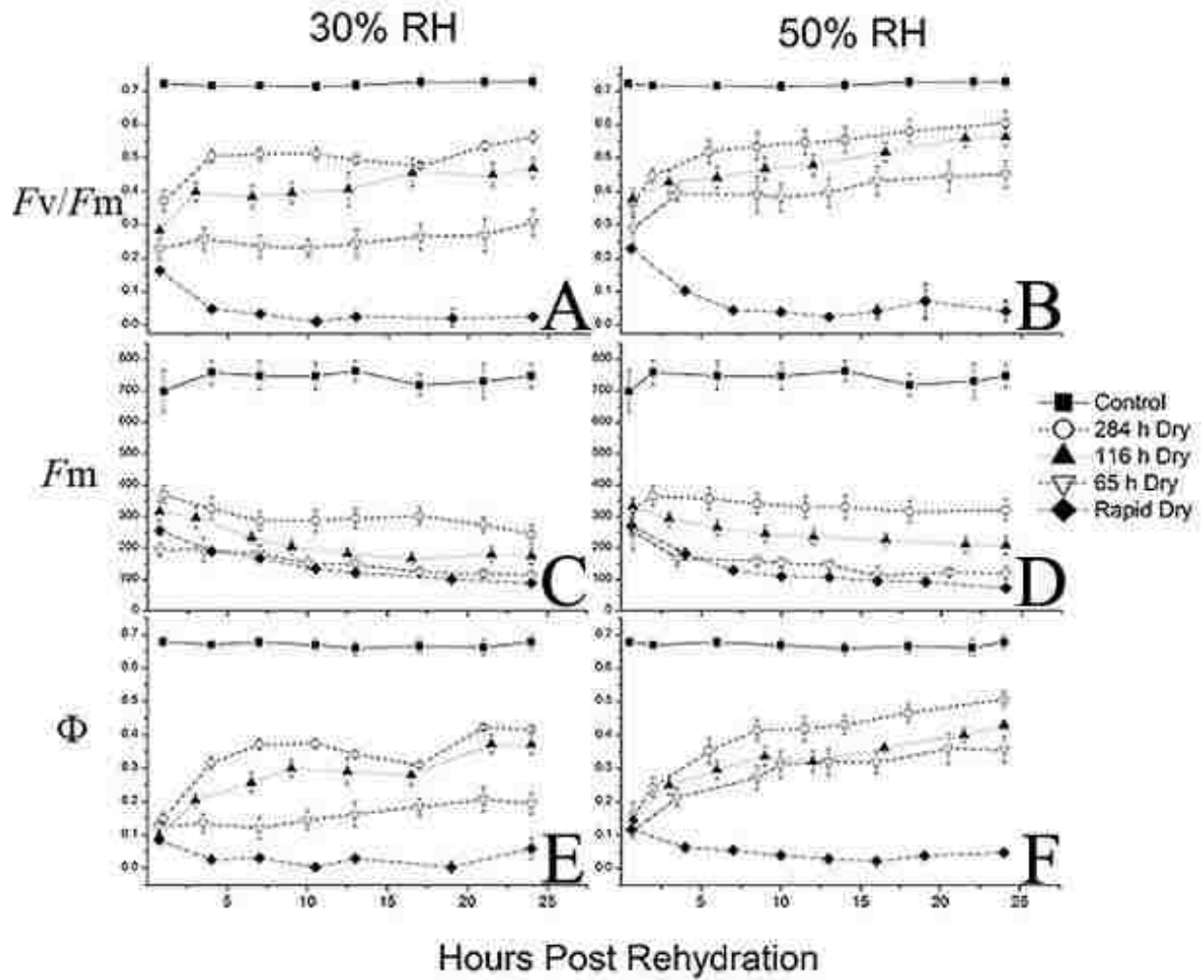
Figure 3. Chlorophyll Fluorescence Parameters Over a 24-h Period Following Re-hydration of Physcomitrella patens.

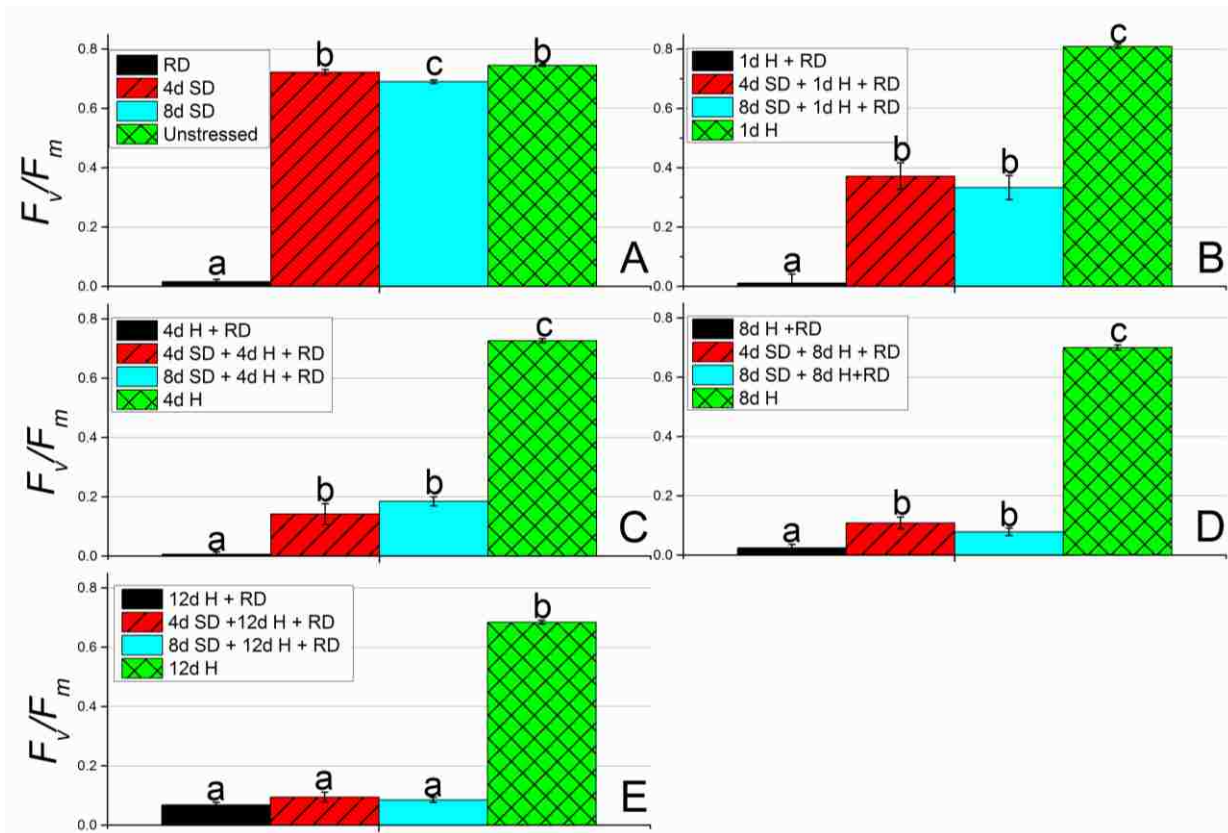Chlorophyll fluorescence parameters over a 24-h period following re-hydration of *Physcomitrella patens*.

Figure 4. Physcomitrella patens F_v/F_m Values 24-h Post Re-hydration Showing Hardening and De-hardening.

Physcomitrella patens F_v/F_m values 24-h post re-hydration, with treatments consisting of a rapid-dry, a 4 or 8 day slow-dry pre-treatment to harden samples, or unstressed all followed by either direct measurement (A) or a 1 (B), 4 (C), 8 (D), or 12 (E) day hydrated de-hardening period followed by a rapid-dry. Values show a protective effect conferred by the initial slow dry (hardening period) lasting 8 d (B,C,D) with protective effects not detectable by 12 d (E). N=15 for all treatments, H=hydrated ,RD=rapid-drying event (<30 m), SD=slow-drying event (4 d or 8 d).
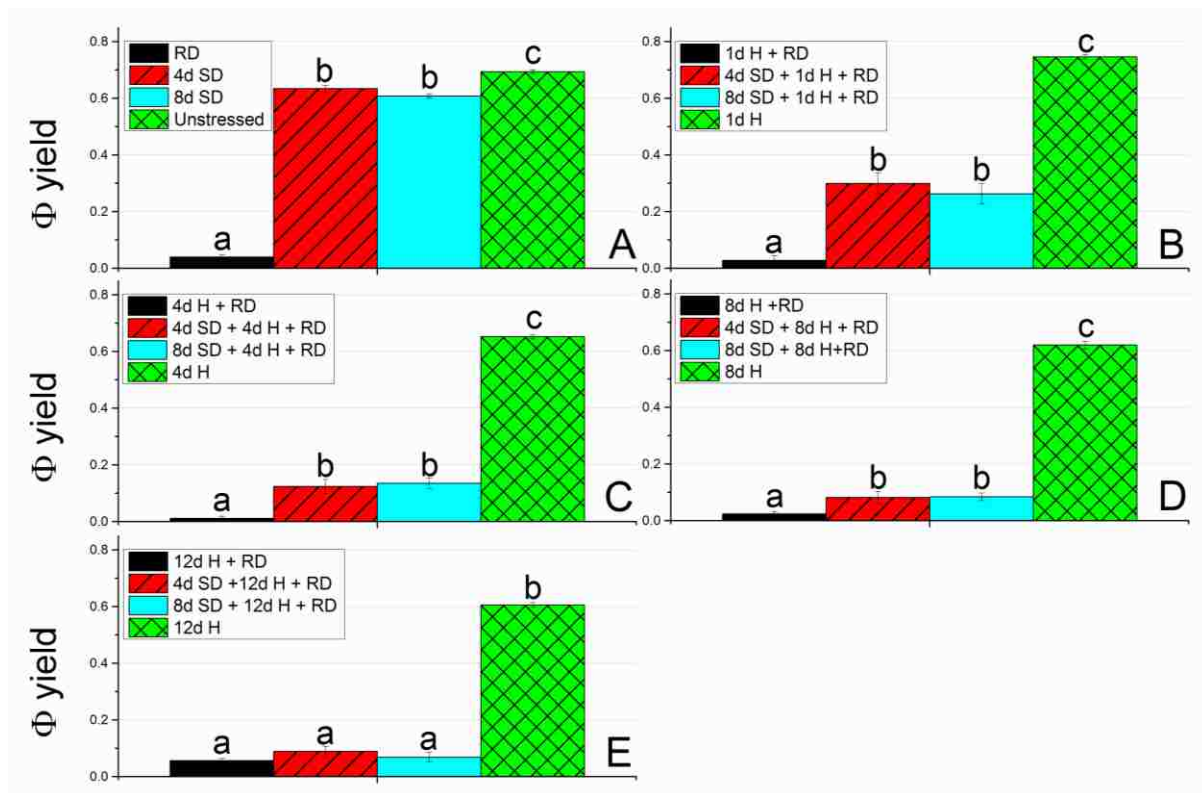
Figure 5. Physcomitrella patens ΦPSII Values 24-h Post Re-hydration Showing Hardening and De-hardening.

Physcomitrella patens ΦPSII values 24 h postrehydration, with treatments consisting of a rapid-dry, a 4 or 8 day slow-dry pre-treatment to harden samples, or unstressed controls all followed by either direct measurement (A) or a 1 (B), 4 (C), 8 (D), or 12 (E) day hydrated de-hardening period followed by a rapid-dry. Values show a protective effect conferred by the initial slow-dry (hardening period) lasting 8 d (B,C,D) with protective effects not detectable by 12 d (E). N=15 for all treatments, H=hydrated, RD=rapid-drying event (<30 min), SD=slow-drying event (4 d or 8 d).
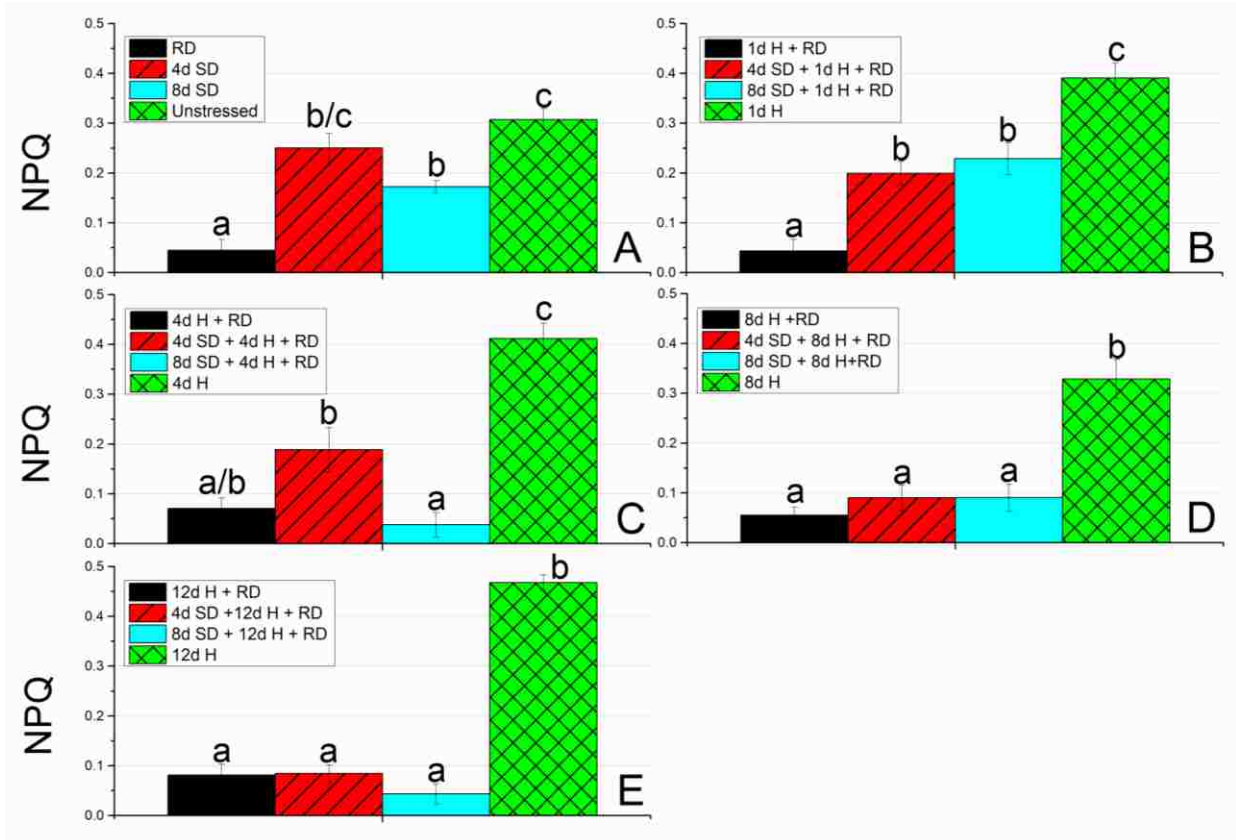
Figure 6. Physcomitrella patens NPQ Values 24-h Post Re-hydration Showing Hardening and De-hardening.

Physcomitrella patens NPQ (non-photochemical quenching) values 24-h postrehydration, with treatments consisting of a rapid-dry, a 4 or 8 day slow-dry pre-treatment to harden samples, or unstressed controls all followed by either direct measurement (A) or a 1 (B), 4 (C), 8 (D), or 12 (E) day hydrated de-hardening period followed by a rapid-dry. Values show a protective effect conferred by the initial slow-dry (hardening period) lasting 8 d (B,C,D) with protective effects not detectable by 12 d (E). N=15 for all treatments, H=hydrated; RD=rapid-drying event (<30 min), SD=slow-drying event (4 d or 8 d).
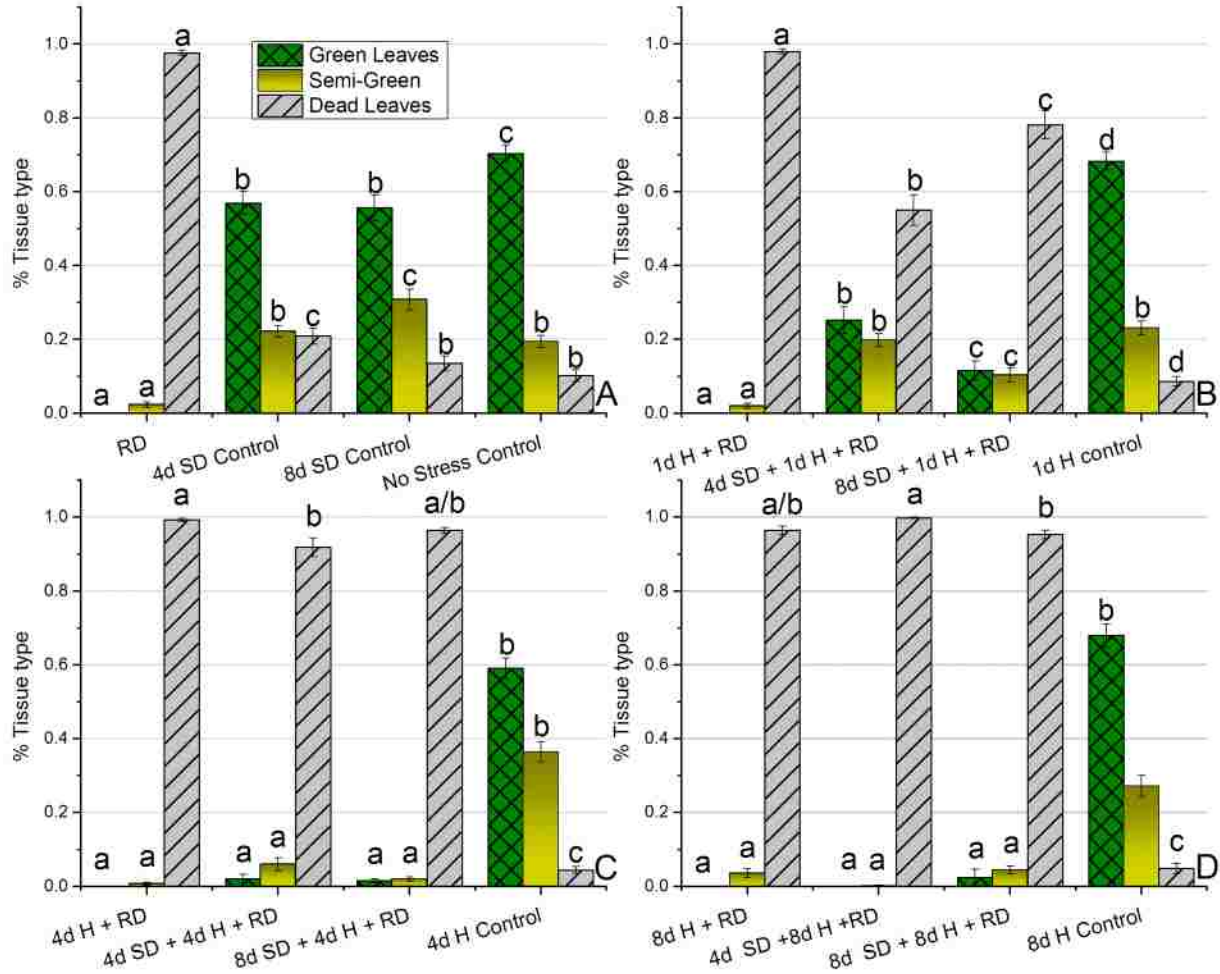
Figure 7. Physcomitrella patens Leaf Damage by Treatment Observed Seven Days Post Re-hydration.
Physcomitrella patens leaf damage by treatment observed seven days post rehydration, control values are shown in A, with treatments consisting of a 4/8 day slow-dry hardening period followed by a 1/4/8 (B, C, D) day hydrated de-hardening period. Protective effects were observed after a 1 d de-hardening period (B) but were lost by 4 d (C). N=45 for all treatments, H=hydrated; RD=rapid-dry event (<30 min), SD=slow-drying event (4 d or 8 d).
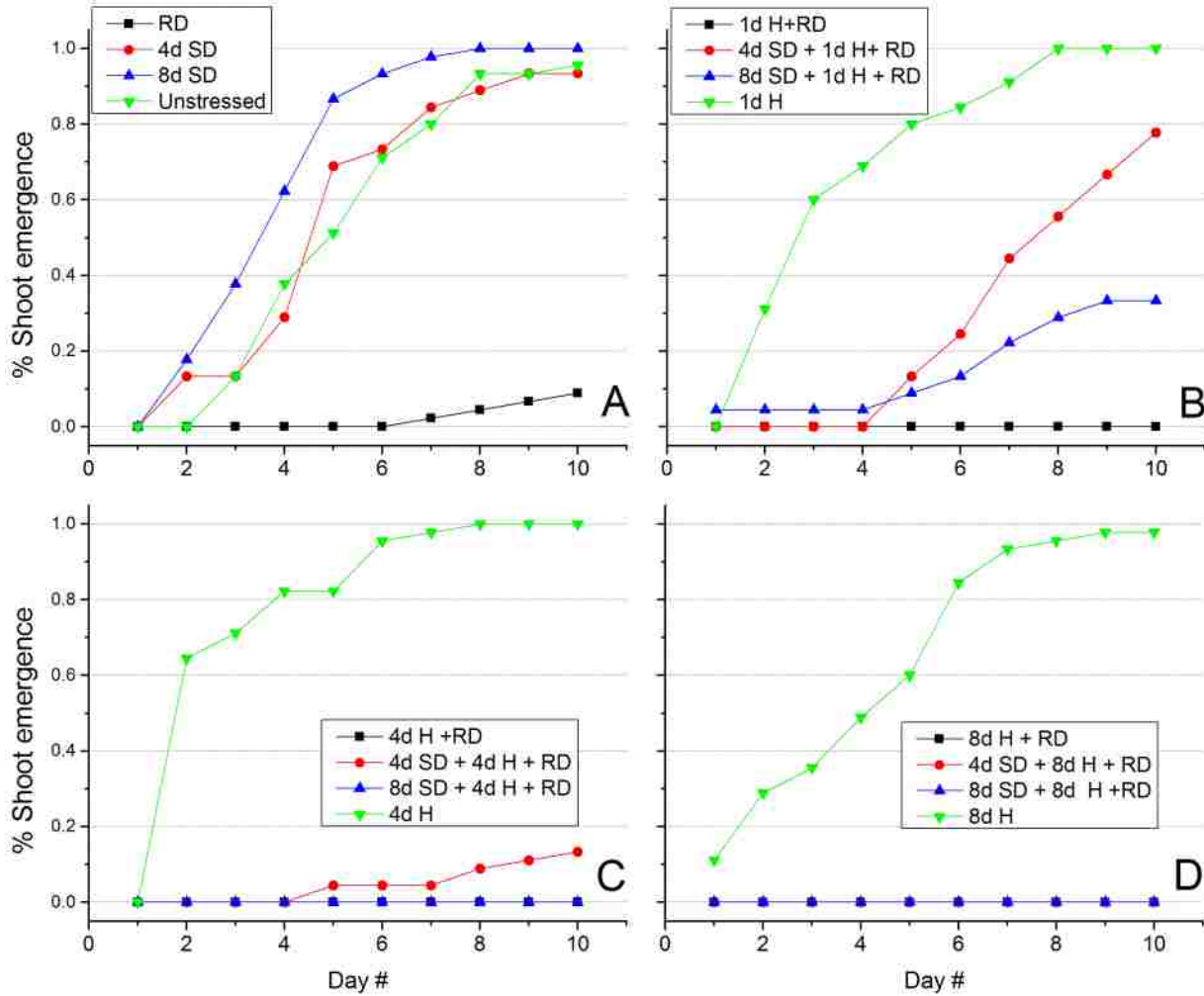
Figure 8. Physcomitrella patens Shoot Emergence Over 10 Days Following Re-hydration.

Physcomitrella patens shoot emergence over 10 days following rehydration, control values are shown in A, with treatments consisting of a 4/8 day slow-dry hardening period followed by a 1/4/8 (B, C, D) day hydrated de-hardening period. Protective effects were observed after a 1 d de-hardening period (B) but were diminished in a 4 d de-hardening period (C) and lost entirely for samples with an 8 d de-hardening period (D). N=45 for all treatments, H=hydrated, RD=rapid-dry event (<30 min), SD=slow-dry event (4 d or 8 d).
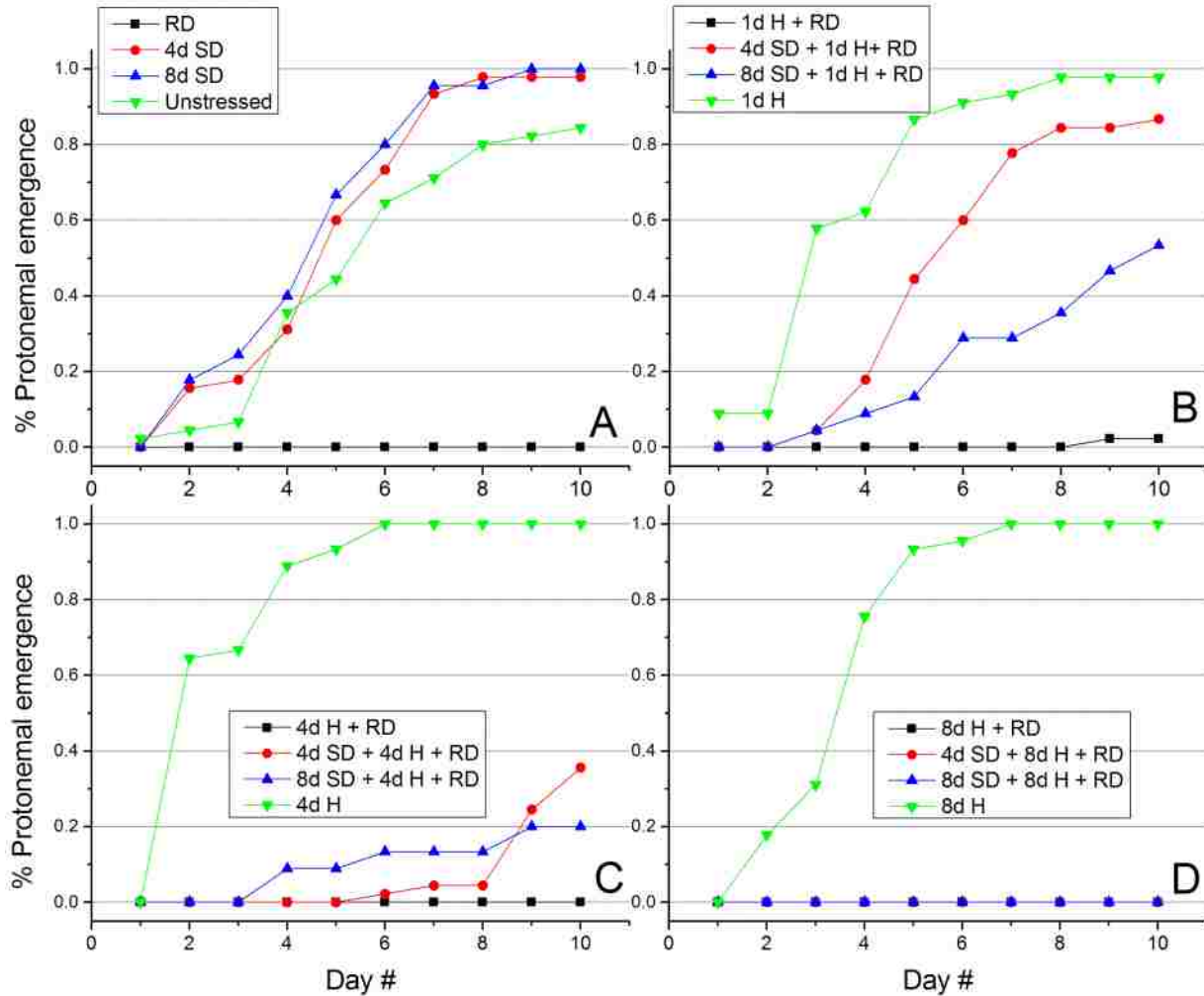
Figure 9. Physcomitrella patens Protonemal Emergence Over 10 Days Following Re-hydration.

Physcomitrella patens protonemal emergence over 10 days following rehydration, control values are shown in A, with treatments consisting of a 4/8 day slow-dry hardening period followed by a 1/4/8 (B, C, D) day hydrated de-hardening period. Protective effects were observed after a 1 d de-hardening period (B) but were lost by 4 d (C) for samples with an 8 d hardening period, and lost entirely with an 8 d de-hardening period (D). N=45 for all treatments, H=hydrated, RD=rapid-dry event (<30 min), SD=slow-dry event (4 d or 8 d).
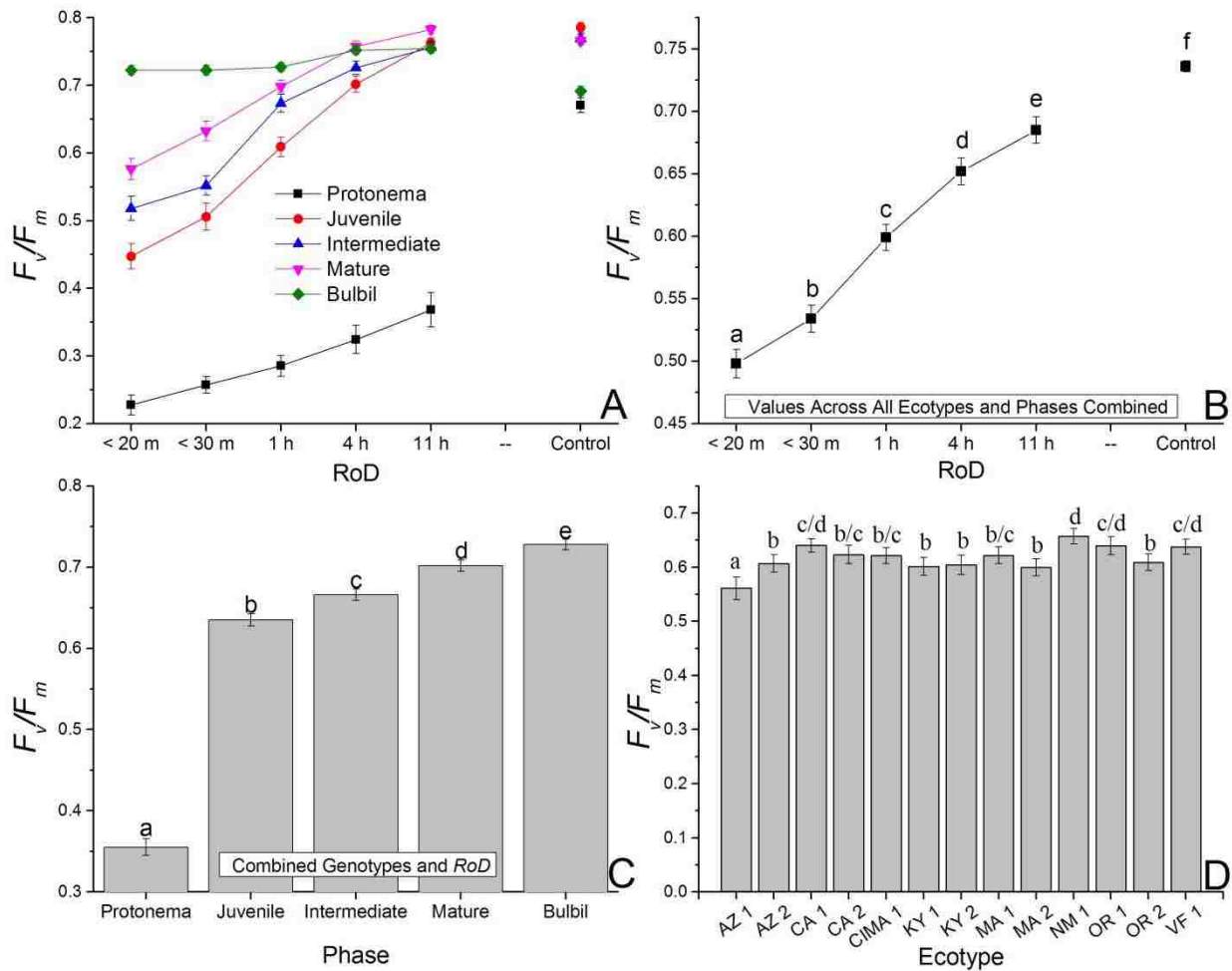
Figure 10. Bryum argenteum F_v/F_m Response Variables.

Panel of images showing *Bryum argenteum F_v/F_m* response variables for the treatment combinations indicated, lowercase letters designate homogeneous subsets within each sub-graph, error bars represent one standard error, and lowercase letters represent homogeneous subsets established at an α of 0.05. A The effect RoD has upon the five life history phases with ecotypes combined (n = 65), B the effect of RoD with all life phases and ecotypes combined (n = 325), C the effect of life history phase with RoD treatments and ecotypes combined (n = 390), D The effect of ecotype with all life phases and RoD treatments combined (n = 150).

Figure 11. Bryum argenteum ɸPSII Response variables.

Panel of images showing *Bryum argenteum* ɸPSII response variables for the treatment combinations indicated; lowercase letters designate homogeneous subsets within each sub-graph, error bars represent one standard error, and lowercase letters represent homogeneous subsets established at an α of 0.05. A The effect RoD has upon the five life history phases with ecotypes combined (n = 65), B the effect of RoD with all life phases and ecotypes combined (n = 325), C the effect of life history phase with RoD treatments and ecotypes combined (n = 390), D The effect of ecotype with all life phases and RoD treatments combined (n = 150).
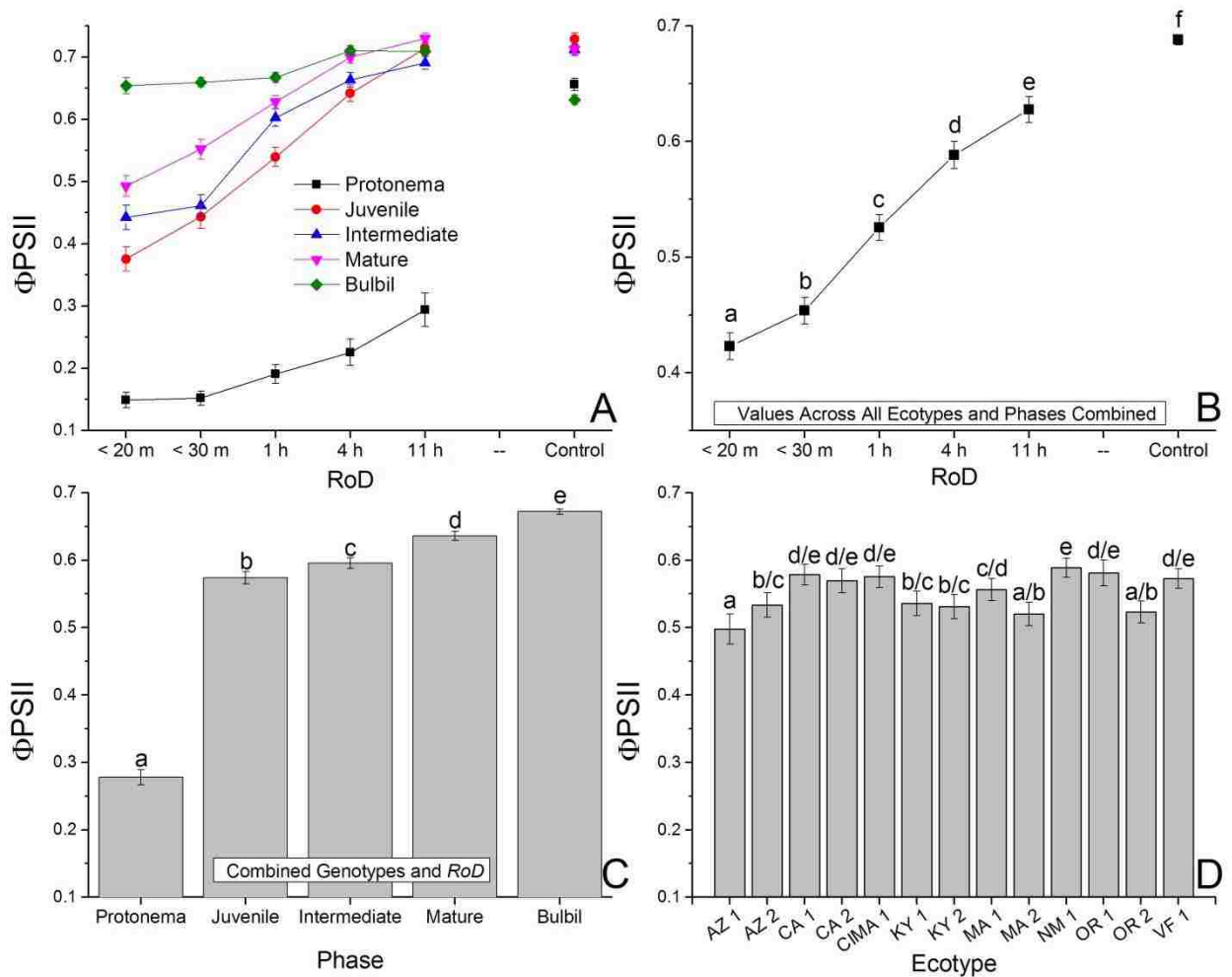
Figure 12. Bryum argenteum NPQ Response Variables.

Panel of images showing *Bryum argenteum* NPQ response variables for the treatment combinations indicated, lowercase letters designate homogeneous subsets within each sub-graph, error bars represent standard error, and lowercase letters represent homogeneous subsets established at an α of 0.05. A The effect RoD has upon the five life history phases with ecotypes combined (n = 65), B the effect of RoD all other factors combined (n = 325), C the effect of life history phase (n = 390), D The effect of ecotype all other factors combined (n = 150.

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Figure 13. Reaction Norms for Protonema From 13 Ecotypes Exposed to Five Rates of Drying.

Reaction norms for protonema from 13 ecotypes exposed to five rates of drying.

Figure 14. Reaction Norms for Juvenile Shoots From 13 Ecotypes Exposed to Five Rates of Drying.

Reaction norms for juvenile shoots from 13 ecotypes exposed to five rates of drying.

Figure 15. Reaction Norms for Intermediate Shoots From 13 Ecotypes Exposed to Five Rates of Drying.

Reaction norms for intermediate shoots from 13 ecotypes exposed to five rates of drying.

Figure 16. Reaction Norms for Adult Shoots From 13 Ecotypes Exposed to Five Rates of Drying.

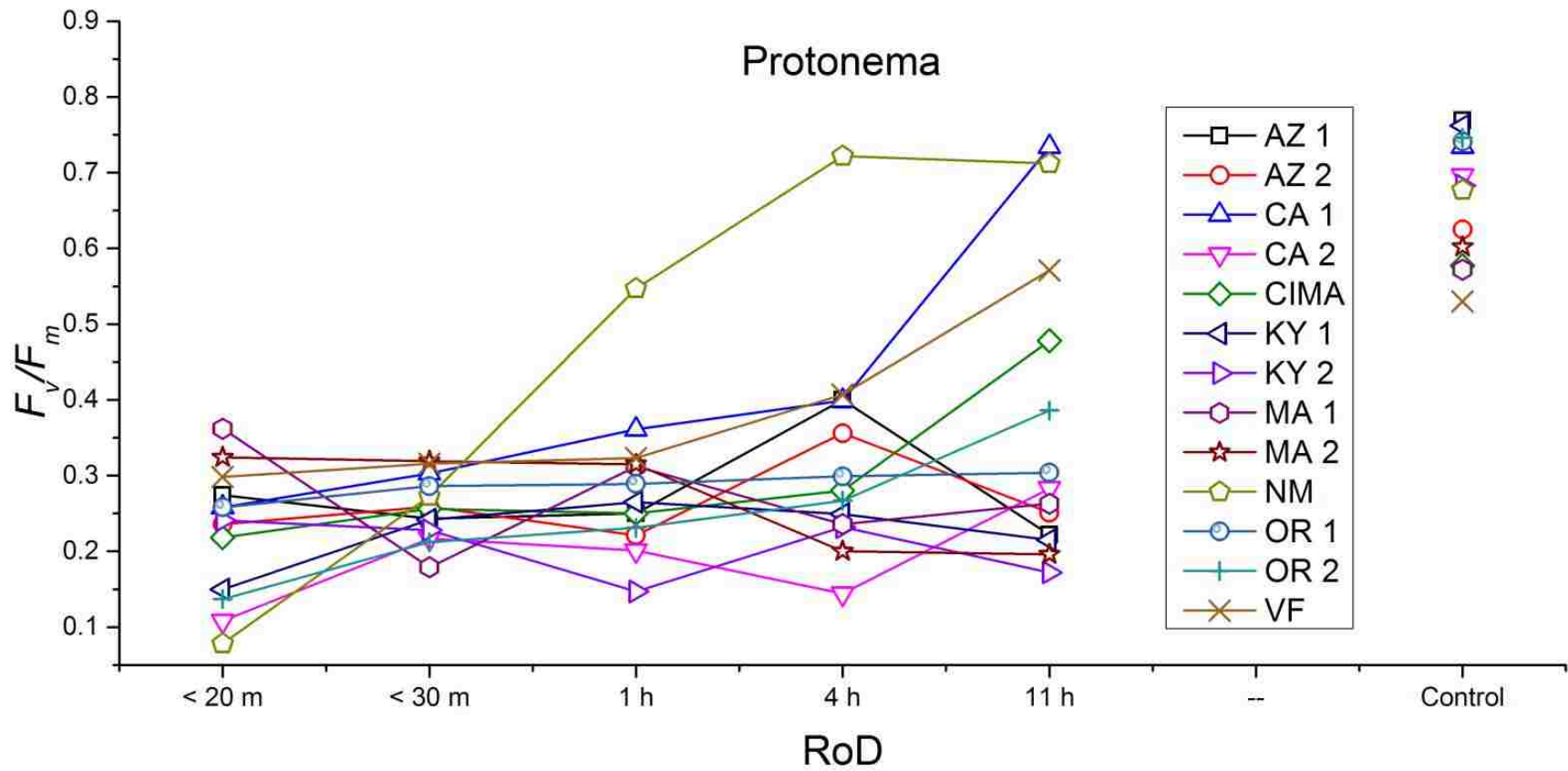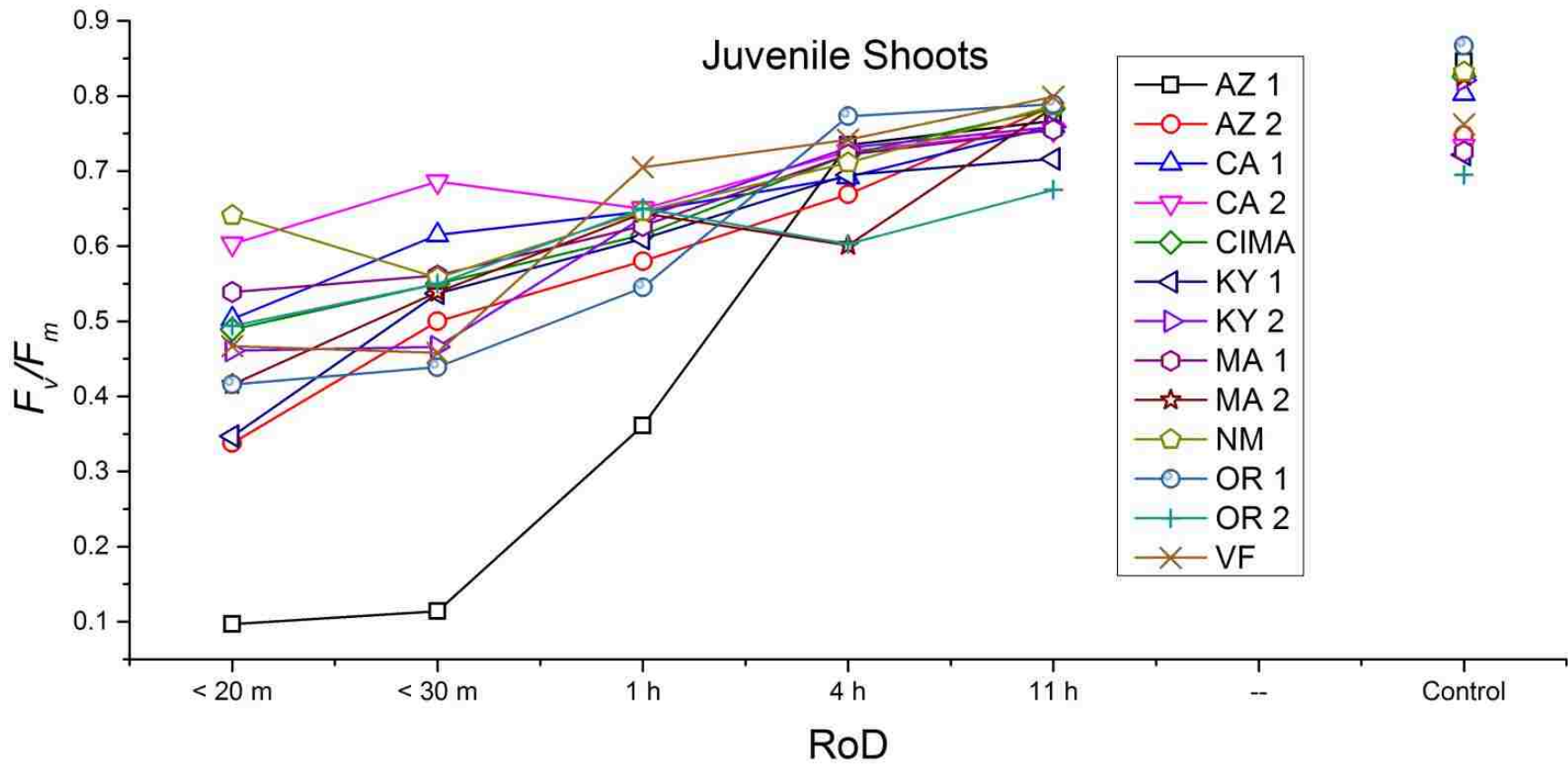Reaction norms for adult shoots from 13 ecotypes exposed to five rates of drying.

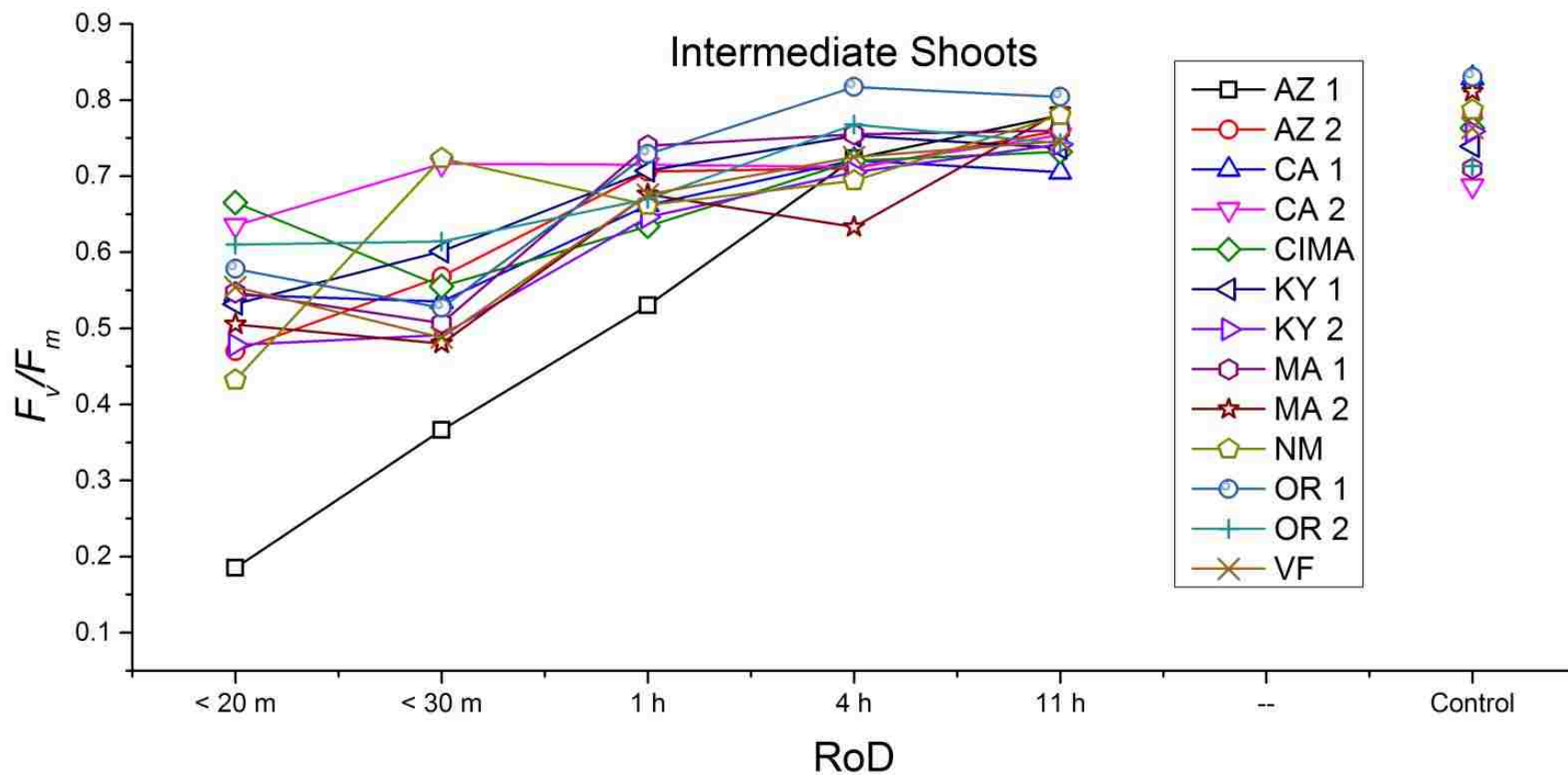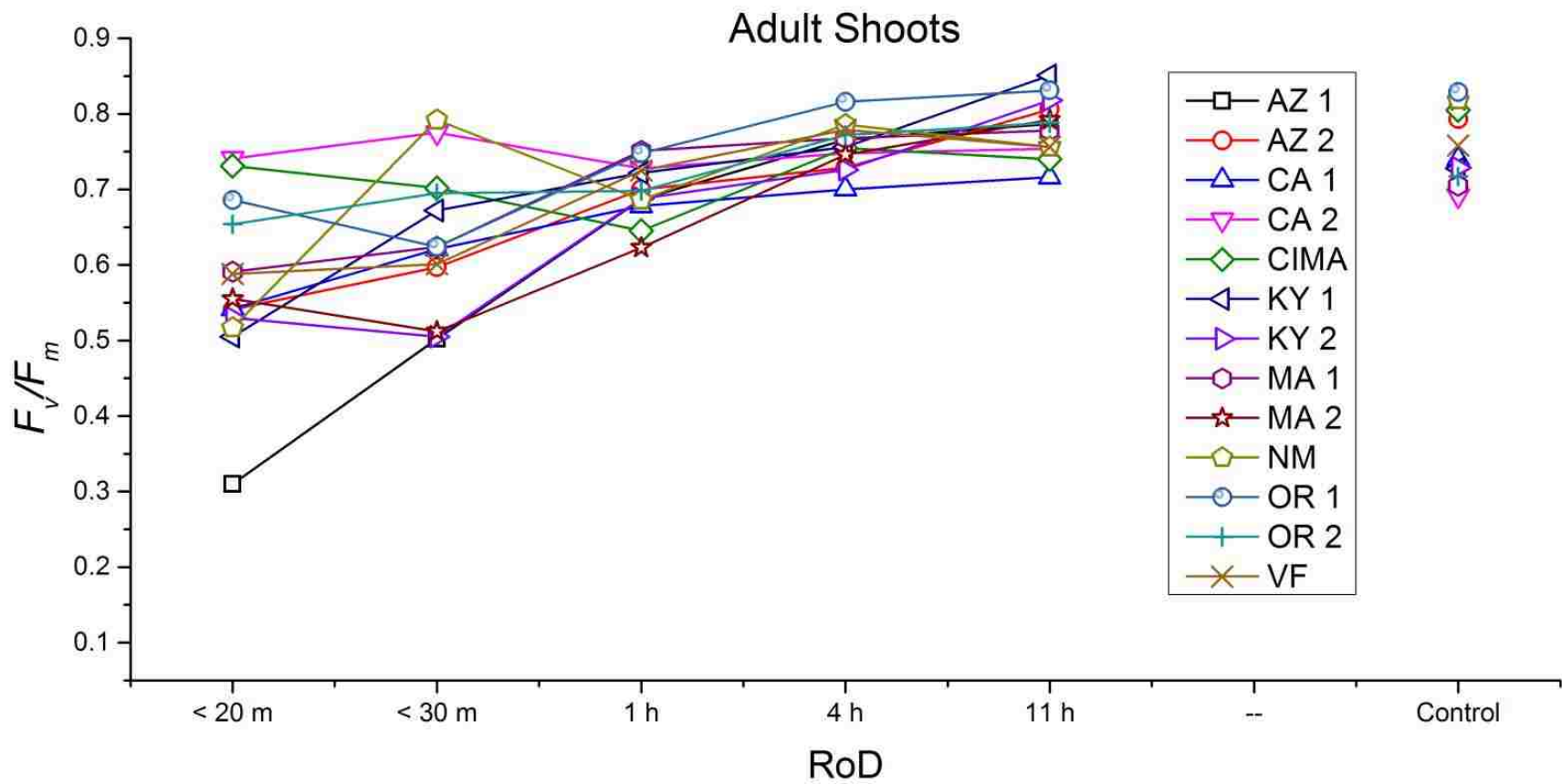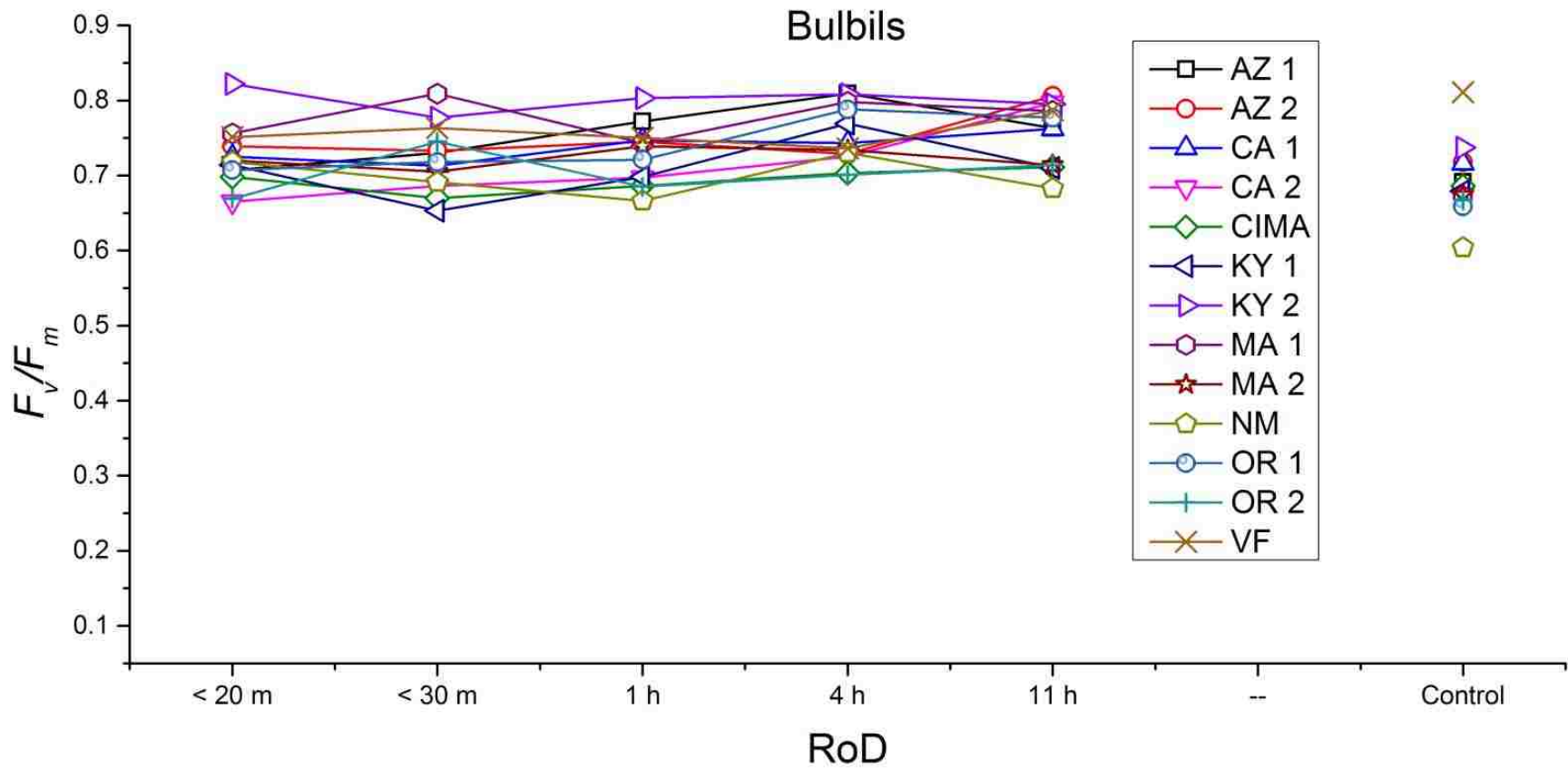*Figure 17. Reaction Norms for Bulbils From 13 Ecotypes Exposed to Five Rates of Drying.*

Reaction norms for bulbils from 13 ecotypes exposed to five rates of drying.

Supplemental Materials

Supplemental Table 1. Kaplan Meier Estimations for Protonema and Shoots of *Physcomitrella patens*, and Chlorophyllous Leaf Percentages.

Kaplan Meier estimations are presented for both protonema and shoots of *Physcomitrella patens*, as well as percentages of leaves that fall into green, partially green, and dead leaves. N=45 for all treatments, H=hydrated, RD=rapid-dry event (<30 min), SD=slow-dry event (4 d or 8 d)

Treatment	Kaplan Meier Estimations				Chllorophyllous Tissue Retention					
	Regrowth									
	Protonema		Shoots		Green leaves		Partially green		Dead leaves	
	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
RD control	10.00	0.00	9.87	0.08	0.00	0.00	0.02	0.01	0.98	0.01
4d SD control	5.13	0.28	5.36	0.32	0.57	0.03	0.22	0.01	0.21	0.02
8d SD Control	4.80	0.28	4.04	0.22	0.56	0.03	0.31	0.03	0.13	0.02
Control No Stress	6.09	0.36	5.60	0.30	0.70	0.02	0.19	0.02	0.10	0.02
1d H + RD	9.98	0.02	10.00	0.00	0.00	0.00	0.02	0.01	0.98	0.01
4d SD + 1d H + RD	6.24	0.30	7.96	0.27	0.25	0.04	0.20	0.02	0.55	0.04
8d SD + 1d H + RD	8.33	0.34	8.76	0.34	0.12	0.03	0.10	0.02	0.78	0.04
1d H Control	3.96	0.28	3.84	0.30	0.68	0.02	0.23	0.02	0.09	0.01
4d H + RD	10.00	0.00	10.00	0.00	0.00	0.00	0.01	0.00	0.99	0.00
4d SD + 4d H + RD	9.64	0.12	9.67	0.17	0.02	0.01	0.06	0.02	0.92	0.03
8d SD + 4d H + RD	9.22	0.28	10.00	0.00	0.02	0.00	0.02	0.01	0.96	0.01
4d H control	2.87	0.19	3.07	0.26	0.59	0.03	0.36	0.03	0.04	0.01
8d H + RD control	10.00	0.00	10.00	0.00	0.00	0.00	0.04	0.01	0.96	0.01
4d SD + 8d H + RD	10.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
8d SD + 8d H + RD	10.00	0.00	10.00	0.00	0.02	0.02	0.05	0.01	0.95	0.01
8d H control	3.87	0.18	4.44	0.34	0.68	0.03	0.27	0.03	0.05	0.01
12d H + RD control	10.00	0.00	10.00	0.00	0.00	0.00	0.02	0.01	0.98	0.01
4d SD + 12d H +RD	10.00	0.00	10.00	0.00	0.00	0.00	0.02	0.01	0.98	0.01
8d SD + 12d H + RD	10.00	0.00	10.00	0.00	0.00	0.00	0.01	0.01	0.99	0.01

| 12d H control | 3.98 | 0.37 | 3.60 | 0.26 | 0.77 | 0.02 | 0.16 | 0.02 | 0.06 | 0.01 |

Supplemental Table 2. Tests of Between Subjects Effects in *Bryum argenteum*

Tests of between subjects effects for the three examined treatment variables in *Bryum argenteum*. Significance was determined against an α of 0.05.

					Tests of Between-Subjects Effects				
Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
Corrected Model	FvFm	68.730a	389	0.177	34.723	0	0.896	13507.253	1
	ϕPSII	79.756c	389	0.205	33.959	0	0.894	13210.071	1
	NPQ	20.281d	389	0.052	2.887	0	0.419	1122.989	1
Intercept	FvFm	742.814	1	742.814	145983.004	0	0.989	145983.004	1
	ϕPSII	591.642	1	591.642	97994.429	0	0.984	97994.429	1
	NPQ	41.775	1	41.775	2313.083	0	0.597	2313.083	1
Phase	FvFm	35.51	4	8.877	1744.644	0	0.817	6978.575	1
	ϕPSII	38.653	4	9.663	1600.549	0	0.804	6402.196	1
	NPQ	1.017	4	0.254	14.074	0	0.035	56.296	1
RoD	FvFm	13.476	5	2.695	529.683	0	0.629	2648.414	1
	ϕPSII	17.135	5	3.427	567.613	0	0.645	2838.064	1
	NPQ	0.67	5	0.134	7.422	0	0.023	37.111	0.999
Ecotype	FvFm	1.08	12	0.09	17.685	0	0.12	212.225	1
	ϕPSII	1.504	12	0.125	20.754	0	0.138	249.053	1
	NPQ	2.705	12	0.225	12.481	0	0.088	149.778	1
Phase * RoD	FvFm	7.455	20	0.373	73.253	0	0.484	1465.058	1
	ϕPSII	9.693	20	0.485	80.27	0	0.507	1605.399	1
	NPQ	0.805	20	0.04	2.228	0.001	0.028	44.553	0.995
Phase * Ecotype	FvFm	2.774	48	0.058	11.356	0	0.259	545.092	1
	ϕPSII	3.326	48	0.069	11.477	0	0.261	550.881	1
	NPQ	6.182	48	0.129	7.132	0	0.18	342.324	1
RoD * Ecotype	FvFm	2.504	60	0.042	8.203	0	0.24	492.166	1
	ϕPSII	2.924	60	0.049	8.072	0	0.237	484.297	1
	NPQ	2.56	60	0.043	2.362	0	0.083	141.738	1

Phase * RoD * Ecotype	FvFm	5.932	240	0.025	4.857	0	0.428	1165.724	1
	φPSII	6.522	240	0.027	4.501	0	0.409	1080.182	1
	NPQ	6.343	240	0.026	1.463	0	0.184	351.189	1
Error	FvFm	7.938	1560	0.005					
	φPSII	9.419	1560	0.006					
	NPQ	28.174	1560	0.018					
Total	FvFm	819.482	1950						
	φPSII	680.817	1950						
	NPQ	90.23	1950						
Corrected Total	FvFm	76.668	1949						
	φPSII	89.174	1949						
	NPQ	48.455	1949						

a R Squared = .896 (Adjusted R Squared = .871)
b R Squared = .871 (Adjusted R Squared = .838)
c R Squared = .894 (Adjusted R Squared = .868)
d R Squared = .419 (Adjusted R Squared = .274)
e Computed using alpha = .05

Supplemental Table 3. Multivariate Tests for Three Treatments in *Bryum argenteum*
Multivariate tests for the three examined treatment variables in *Bryum argenteum*. Significance was determined against an α of 0.05.

| Multivariate Tests[a]									
Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[d]
Intercept	Pillai's Trace	.991	40859.19[b]	4.000	1557	.000	.991	163436.778	1.000
Phase	Pillai's Trace	.847	104.76	16.000	6240	.000	.212	1676.268	1.000
RoD	Pillai's Trace	.695	65.65	20.000	6240.	.000	.174	1312.991	1.000
Ecotype	Pillai's Trace	.297	10.44	48.000	6240	.000	.074	501.204	1.000
Phase* RoD	Pillai's Trace	.607	13.94	80.000	6240	.000	.152	1115.804	1.000
Phase* Ecotype	Pillai's Trace	.608	5.82	192.000	6240	.000	.152	1118.590	1.000
RoD* Ecotype	Pillai's Trace	.456	3.34	240.000	6240	.000	.114	803.371	1.000
Phase* RoD* Ecotype	Pillai's Trace	1.000	2.16	960.000	6240	.000	.250	2079.289	1.000

a. Design: Intercept + Phase + RoD + Ecotype + Phase * RoD + Phase * Ecotype + RoD * Ecotype + Phase * RoD * Ecotype
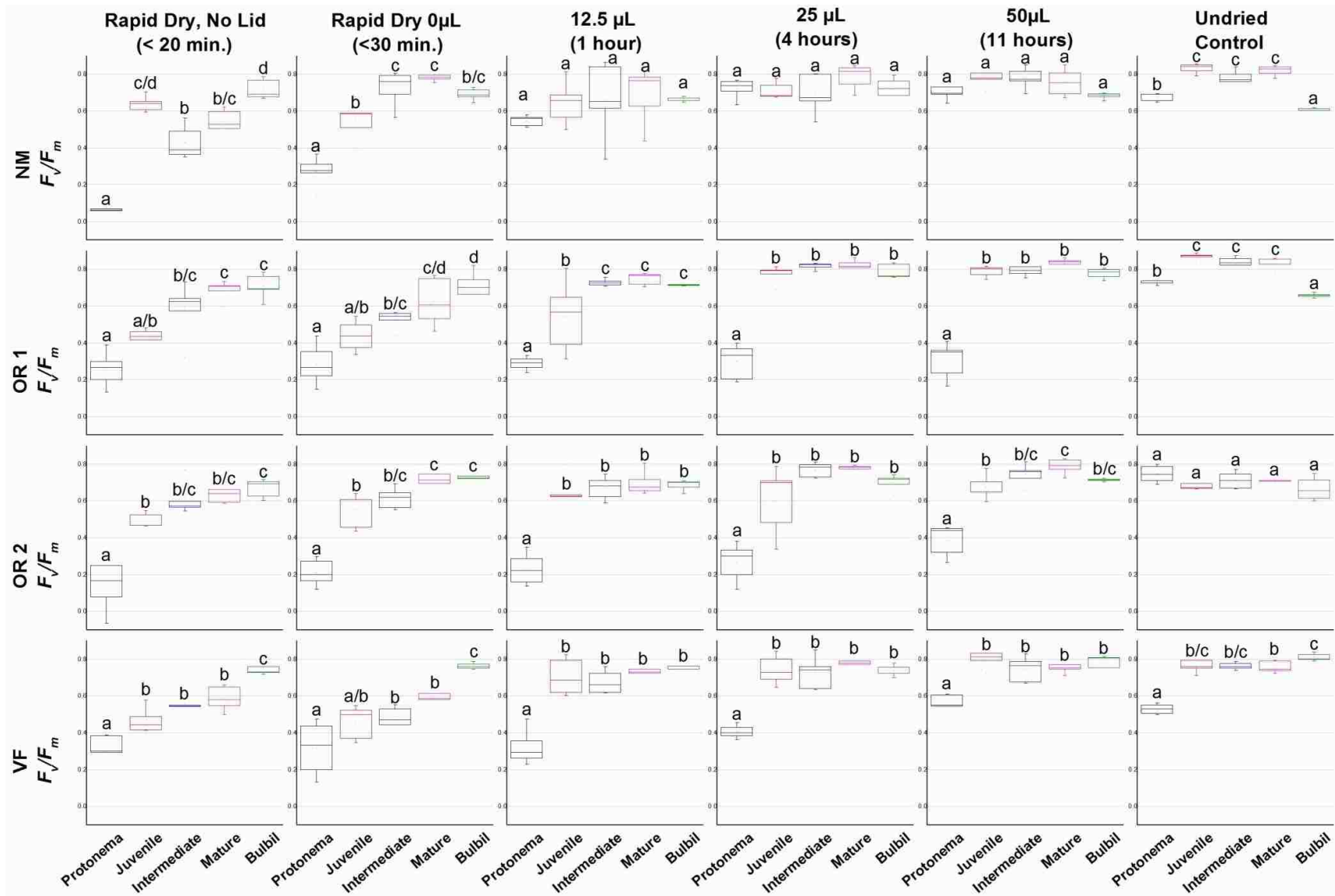
b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.
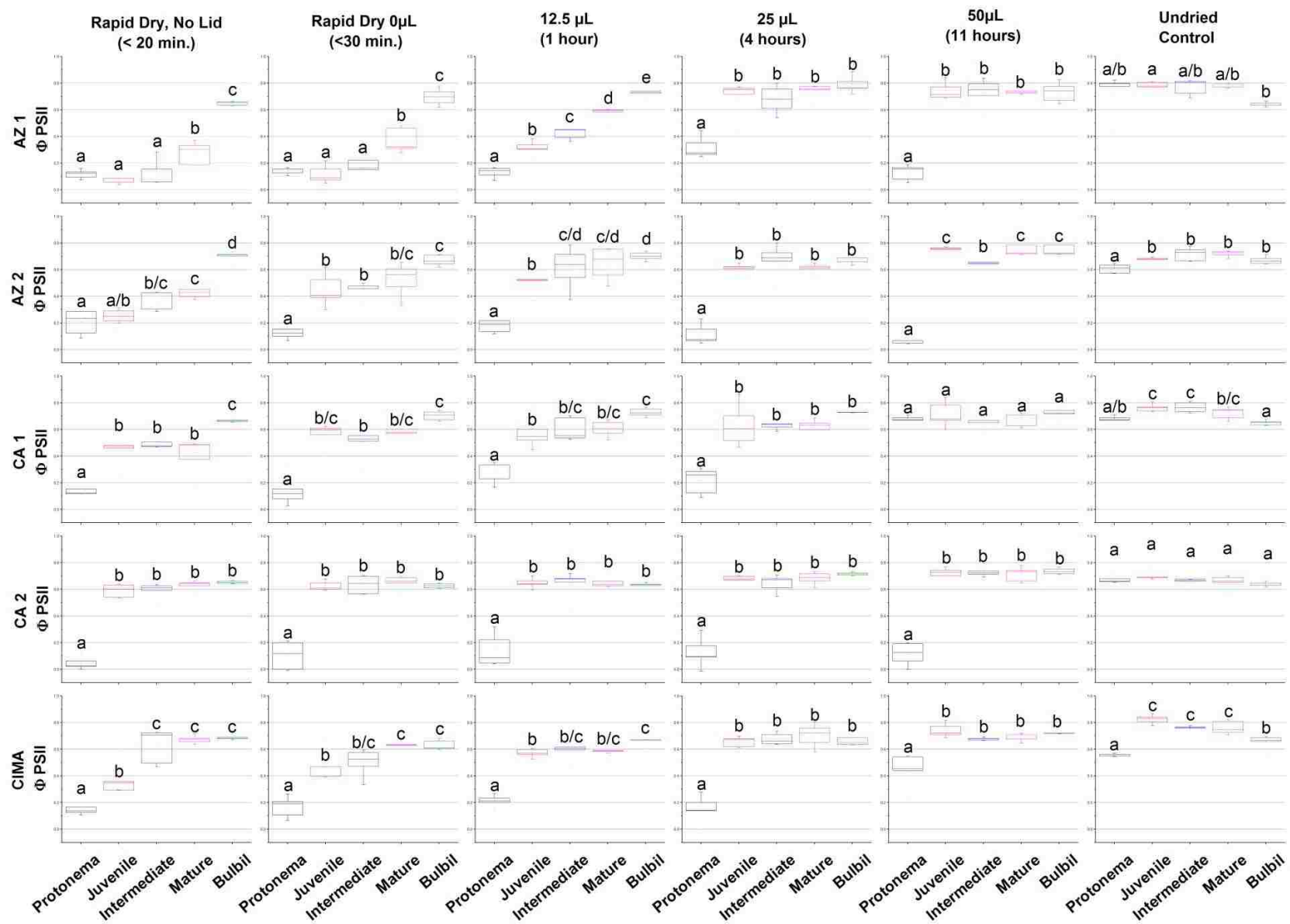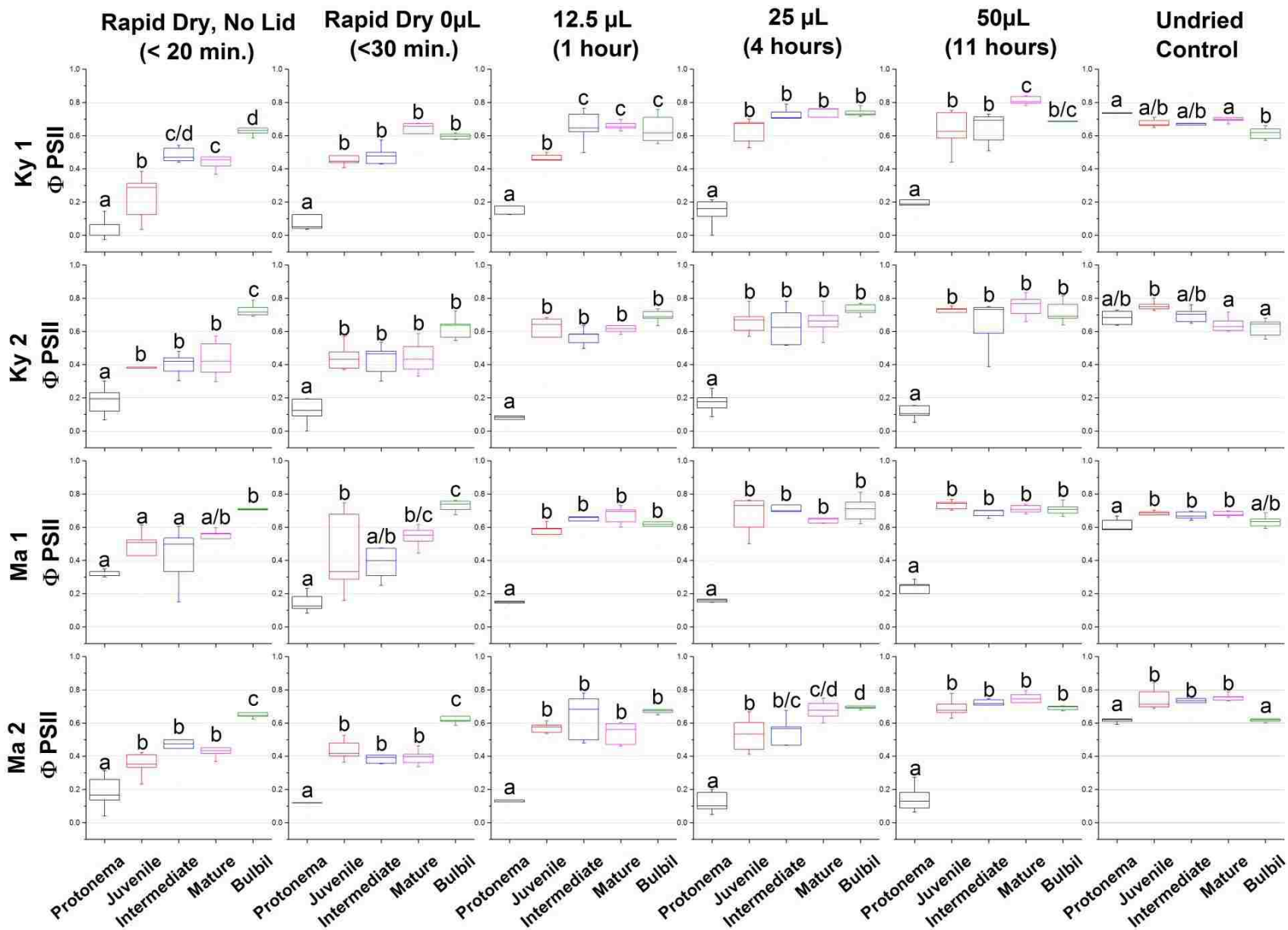
d. Computed using alpha = 0.05
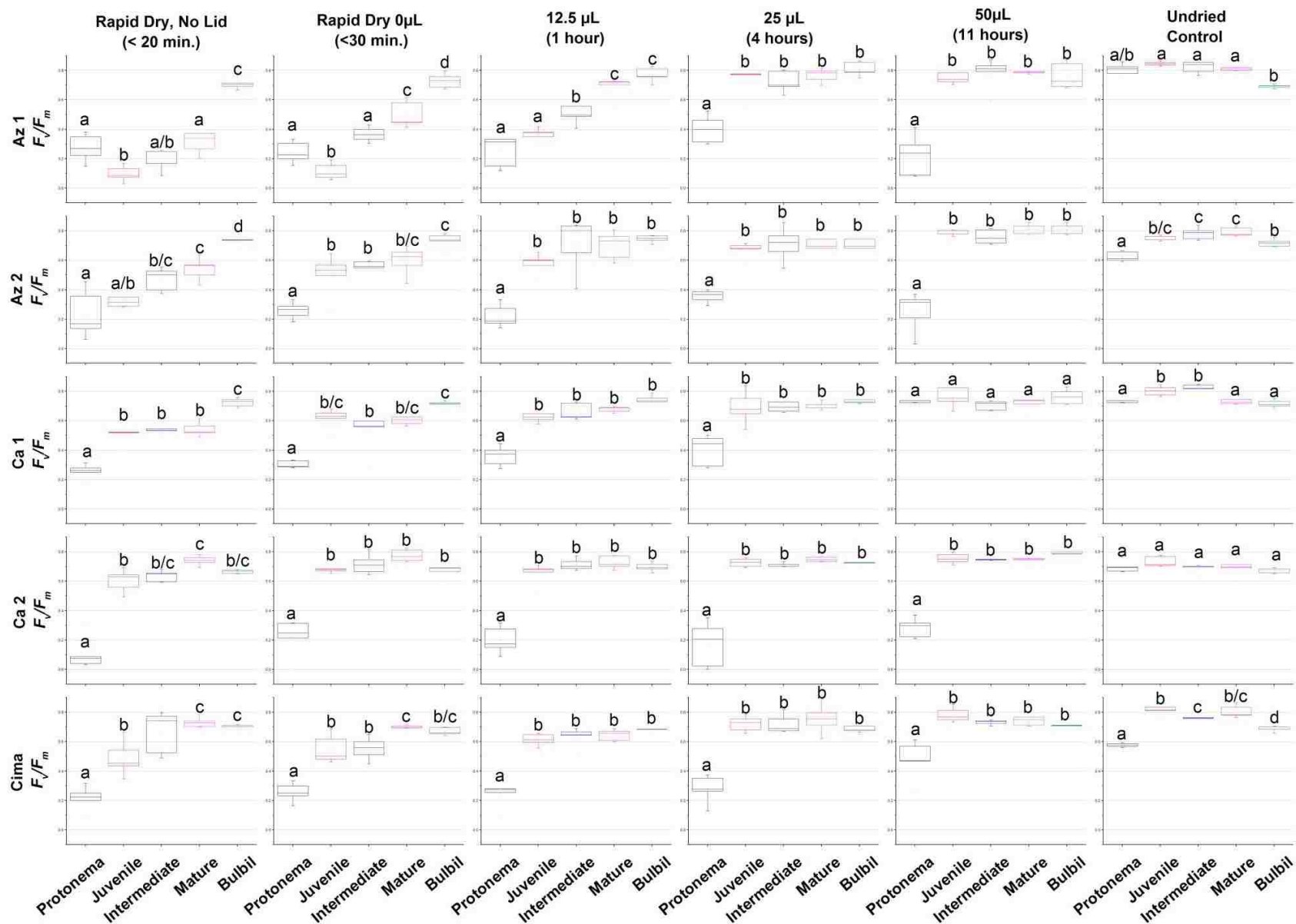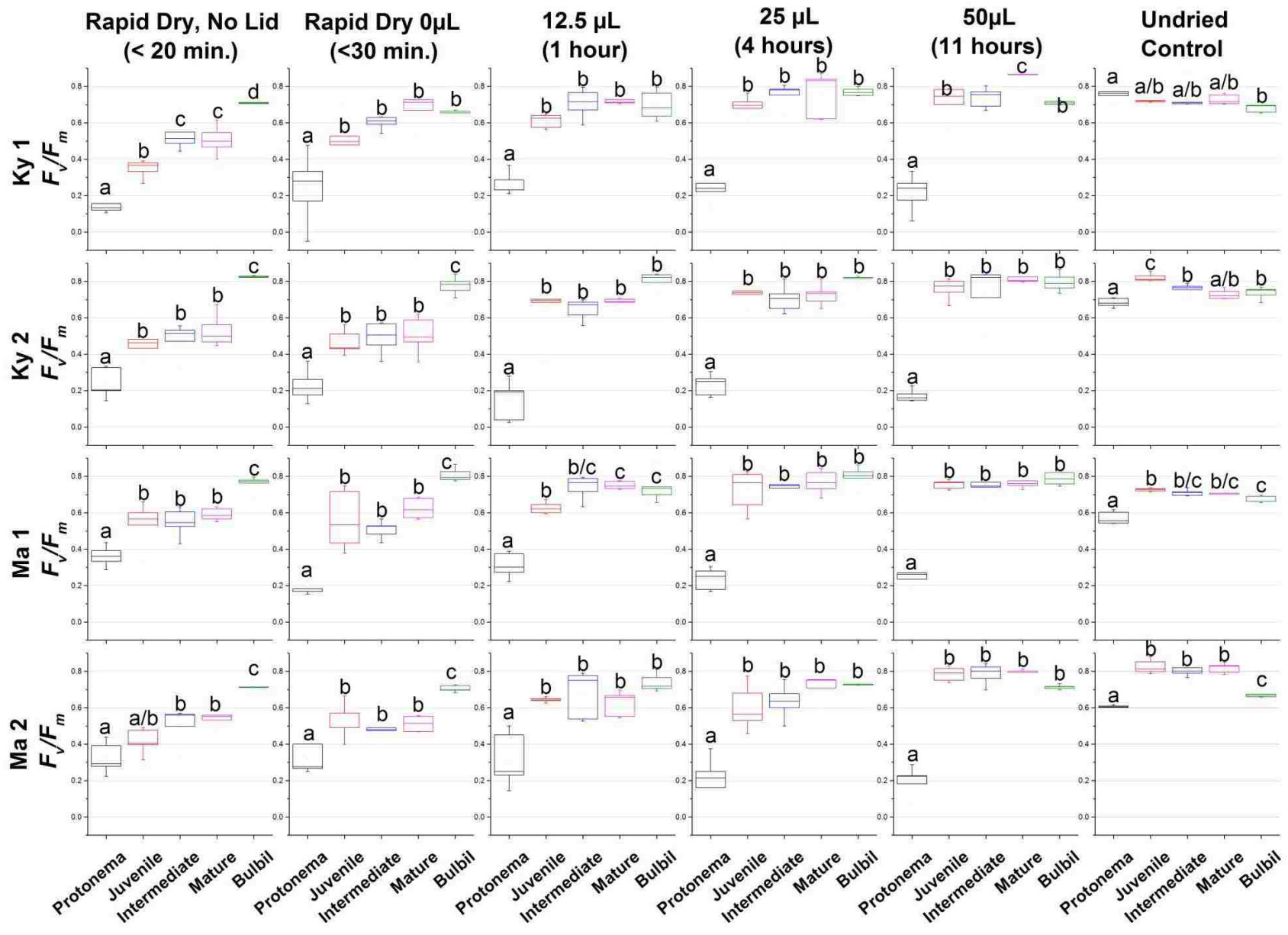
Supplemental Figure 1. Box and Whisker Plots for Effects of Individual Treatment Combinations Upon Fv/Fm in Bryum argenteum.

Box and whisker plots displaying the effect of individual treatment combinations upon *Bryum argenteum* for F_v/F_m. Columns represent the six RoD, rows represent the 13 ecotypes. Within each plot, values and homogeneous subsets for the five phases are shown in accordance with their respective columns (RoD), and row (ecotype). Whiskers represent 5th —95th percentile, while the box itself represents the 25th —75th percentiles, lowercase letters represent homogeneous subsets established at an α of 0.05.
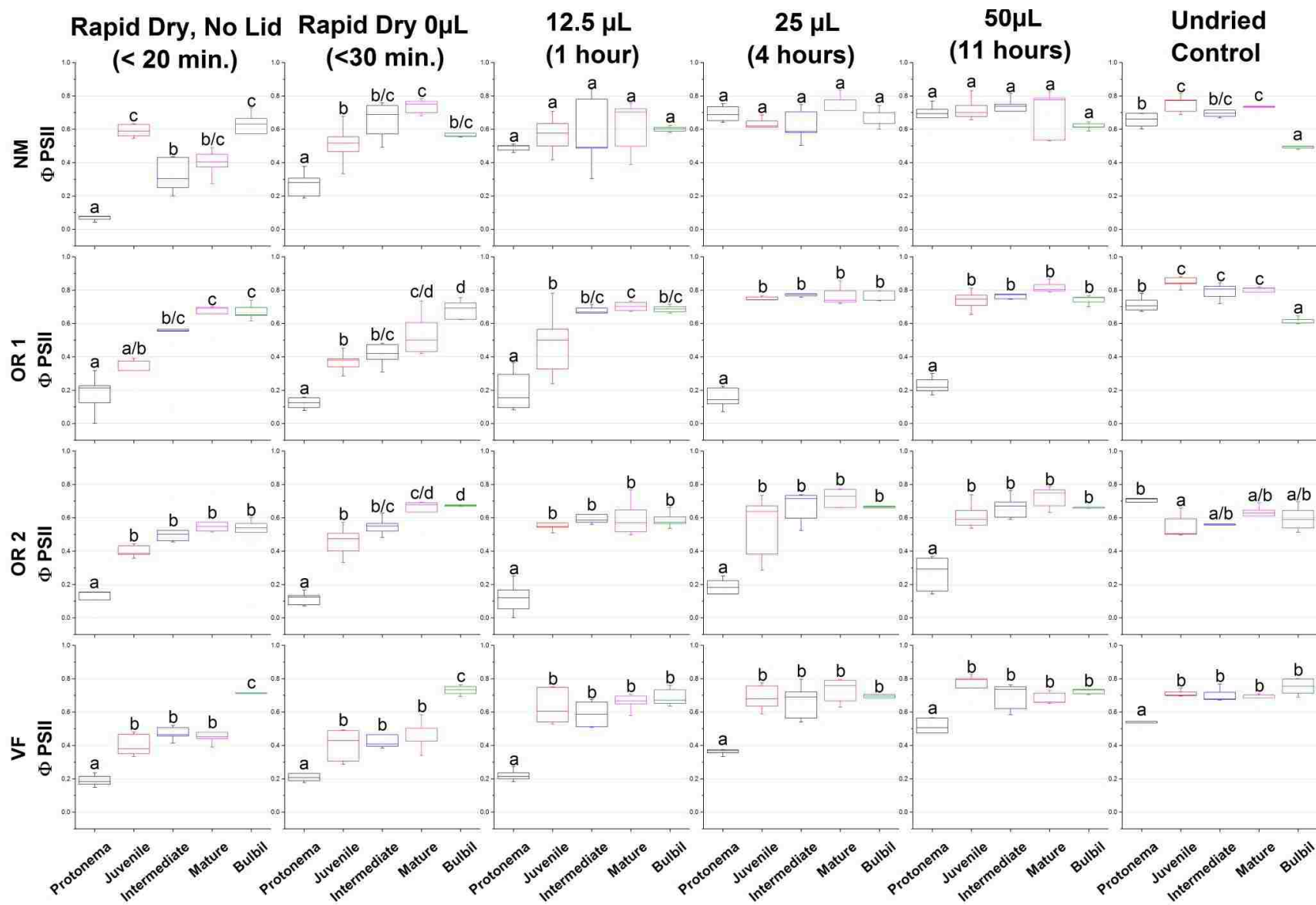
Supplemental Figure 2. Box and Whisker Plots for Effects of Individual Treatment Combinations Upon ϕPSII in Bryum argenteum.

Box and whisker plots displayng the effect of individual treatment combinations upon *Bryum argenteum* for ϕPSII. Columns represent the six RoD, rows represent the 13 ecotypes. Within each plot, values and homogeneous subsets for the five phases are shown in accordance with their respective columns (RoD), and row (ecotype). Whiskers represent 5th —95th percentile, while the box itself represents the 25th —75th percentiles, lowercase letters represent homogeneous subsets established at an α of 0.05.

Rapid Dry, No Lid (< 20 min.) | Rapid Dry 0µL (<30 min.) | 12.5 µL (1 hour) | 25 µL (4 hours) | 50µL (11 hours) | Undried Control

Az 1 NPQ

Az 2 NPQ

Ca 1 NPQ

Ca 2 NPQ

Cima NPQ

Protonema Juvenile Intermediate Mature Bulbil
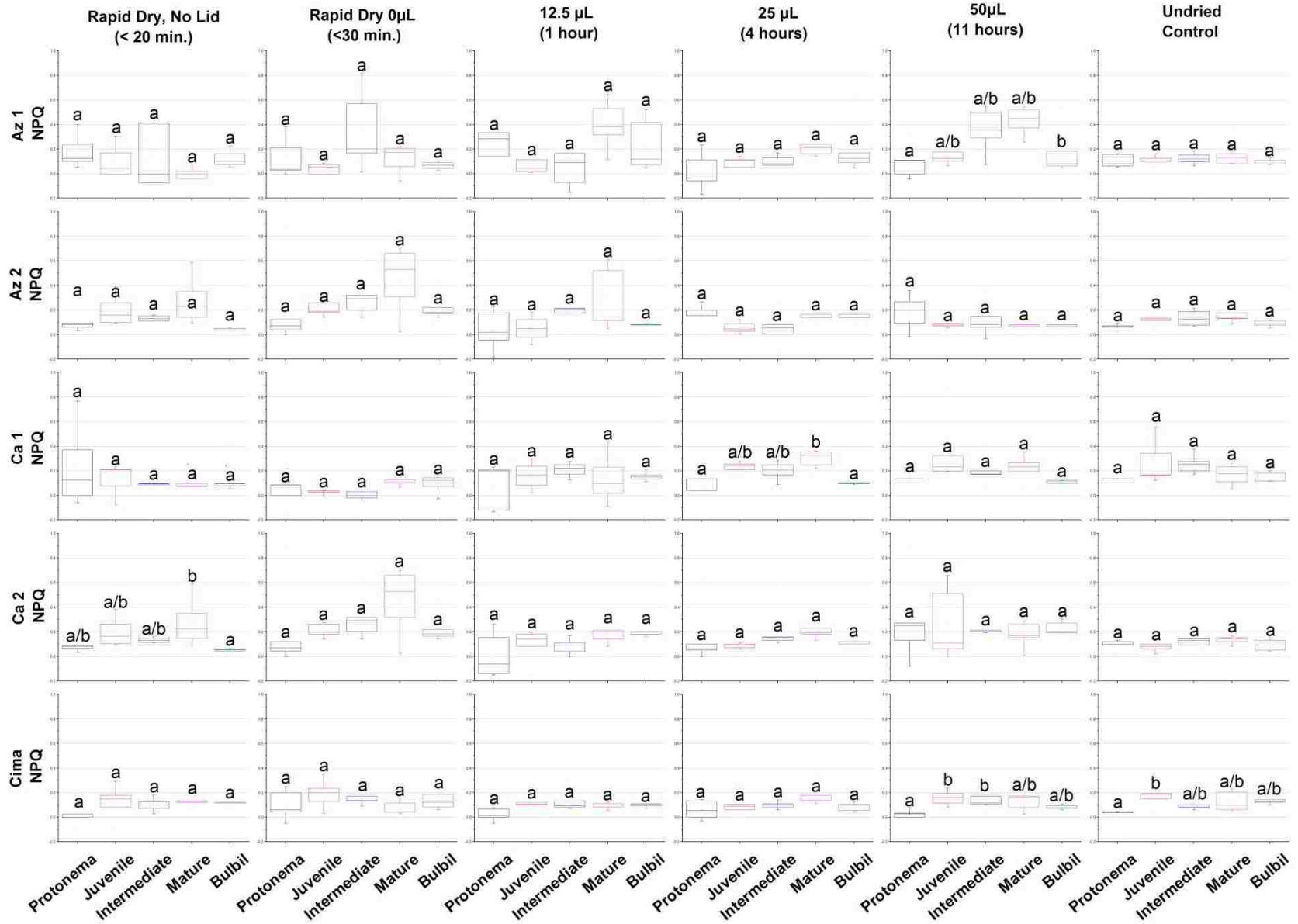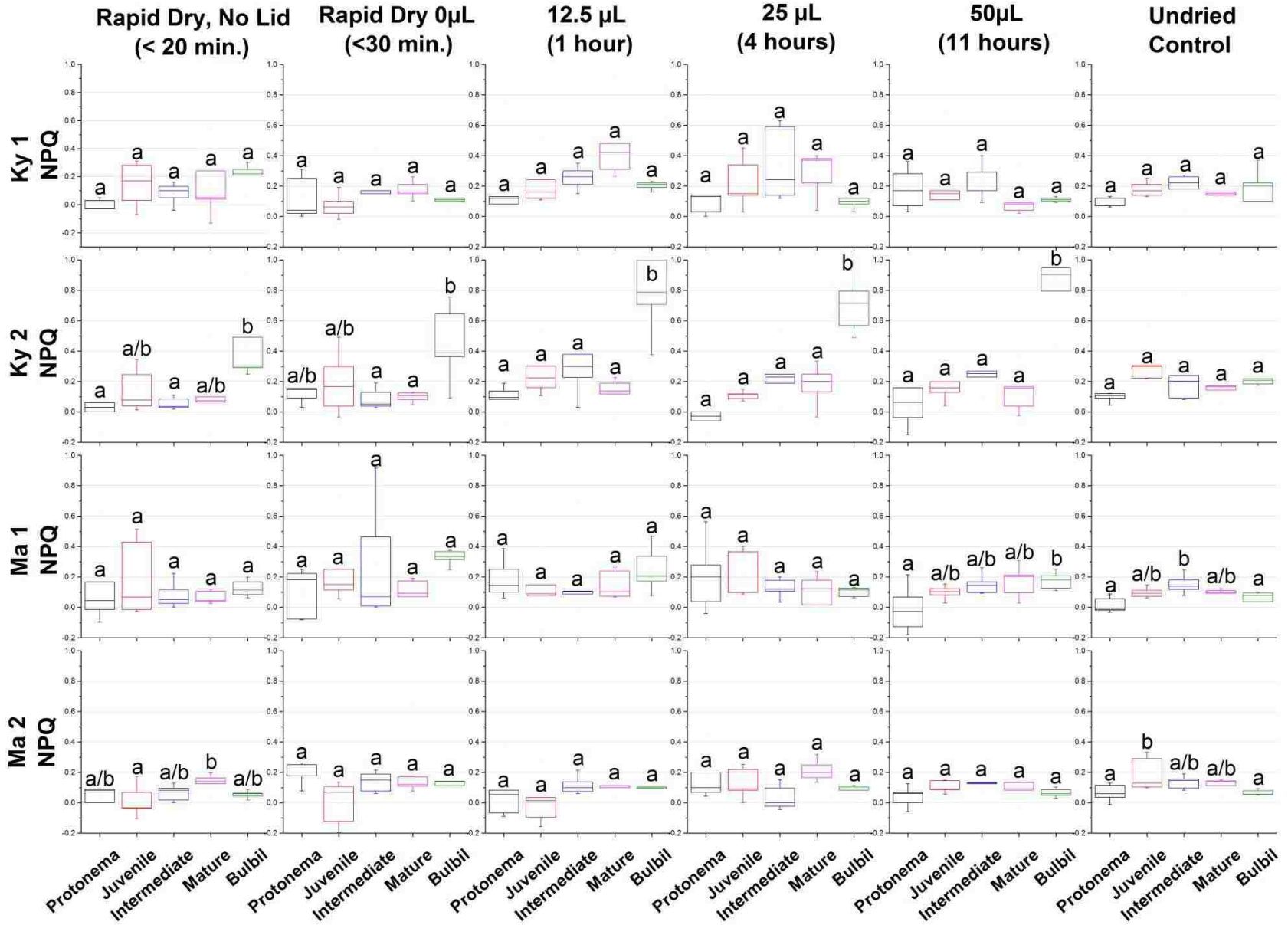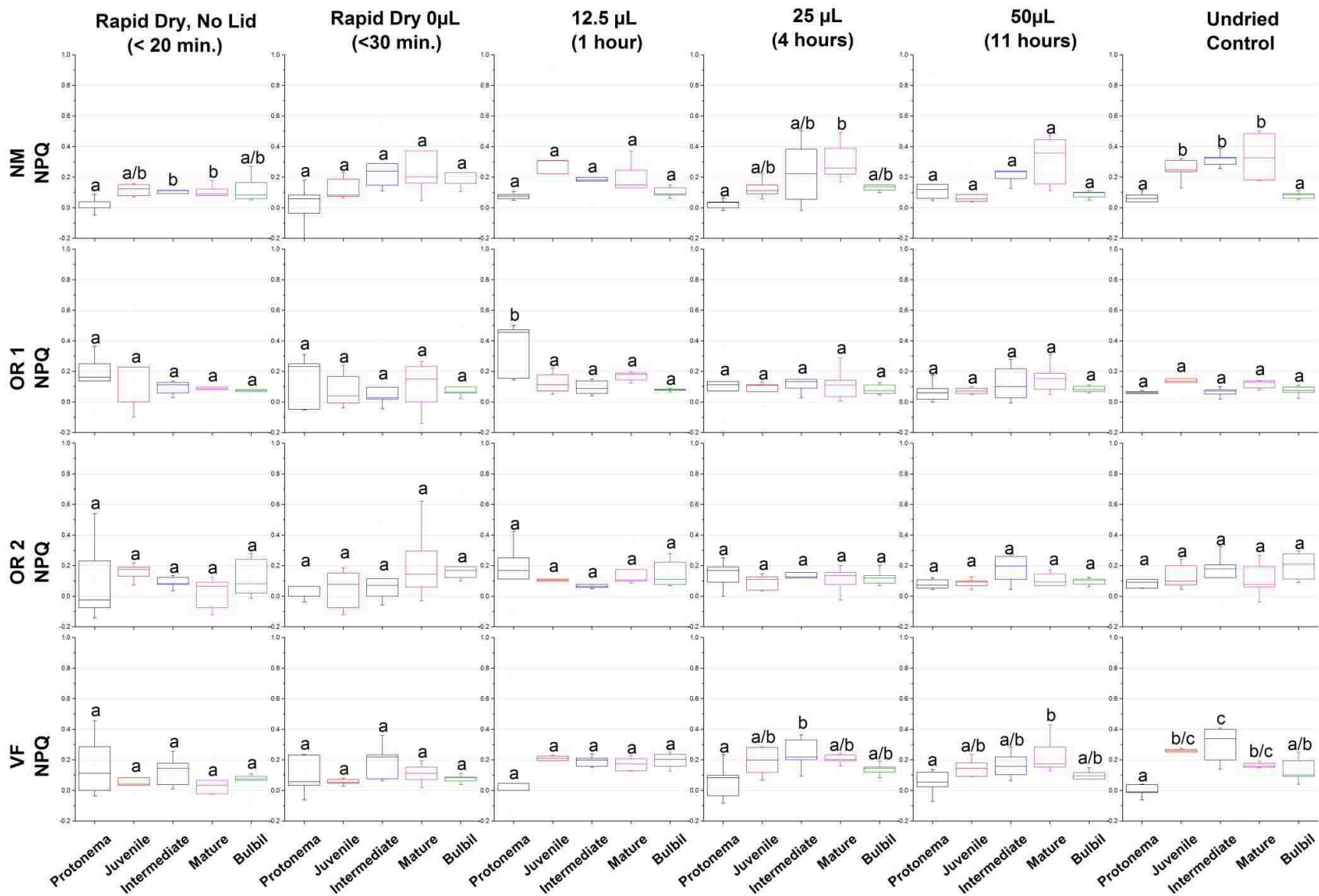
Supplemental Figure 3. Box and Whisker Plots for Effects of Individual Treatment Combinations Upon NPQ in Bryum argenteum.

Box and whisker plots displayng the effect of individual treatment combinations upon *Bryum argenteum* for NPQ (non-photochemical quenching). Columns represent the six *RoD*, rows represent the 13 ecotypes. Within each plot, values and homogeneous subsets for the five phases are shown in accordance with their respective columns (*RoD*), and row (ecotype). Whiskers represent 5th —95th percentile, while the box itself represents the 25th —75th percentiles, lowercase letters represent homogeneous subsets established at an α of 0.05.

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Curriculum Vitae

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EDUCATION

08/2009- *Current*   University of Nevada, Las Vegas, School of Life Sciences, Las Vegas, NV

Ph.D. Candidate, concentration in Ecology and Evolutionary Biology,

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Cumulative GPA: 3.93/4.00

Dissertation: *In progress,* Factors influencing induction of desiccation

tolerance in bryophytes: redefining fundamental aspects of the organism's

relationship with the environment in xeric habitats.

08/2004-12/2007   West Virginia State University, College of Natural Sciences and

Mathematics, Institute, WV

M.S. - Biotechnology

Cumulative GPA: 3.90/4.0

Thesis: The evolution and interrelationships of *Tetraphylledian* cestodes of sharks and stingrays as shown by sequencing of the entire LSU of the ribosome.

08/2001-05/2003   Marshall University, Biological Sciences, Huntington, WV

Post-baccalaureate – phylogeny and applications in biofuel development

Projects: Phylogeny of dry desert scorpions of Kazakhstan;

Biofuel applications: H_2 production in the green alga *Chlamydomonas reinhardtii*

08/1996-06/2000   West Virginia State College, College of Natural Sciences and Mathematics Institute, WV

B.S. - Biology

RESEARCH FOCUS

Interaction between ecotypic variation, sex, rate at which desiccation is applied, developmental phase upon the capacity to survive desiccation in bryophytes. Physiological hardening its establishment and retention in bryophytes as it applies to desiccation. Control of invasive bryophytes in managed habitats and urban environments. Future research directions include genetic variation across North America in the *Syntrichia* clade and the genetic underpinnings of

evolutionary factors, and stress related genes as it relates to habitat suitability and tolerance of desiccation.

JOURNAL PUBLICATIONS

Stark, L. R., Brinda, J. C., & Greenwood, J. L. (2016). Propagula and shoots of *Syntrichia pagorum* (Pottiaceae) exhibit different ecological strategies of desiccation tolerance. *The Bryologist*, *119*(2), 181-192.

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Stark, L. R., Greenwood, J. L., Brinda, J. C., & Oliver, M. J. (2014). Physiological history may

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    crassinerve. *Plant Biology*, *16*(5), 935-946.

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    desiccation tolerance: Effects of rate of drying on shoot damage and

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Markov, S. A., Eivazova, E. R., & Greenwood, J. (2006). Photostimulation of H_2 production in

    the green alga *Chlamydomonas reinhardtii* upon photoinhibition of its O_2-evolving

    system. *International Journal of Hydrogen Energy*, *31*(10), 1314-1317.

PRESENTATIONS

Greenwood, J. L. Exposure to slowly applied desiccating conditions hardens *Physcomitrella*

    patens against subsequent rapidly applied desiccation stress. Botany 2016, Botanical

    Society of America, Savannah, Georgia, July 30-August 3, 2016.

Stark, L. R., Greenwood J. L., Brinda J.C., & Slate, M. Suprasaturated shoots of *Syntrichia*

    norvegica are not constitutively desiccation tolerant: assessing rate of drying and

    equilibrating relative humidity. Botany 2016, Botanical Society of America, Savannah,

    Georgia, July 30-August 3, 2016.

Greenwood, J. L. *(Invited)* Influence of life history phase, genotype, and rate of drying upon

    desiccation tolerance in *Bryum argenteum*. Botany 2015, Botanical Society of America,

    Edmonton, Alberta, Canada, July 25-29, 2015.

Greenwood, J. L. Influence of life history phase, genotype, and rate of drying upon desiccation tolerance in *Bryum argenteum*. SoBeFree March 27-30, 2015.

Greenwood, J. L. Interactive effects of genotype, life history phase, sex, and rate of drying upon survival in a desiccated state in the cosmopolitan moss *Bryum argenteum*. Botany 2014, Botanical Society of America, Boise, Idaho, July 26-30, 2014.

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Greenwood, J. L. (Poster) Photostimulation of H_2 production in the green alga *Chlamydomonas reinhardtii* upon photoinhibition of its O_2-evolving system. 24th Biotechnology Symposium for Fuels and Chemicals, Gatlinburg, TN, April 28-May, 2002.

EXPERIENCE

2008-2010   University of Nevada, Las Vegas, Las Vegas, NV

Research Technician

Conducted desert bighorn sheep surveys, relict leopard frog surveys, amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism generation and analysis of bighorn sheep samples, amplification via PCR of small subunit rRNA for bighorn sheep and relict leopard frog samples, microsatellite amplification and analysis of bighorn sheep.

2004-2007   West Virginia State University, Institute, WV

Research Assistant

DNA extraction from animal tissues; polymerase chain reaction (PCR); electrophoresis; extraction of DNA; nested PCR; real time PCR; quantification and analysis of DNA via spectrophometry sequencing; DNA precipitation; primer design; AFLP.

2003-2007   West Virginia Department of Agriculture, Charleston, WV

PCR technician/FHP specialist

Molecular identification of various agricultural and forest pathogens with an emphasis on *Phytophthora ramorum*; DNA extraction from plant tissues; PCR; nested PCR; electrophoresis; preparation of microbial media; ELISA reactions upon suspected plant samples; isolation and culturing of fungi, oomyceetes, and bacteria; creation of laboratory protocols; collection and storage of MSDS sheets; autoclaving of all needed laboratory materials; database creation and maintenance. Surveying for new state records of invasive species; assistance with pesticide spray programs to control invasive species; release of beneficial predatory insects to exterminate invasive species. Safety committee member, 2004-2007 with aims to reduce the level of risk all department employees are subject to, and emphasizes on laboratory safety techniques and maintenance of O.S.H.A. compliance.

2001-2002   Marshall University, Huntington, WV

Research Assistant

DNA extraction from animal tissues; PCR; electrophoresis; extraction of DNA; preparation of microbial media; culturing of algae; gas chromatography; DNA precipitation.

1997-2000   West Virginia State University, Institute, WV

Research Assistant

Extraction of DNA from animal tissues; PCR; electrophoresis; gel extraction of DNA; DNA precipitation; autoclaving of laboratory materials.

TEACHING

University of Nevada, Las Vegas

Class Lecturer

BIO 197 (30 lectures, 6 semesters) guest lecturer covering animal evolution, diversity, and physiology, 2014-present

BIO 197 Primary instructor, summer 2014

Laboratory Lecturer and Instructor

BIO 197 Labs (Lower division biology, 2nd semester) - 4 sections, 2016- present

BIO 196 Labs (Lower division biology, 1st semester) - 14 sections, 2011, 2014-2016

BIO 189 Labs (Introductory biology for non-majors) - 2 sections, 2014

BIO 224 Labs (Human anatomy and physiology, 2nd semester) - 12 sections, 2011-2013

West Virginia State University

BIO 101 Labs, 2 sections, 2005

Marshall University

BIO 101 Labs, 8 sections, 2001-2002

GRANTS & FELLOWSHIPS

Graduate and professional student association, Travel Grant -2014

Graduate and professional student association, Travel Grant -2016

NASA Scholarship Recipient -2005

NASA Scholarship Recipient -2002

NASA Scholarship Recipient -1999

CURRENT MEMBERSHIP

American Bryological and Lichenological Society