UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

EFFECTS OF HIGH LIGHT INTENSITY AND DESICCATION STRESS ON MOSS SPECIES

By

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Preface

The experimental work described in this thesis was carried out by the candidate while based in the Discipline of Biological Sciences School of Life Sciences, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, under the supervision of Professor Richard P. Beckett, from July 2019 to July 2021.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Date: 30/07/2021

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Abstract

Effects of high light intensity and desiccation stress on moss species

Bryophytes are desiccant tolerant non-vascular plants, capable of growing and surviving in extreme conditions. They are divided into three groups: liverworts, hornworts, and mosses. Most mosses grow in shady and moist environments, although some form part of arid soil crusts where they protect soil from erosion. The moss flora of the Afromontane vegetation around Pietermaritzburg is dominated by two acrocarpous mosses, *Atrichum androgynum,* and *Dicranella subsubulata*. *A. androgynum* tends to grow in wetter, more shaded habitats and is a rather delicate species, while *D. subsubulata* grow in open, drier habitats and is more robust. Rarely, the species grow together, for example at the transition of a shaded indigenous woodland to plantations. We hypothesized that the more robust species has higher stress tolerance and has largely constitutive stress tolerance mechanisms. By contrast, we hypothesized that the more delicate species is less tolerant and may have inducible tolerance mechanisms. In the present study, desiccation tolerance and tolerance to high light stress were investigated in *A. androgynum* and *D. subsubulata*. Results confirmed that *D. subsubulata* was more tolerant of high light stress than *A. androgynum*. Exposure to moderate light intensities did not increase tolerance to subsequent high light stress in either species. Similarly, *D. subsubulata* was more desiccation tolerant than *A. androgynum*. Not consistent with our original hypothesis, mild desiccation, and treatment ABA-induced tolerance to desiccation in both species. Furthermore, detailed studies of the antioxidant enzyme peroxidase showed that enzyme activity was induced during slow drying in both *D. subsubulata* than *A. androgynum*. It appears that inducible tolerance mechanisms are present in both species. The work presented here represents a contribution to the autecology of two important mosses in the KwaZulu-Natal midlands Afromontane vegetation.

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Abbreviations

RWC, Relative water content

WC, water content

min, minute

h, hour

d, day

ROS, reactive oxygen species

ABA, Abscisic acid

O_2, Oxygen

H_2O_2, hydrogen peroxide

·OH, hydroxyl radical

H_2O, water

MDA,malondialdehyde

POX, peroxidase

SOD, superoxide dismutase

GR, glutathione reductase

LEA, late embryogenesis abundant genes or proteins

A or Abs, absorbance

PYL, prybactin resistance

RoR, rate of rehydration

RoD, Rate of dehydration

F_o, minimum modulated chlorophyll fluorescence, QA in the RC of PSII oxidized

$F_{o'}$, minimum modulated fluorescence in the absence of water

Fm, maximum modulated fluorescence, QA reduced

NPQ, non-photochemical fluorescence quenching, usually calculated as ($F_M/F_{M'}$-1), but (Fm/Fo´-1) after desiccation of lichens

PSI, photosystem I

PSII, photosystem II

PPFD, photosynthetically active photon flux density

$F_V/F_M = (F_M-F_o)/F_M$, quantum efficiency of (transiently stable) charge separation in PSII

mL, millilitre

mM, millimolar

µM,micromolar

PAR, photosynthetically active radiation

RH, relative humidity

Native PAGE, non-denaturing gel electrophoresis

RFO, raffinose

DT, desiccation tolerance

Chapter 1: Literature Review

1.1. Bryophytes

Bryophytes are desiccation tolerant organisms that can survive and grow in dry, high light conditions. Although there is some debate, many consider that they were the "first land plants", probably because of their desiccation tolerance. Bryophytes are referred to as "ectohydric" organisms and can receive not only water from the atmosphere but also inorganic nutrients (Shaw and Renzaglia, 2004; Green et al., 2011). Today, there are 23000 species of bryophytes, and they occur in a great diversity of forms, depending on the environment in which they grow (Bahuguna et al., 2013). When studying the "autecology" of a specific species, it is important to consider the response of the species to abiotic factors such as high light stress, desiccation, and nutrient supply. It is also important to consider how these stresses vary throughout the seasons. Understanding the extraordinary tolerance of mosses to some stresses could potentially contribute to programs aimed at improving the tolerance of crop plants to abiotic stress (Stark, 2017).

Bryophytes reach their greatest development as mats under cool and moist conditions, often on the canopy floor. They can affect the nutrient cycling of an ecosystem by absorbing more energy deposition and nutrients leached from dripping vegetation above the ground (Bahuguna et al., 2013). Bryophytes are divided into three classes, namely hornworts, liverworts, and mosses. They all play a significant role in the regulation of ecosystem nutrient cycling. Mosses account for 25% of the annual accumulation of phosphorus from precipitation, and act as competitors to other organisms reducing the number of minerals being shared (Bahuguna et al., 2013). They are also desiccation tolerant (DT). Briefly, DT is the ability of an organism to recover from being "air-dried" (Gaff and Oliver, 2013), a trait that was subsequently lost during the evolution of angiosperms. Bryophytes have no control over water uptake and release, and the great majority of species lack features such as a cuticle, stomata, and xylem present in almost all vascular plants. Desiccation tolerance can be conceived of being true "tolerance" to water stress; by comparison most vascular plants use a variety of "avoidance" strategies to avoid drought, e.g., thick cuticles, sunken stomata, water storage, and rapid stomatal closure (Gaff and Oliver, 2013).

Many bryophytes need to display tolerance to high light. Excess light can cause photoinhibition, a reversible reduction in photosynthetic activities. Specific mechanisms are

used to avoid or repair damages caused by light stress. Some damage seems to occur to all photosynthetic organisms even under moderate light intensities, and when the rate of repair matches the rates of damages there is no net loss of photosynthetic activity (Keren and Krieger-Liszkay, 2011). Photosynthetic organisms from cyanobacteria to vascular plants have various mechanisms responsible for decreasing photoinhibitory damage (Keren and Krieger-Liszkay, 2011). These include the short-term acclimation that influences the structure and functions of antennae complexes, for example, non-photochemical quenching (NPQ), state transitions, reaction center quenching, alternative electron transport processes, and movement of chloroplasts, leaves, or whole organisms away from intense light (Kehoe, 2010). Long-term acclimatization also occurs and involves changes in the ratios of photosynthetic pigments, and changes in the ratio of PSII to PSI (Kehoe, 2010). These mechanisms are found in all photosynthetic organisms, including crop species that normally grow in less extreme environments, but also cyanobacteria, lichens, and mosses that grow in extremely exposed soil crusts (Keren and Krieger-Liszkay, 2011). If not prevented, photoinhibition will reduce photosynthetic productivity. The main aim of the Introduction is to review tolerance to desiccation and high light stress in plants, with an emphasis on bryophytes, as a way of introducing the present study.

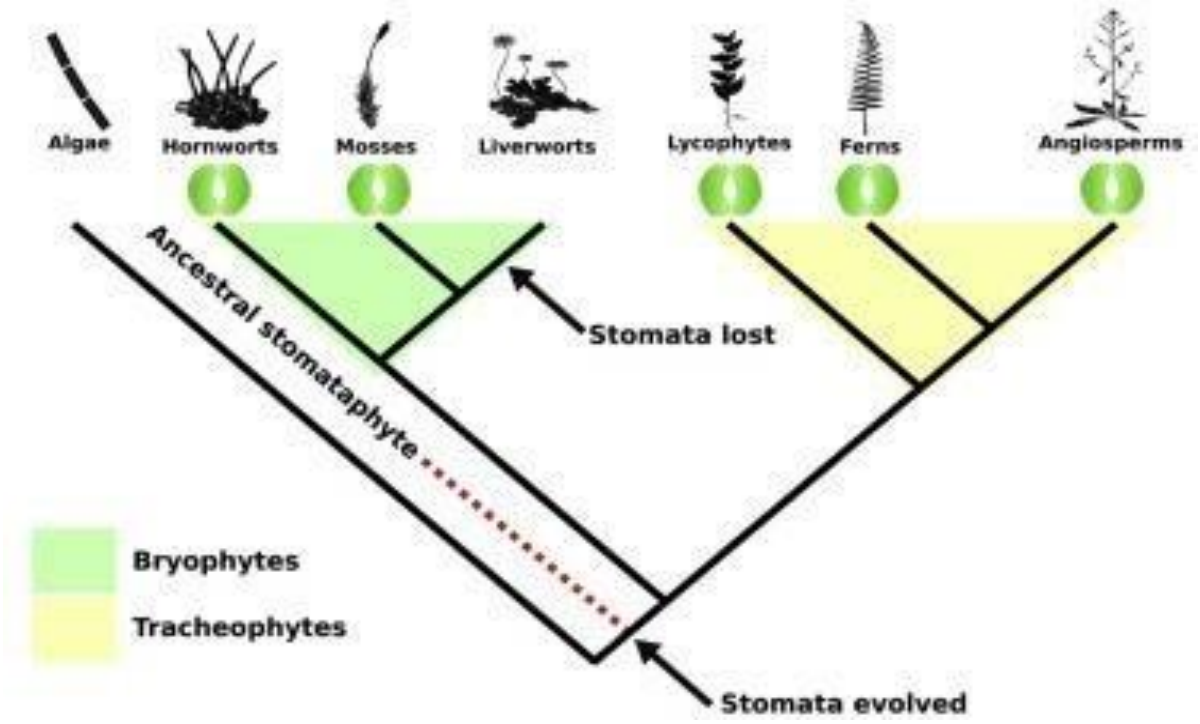


Fig 1: Evolutionary relationship between land plants and their algae relatives (Harris et al., 2020).

1.2. Desiccation tolerance in bryophytes

As discussed above, almost all bryophytes are desiccation tolerant. A simple definition of DT is an ability to survive at RWC below 10%, which they typically reach in an air-dried state (Proctor et al., 2007). Although a bryophyte-like plant was probably the ancestor of present-day angiosperms, later land plants developed reproductive structures such as pollen, spores, and seeds that reduced the requirement for liquid water (Fig 1; Norwood et al., 2003; Gaff and Oliver, 2013). Furthermore, Angiosperms have evolved cuticles, stomata, and conducting tissues that buffer tissues relative to water contents against changes in the environment (Gaff and Oliver, 2013). DT can be either constitutive or induced. Although one might think that some species from for example those that form soil crusts would have exclusively constitutive mechanisms, as they dry too quickly for mechanisms to be induced (and therefore need high inherent tolerance) actually even in some of these species some DT can be induced (Stark, 2017).

The severity of the effect of desiccation on bryophytes depends on the four factors according to McLetchie and Stark (2019):

1. The duration a moss plant spends dry
2. The rate of rehydration (RoR)
3. The rate of dehydration (RoD)
4. The relative water content (RWC) in the dry state.

As discussed above, plants can be considered to be DT when an organism can return to normal metabolism from the air-dry state. Typically, an air-dry state is achieved when the plant material is in equilibrium with air of 50% RH or less and the plant material has reached a relative water content c. 10%. The lack of a cuticle in most bryophytes allows their tissues to equilibrate with the environment during dry conditions (McLetchie and Stark, 2019; Green et al., 2011). As could be predicted, the highest DT is found within the species that are from dry habitats (Green et al., 2011). One of the aims of studying DT bryophytes is to potentially increase stress tolerance in higher plants. Liverworts and mosses are true green plants, and studies on them can potentially be extrapolated to higher plants more easily than, for example, lichens.

1.3. Morphological and anatomical changes in bryophytes during desiccation

During the loss of water content, leaves and stems shrink or folds to protect intracellular metabolism against mechanical stress (Cruz de Carvalho et al., 2014). *Atrichum undulatum*, which is morphologically very similar to *A. androgynum* used in the present study, shows leaf shrinkage and downwards curling starting from the leaf apex. Leaves change their colour from green to dark green. During rehydration, gametophytes take up water fast; the shrunken leaves expand and the dark green colour disappears (Hu et al., 2016). In *A. undulatum* the cell wall depresses as the protoplasts shrink. During desiccation lipid droplets decompose and chromatin becomes condensed and binds to the nuclear membrane, presumably so that it can be preserved (Hu et al., 2016). Chloroplasts remains present during dehydration, and bryophytes are "homiochlorophyllous", although the stroma and lamella become scattered when dehydration starts and later during dehydration they disappear. It has been suggested that bryophytes need to go under these changes to protects the cellular structure against ROS damage (Hu et al., 2016).

1.4. Changes in physiology and biochemistry of bryophytes during desiccation

An important tolerance mechanism appears to be the accumulation during dehydration of mRNA transcripts that became sequestered in messenger ribonuclear protein particles (mRNAPs) (Oliver et al., 2020). These particles are maintained in tissues during desiccation. Although the roles of most of these transcripts is unknown, it is assumed that they code for proteins that are responsible for repairs (Gao et al., 2017). Photosynthesis decreases during dehydration due to the reduction in the activity of enzymes required to activate the carbon cycle for example, rubisco and chloroplastic GAPDH (Cruz de Carvalho et al., 2014). Cytosolic GAPDH is accumulated within the leaves of the poikilohydric angiosperm *Craterostima plantgineum* during dehydration. This appears to be a common reaction in most DT species, and there is evidence that GAPDH is involved in preparation for desiccation by increasing the level of sucrose to protects the cellular structure against mechanical shock (Cruz de Carvalho et al., 2014).

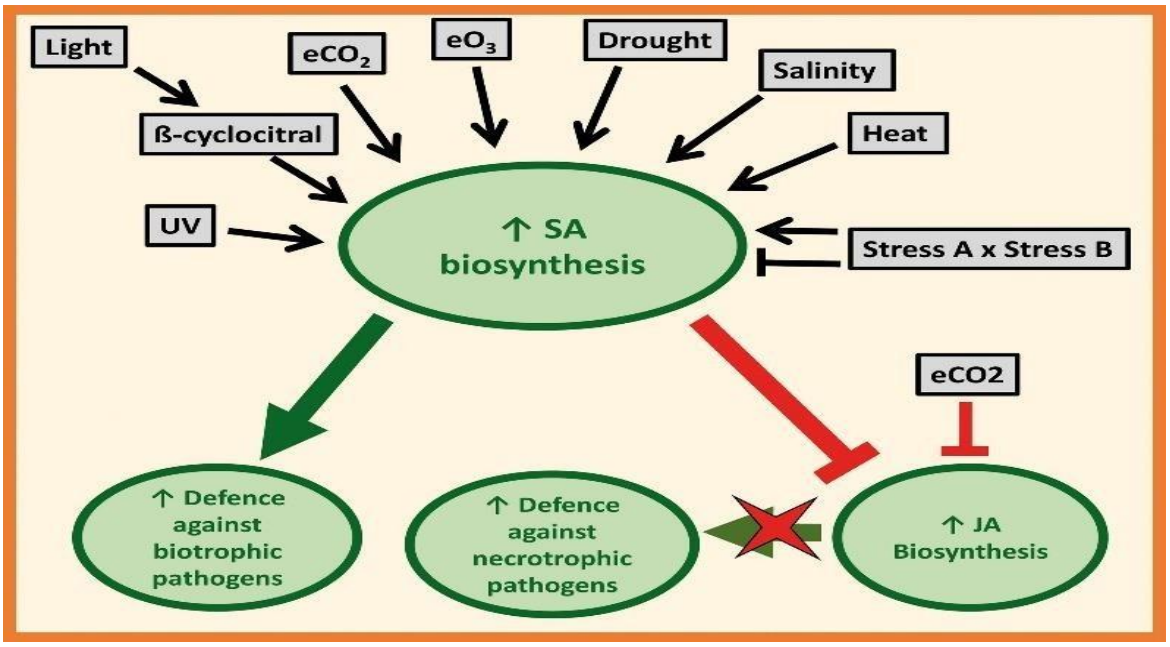


Fig 2: Diagrammatic representation of the sensing of stress by bryophytes, and signalling during environmental stress (Bigot et al., 2018).

1.4.1. ROS formation during desiccation

ROS generation is often the first biochemical response for plant and animal tissues subjected to stress (Anjum et al., 2011). Specifically, desiccation stress causes the formation of ROS in plant tissues (Smirnoff, 1993). The most important ROS are $O_2^{\cdot-}$ (the superoxide radical), H_2O_2 (hydrogen peroxide), and •OH (the hydroxyl radical). Hydroxyl radicals are the radical that can result in lipid peroxidation. However, ROS can also act as the second messengers to trigger defense reactions in plants (Anjum et al., 2011). During stress, the levels of ROS can increase beyond any role in signaling, and cause damage to proteins, DNA, and lipids (Apel and Hirt, 2004; Ntuli 2012). ROS overproduction during drought stress increases the level of malondialdehyde (MDA) (Møller et al., 2007; Anjum et al., 2011), a marker for lipid peroxidation (Møller et al., 2007). In general, lipid peroxides act as good indicators that ROS has damaged plant tissues (Anjum et al., 2011).

1.4.2. Desiccation mechanism based on enzymes and non-enzymatic antioxidants

It is a common response in most organisms that the antioxidant defence system is activated in response to a wide variety of stresses. The antioxidant system involves both enzymatic and non-enzymatic components (Mitra et al., 2013). Enzymatic antioxidants include the class I (ascorbic, APX) peroxidases, class III peroxidases (POX), superoxide dismutase (SOD),

catalase (CAT), and auxiliary enzymes, for example, mono- and dehydroascorbate reductases and glutathione reductase (GR). Peroxidases are highly efficient scavengers of ROS, specifically H_2O_2, but in addition peroxidases can also have a role in producing H_2O_2 which may form part of the so-called "oxidative burst", possibly to act in pathogen defence (Mayaba et al. 2001). Catalases have lower affinity for H_2O_2 and are therefore less effective compared to peroxidases in breaking down H_2O_2 with low concentration (Mayaba et al. 2001). Peroxidases use different substrates such as ascorbate to breakdown H_2O_2 to H_2O. It is well known that an interplay exists between enzymatic and non-enzymatic antioxidants to scavenge ROS in plants (Foyer and Halliwell, 1976).

Non-enzymatic antioxidants probably play a smaller role than enzymes but help in controlling levels of ROS (Caverzan et al., 2016). Mitochondria synthesize ascorbate that is transported across the cell membrane to other cell organelles (Ahmad et al., 2009). Ascorbate is probably the most important antioxidant compound, and in addition to its role as a substrate for peroxidase it can react directly with ROS. Another non-enzymatic ROS scavenger is tocopherol. Tocopherol can scavenge peroxyl radicals that are found in membranes, including those in the chloroplast (Caverzan et al., 2016). Perhaps the most important molecule responsible for maintaining redox balance within the cellular compartments is glutathione (GSH). GSH biosynthesis enzymes are upregulated during the abiotic stress (Apel and Hirt, 2004). Oxidized glutathione (GSSG) can be calculated as the percentage of total glutathione, and the balance between GSSG and GSH is arguably the best indicator of redox status. Damage caused by desiccation on protein synthesis are shown during rehydration. Carotenoids produced by *Marchantia polymorpha L.* during desiccation can reduce the level of ROS produced by desiccation stress (Kavitha and Murugan, 2016).

In humans, the imbalance of ROS and antioxidants is at least in part responsible for causing many degenerative diseases (Ahmad et al., 2009; Gahtori and Chaturvedi, 2019). Interestingly, some bryophytes are known as a good source of efficient natural antioxidants including phenols and ferulic acid. It is therefore not surprising that bryophytes have been widely used as medicinal plants. In China, bryophytes were traditionally used as medicines before they were known as good source of antioxidants. Recently, a study was done using *Oxytegus tenuirostris*, *Eurhynchium striatum*, and *Rhynchostegium murale* to investigates the activity of antioxidants that showed that ecological factors (climate) plays a huge role in determining the properties of antioxidant molecules in mosses (Gahtori and Chaturvedi, 2019).

1.4.3. Desiccation tolerance mechanisms based on sugar

An important component of DT in many organisms is the accumulation of sugars, specifically sucrose and trehalose. Sucrose and trehalose stabilize the structure of macromolecules, protect the biological membranes, stop diffusion, preserve the cytoskeleton, and possibly act as antioxidants (Norwood et al., 2003). Trehalose-6-phosphate and trehalose are central metabolite regulators impacting the status of carbohydrates energy and growth metabolism (Gechev et al., 2012). During severe desiccation, carbon from carbohydrates such as starch and stachyose can be metabolized to sucrose for extra osmoprotectant activity (Smeekens et al., 2010; Gechev et al., 2012). Raffinose is made by raffinose synthase from sucrose and galactinol (Smeekens et al., 2010).

1.4.4. Desiccation tolerance mechanisms based on LEA and other proteins

Late embryogenesis abundant genes or proteins (LEA) are proteins that are present both in vegetative tissues and seeds. LEA proteins may supply a water hydration "shell" to specific macromolecules and proteins to protect them against damage caused by dehydration (Ntuli, 2012). In addition to LEA proteins, small heat shock proteins (sHSPs) are expressed in a desiccation-tolerant plant without stress and are also induced during drought stress (Gechev et al., 2012). sHSPs can act as molecular chaperones for some proteins, protecting them from denaturation and aggregation (Farrant et al., 2007). Rapid induction of heat shock genes often occurs during dehydration in resurrection plants (Hundertmark and Hincha, 2008). Studies on the "model moss" *Physcomitrium patens* have shown the importance of dehydrin genes such as PpDHNA in mosses subjected to salt and osmotic stress (Saavedra et al., 2006). While the precise function of LEA and sHSP proteins remain unclear, there is enough evidence to suggest that they play an important role in the mechanism of DT in resurrection plants.

1.5. Abscisic acid

Abscisic acid (ABA) is an important plant phytohormone that plays a significant role in the response of plants to abiotic stress, senescence, seed growth, and generally in plant development (Kuromori et al., 2010). The most important role of ABA in vascular plants is probably stomatal regulation, but the hormone is involved in many other stresses and normal plant development. Land plants have multiple receptors for ABA, nucleocytoplasmic center is the most important with two with specific receptors, PYR (pyrabactin resistance) or PYR-like receptor. Although ABA is produced in plant roots, it can be translocated to the guard cells in the xylem. The receptor AtABCG25 is responsible for intracellular ABA transport (Kuromori

et al., 2010). ABA regulates the synthesis of Late Embryogenesis Abundant protein genes during the desiccation that accompanies seed maturation. LEA proteins are also responsible for increasing dehydration tolerance in vascular plants (Koster et al., 2010).

Hartung (2010) reported that at that time, nine mosses species were known to possess endogenous ABA (Hartung, 2010). The amount of ABA varies greatly; for example, *Riccia fluitans* has high levels of ABA that may be related to its high DT (Hartung and Gimmler, 1994). There are only a few reports on the biosynthesis and metabolism of ABA in Bryophytes. In lower plants, ABA is involved in signalling the upregulation of genes that are needed for DT. For example, pre-treatment of the moss *Atrichum androgynum* increased the rate of recovery the photosynthesis following desiccation, and also doubled NPQ, which may reduce ROS formation (Mayaba et al. 2001). In the same work, ABA was shown to slightly increase the accumulation of sugar, which as discussed above may be responsible for the protection of the membrane during desiccation (Mayaba et al., 2001).

1.6. Tolerance to light stress by avoiding ROS formation.

1.6.1. Photoinhibition in mosses

High light can damage plants, and a major reason for the damage is the formation of ROS (Pospíšil., 2016). When the light energy exceeds the maximum capacity of the photosystem to utilize it, ROS formation in PSII can induce photoinhibition (Pospíšil et al., 2016). Chlorophylls are found in PS antennae complex. When singlet state chlorophyll absorbs excess energy, it is converted to the potentially harmful triplet state chlorophyll. Xanthophylls directly quench excited chlorophyll preventing the formation of triplet chlorophyll (Pospíšil et al., 2016). Indirect prevention of the formation of triplet chlorophyll involves the rearrangement of Lhcb protein controlled by Psbs (Ruban et al., 2012). Quenching singlet chlorophyll can be insufficient leading to conversion to triplet chlorophyll thus transferring energy from O_2 forming $^1O_2$ and other ROS, responsible for most photoinhibition (Pospíšil et al., 2016).

PSI is sensitive to inhibition induced by high light, particularly at low temperatures (Foyer, 2018). Light during the summer season is not regarded as the determining factor for low productivity of bryophytes. Nutrient availability and water stress are often cited as having large effects on productivity. However, photoinhibition can also limit the productivity of moss at high latitudes where it is cold, possibly in part due to inhibition of PSI. In general, the optimal light intensity of photosynthesis in Bryophytes is rather low in high latitudes (Barták et al.,

2012). Damage caused by high light in photosynthetic apparatus are limiting factors to the productivity of Antarctic bryophytes (Adamson et al., 1988). Mosses experience stress in polar habitats, where the combined effects of low temperature and bright light are extremely stressful. (Adamson et., 1988). Interestingly, it has been observed that mosses growing in shaded habitats can grow faster than mosses without a canopy above them (Murray et al., 1993). High light and desiccation stress induces similar physiological effects on the photosynthetic apparatus of bryophytes; both can promote ROS formation, particularly around PSII (Barták et al., 2012). It seems likely that ROS formation is the major cause of damage to the photosynthetic apparatus under the effects of dehydration and high light stress (Barták et al., 2012).

1.6.2. Light screening by flavonoids and phenols in bryophytes

Flavonoid biosynthesis is an important response of plants to their environment. Many stresses such as cold, UV-B light (UVB), desiccation, and salinity induce the production of flavonoids (Davies et al., 2020). Flavonoid *o*-glycosides are the main important flavonoids acting to provide UVB tolerance in both *Marchantia* and *Arabidopsis* (Yin and Ulm, 2017; Davies et al., 2020). If for any reason (e.g. mutation) the production of flavonoids is reduced, the susceptibility of *Marchantia* and *Arabidopsis* to UV increases. The production of flavonoids can be reduced by excessive UV light. Interestingly, mutants with elevated levels of flavonoids have high UVB tolerance (Soriano et al., 2018). However, in mosses and lycophytes the flavonoid biosynthsis pathway that protects against UVB light appears to be absent. In mosses, phenolics appear to be the most important compounds for UVB tolerance (Soriano et al., 2018). Ultraviolet (UV) radiation is the noticeable environmental factors with an influence on photosynthetic organism and water availability is the important environmental factor in mosses. Two compounds *p*-coumaric and ferulic acid are induced by UV radiation in *Plagiochila asplenioides.* In Bryophytes, unlike lichens, pigments that screen the photosynthetic apparatus from high PAR are rare, rather these compounds and appear to be produced more to stop UV light. Some bryophytes became pigmented during the exposure to high light (Waterman et al., 2017), but more work is needed to assess whether these pigments offer any photoprotection (Waterman et al., 2017; Soriano et al., 2018). Nevertheless, in the context of the present study, neither *D. subsubulata* nor *A. androgynum* have ever been observed to synthesize pigments.

1.6.3. Induction of Non-Photochemical quenching (NPQ) by light

Non-Photochemical quenching (NPQ) can protect plants from oxidative stress caused by high light by dissipating excess energy radiating as heat (Gerotto et al., 2012). Photosynthetic organisms harvest light from sunlight and convert it into chemical energy. During the evolution of first land plants, plant needed to develop suitable mechanisms to protect plants from high light and excessive UV radiation, first land plants needed to survive combinations of desiccation and light stress. As the atmosphere became oxygenated by photosynthesis, it seems likely that the risk of ROS production increased (Niyogi, 1999; Gerotto et al., 2012). NPQ is a short-term method of protecting plants from excess light (Gerotto et al., 2012). NPQ has two main components, energy (qE) and inhibitory quenching (qI). qE is generally believed to be caused by the enzyme-catalyzed xanthophyll cycle, whereby the carotenoid violaxanthin is enzymatically converted to zeaxanthin in a pH-regulated process that occurs during increases in light intensity (Demmig-Adams et al. 2020). LHCSR 1, 2, and Psbs are involved in the induction of NPQ, but the precise mechanism remains unclear (Gerotto et al., 2012). Using the portable and lab chlorophyll fluorescence devices, NPQ can be measured in the field and in more controlled environments. Studies showed that in general highly desiccation tolerant bryophytes have higher levels of NPQ than more sensitive species (Deltoro et al., 1998). Beckett et al. (2005) showed that in *A. androgynum* slow drying increases qE. However, NPQ has not been studied in *D. subsubulata.*

1.7. Class I and Class III Peroxidases in mosses

As discussed above, antioxidant enzymes form an important part of the defence of organisms against oxidative stress, irrespective of how such stress occurs. One of the most important families of antioxidant enzymes are the peroxidases. Peroxidases are found in almost all organisms from plants, animals to bacteria (Pandey et al., 2017). Peroxidases play the important role of breaking down H_2O_2 to water (H_2O) and oxygen (O_2) (Pandey et al., 2017). Hydrogen peroxide is a toxic product that is produced in multiple sites because of stress. In vascular plants, the ascorbate-glutathione (or Halliwell-Asada) cycle removes harmful ROS, particularly in the chloroplasts. Here, superoxide dismutase (SOD) dismutates $O_2^{\cdot -}$ to H_2O_2 which is broken down to H_2O and O_2 with reaction catalyzed by ascorbate peroxidase (APX) or Class I peroxidases within the stroma. In the process, ascorbate is converted to monodehydroascorbate (MDHA), some of which disproportionates to dehydroascorbate (DHA). Ascorbate is regenerated from DHA by dehydroascorbate reductase (DHAR) in a

reaction involving the oxidation of GSH to GSSG. GSH is recovered from GSSG by glutathione reductase (GR), with NADPH providing the reducing power. MDHAR reduces any MDHA that has not disproportionated directly back to ascorbate. However, the location of the ascorbate-glutathione pathway (also called the Halliwell-Asada pathway) in bryophytes is less clear. For example, the moss *Physcomitrium patens* does not have chloroplastic isoforms of MDHAR indicating that ascorbate reduction takes place in cytoplasm (Parvin et al. 2019; Pandey et al. 2017).

While APX doubtless plays a key role in tolerance to high light, the enzyme is likely to have much wider roles in stress tolerance in Bryophytes. Recently, Onele et al. (2021) showed that APX activity is increased by desiccation stress in the boreal moss *Dicranum scoparium*. In addition to the APX enzymes, Class III peroxidases are likely to be involved in reducing levels of stress-induced H_2O_2. For example, Onele et al. (2019) showed that slow drying induces the activity of Class III peroxidases in the moss *D. scoparium*. Bryophyte peroxidase enzymes are structurally close to higher plant enzymes, and often have similar responses to biotic and abiotic stress (Ros et al. 2007).

1.8 Inducibility of tolerance

Bryophytes live in a variety of niches, and their stress tolerance mechanisms are likely to reflect unique strategies based on the prevailing environment of the plants. For example, for DT, some microenvironments are characterized by rapid drying (e.g., growing epiphytically on trees or rocks), while others will involve slower rates of desiccation (e.g., growing on moist, shaded soil). Drying more slowly allows for induction of physiological protective mechanisms to a greater extent than drying rapidly (Tuba et al., 1998). At the two extremes therefore will be bryophytes that can tolerate rapid drying (constitutively DT), and at the other those that can only tolerate slow drying (inducibly DT). However, in reality, ecological strategies for DT are perhaps best viewed along a gradient of inducibility, with many species exhibiting a "mixed strategy" of DT (Stark and Brinda, 2015; Stark, 2017). To test for this, in theory bryophytes should be maintained at full turgor to de-harden them from any field stress, and then rapidly dried. If there is little damage observed during rehydration (or, alternatively, 24 h post-rehydration), then it seems reasonable to assume that the species depends largely on constitutive DT mechanisms. If, however, significant damage is evident upon rehydration, and this damage is reduced when plants are dried more slowly (SD), then it seems likely that the species possesses mostly inducible DT mechanisms (Coe et al. 2021). It therefore follows that to characterize a DT strategy, at the very least, material should be subjected to slow and fast

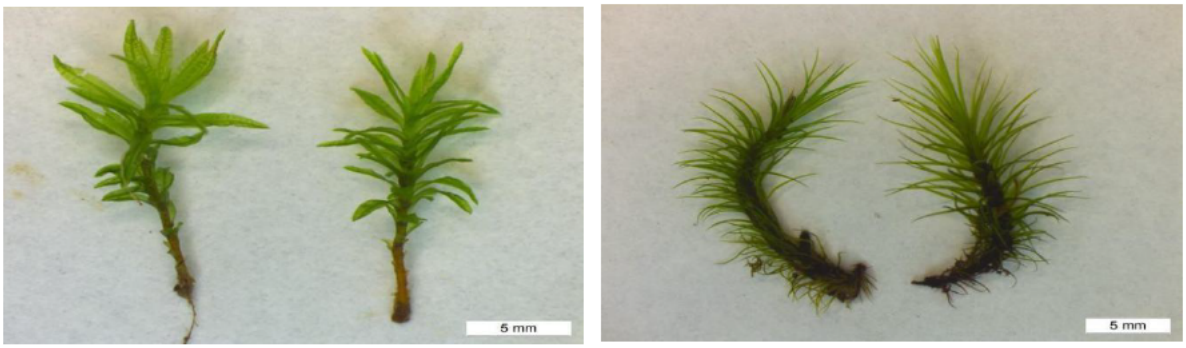
drying, and the recovery from stress quantified. Constitutively tolerant species will recover well irrespective of the speed of drying, while species possessing largely inducible mechanisms will recover much better when drying is slow rather than fast.

1.9. Introduction to the present study

The moss flora of the Afromontane vegetation around Pietermaritzburg is dominated by two large acrocarpous mosses, *Atrichum androgynum,* and *Dicranella subsubulata*. *A. androgynum* tends to grow in wetter, more shaded habitats and is a rather delicate species, while *D. subsubulata* grow in open, drier habitats and are more robust. Rarely, the species grow together, for example at the transition of a shaded indigenous woodland to plantations. We hypothesized that the more robust species have higher stress tolerance and has largely constitutive stress tolerance mechanisms. By contrast, we hypothesized that the more delicate species are less tolerant and may have inducible tolerance mechanisms. The present study is an autecological investigation that attempts to understand the distribution of these species by comparing their DT and tolerance to high light stress. We also studied fundamental questions about stress tolerance in mosses by testing whether stress tolerance can be induced in the two contrasting species.

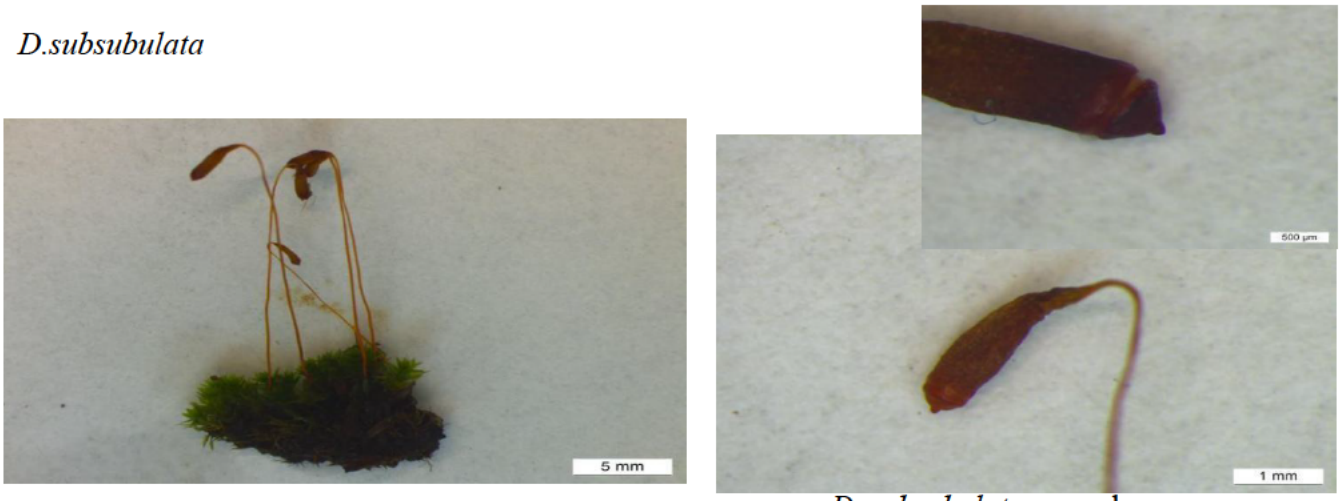
2.1. Plant Material

Dicranella subsubulata was collected at Worlds View, Pietermaritzburg. The area is predominantly covered by commercial plantations but there are small pockets of undisturbed, original grassland and indigenous forest (29°58’3866” S, 30°. 32’79’97” E). *Atrichum androgynum* was collected from boulders in the understorey of an Afromontane forest in Ferncliffe Nature Reserve (29°33'02.2"S 30°20'29.6"E), along the riverbanks at Fort Nottingham nature reserve (29°41’5791” S, 29°. 91’49’30” E) and in Afromontane forest in Chase Valley, next to Cascade Mountain Bike Park (29°56’5738” S, 29°. 34’67’78” E). Fresh material was cleaned with tap water followed distilled water to remove all debris from collection sites and rehydrated by storing on wet filter paper at 10^0C and 30 µmol m^{-2} s^{-1} dim light in a growth cabinet for several days before experiments, to “de-harden” and remove any residual effects offield stress.



A. androgynum D. subsubulata

D.subsubulata



D. subsubulata, capsule

Fig 3: *A. androgynum* and *D. subsubulata* stem segments and *D. subsubulata* growth form and reproduction structure spores.

Fig 4: Rare example of *A. androgynum* growing with *D. subsubulata* from Cascades, Pietermaritzburg.

2.2. Chlorophyll fluorescence on fresh and stored mosses

For both *D. subsubulata* and *A. androgynum* chlorophyll fluorescence was measured using a Hansatech Instruments FMS 2 modulated fluorometer. Stem segments were clamped in standard Hansatech leaf-clips. To take a measurement, each replicate was pretreated in darkness for a minimum of 10 min, and then F_o and F_M were recorded. The actinic light was then switched on, and F_t and $F_{M'}$ measured after several minutes (when F_t had become stable). To test the effect of storage, chlorophyll fluorescence measurements were taken for fresh material before the material was stored for 7 d at 10^0C 30 µmol m^{-2} s^{-1}. The maximal efficiency of PSII (F_V/F_M)was calculated as:

$F_V/F_M = (F_M – F_o)/F_M$

where F_M is the maximum fluorescence (photosynthetic reaction centres closed) and F_o is the minimum fluorescence (photosynthetic reaction centres open).

The electron transfer rate (ETR) was estimated as:

ETR = ΦPSII x 0.5 x PFD

where ΦPSII (the operating efficiency of PSII) = $(F_{M'} – F_t)/F_{M'}$

F_t is the stable fluorescence signal in the light, and $F_{M'}$ is the maximum fluorescence with a saturating light intensity pulse in the light.

Non-photochemical quenching (NPQ) was estimated using the Stern-Volmer quotient:

$NPQ = (F_M - F_{M'})/F_{M'}$

2.3. The effect of light on recovery following photoinhibition

For both *D. subsubulata* and *A. androgynum* fresh 2 cm apical stem segments were used. Mosses were dark-adapted for 10 min before fluorescence measurements. F_V/F_M, ETR, and NPQ at 46 µ mol $m^{-2}s^{-1}$ were measured using a Hansatech Instruments (King's Lynn, UK) FMS 2 modulated fluorometer. Apical stem segments were placed in a petri dish with wet filter paper, with each treatment comprising 10 replicates, and stored overnight at 10^0C 30 µmol m^{-2} s^{-1}, in growth chamber. Two open petri dishes with 10 stem segments were treated with 1200 µmol $m^{-2}s^{-1}$ bright lab light for 4 h. High-light stress was applied by a white LED panel (Model SL-3500, Photon System Instruments, Brno, Czech Republic). During recovery, one petri dish was kept in the dark, while the other was kept in the growth chamber. Measurements were

taken at 4, 5, 6, 7 8, and 10 h after the start of the experiment, and then again 24 and 25 h.

2.4. The effect of slow desiccation using $CaCl_2$

Materials (10 segments from each species) were slowly dried above a saturated solution of $CaCl_2$ giving RH of c. 40% for 48 h and 1 week at 10°C, 30 µmol m^{-2} s^{-1}. Segments were rapidly rehydrated in the dark and chlorophyll fluorescence parameters were estimated every 10 min for 120 min and again after 1440 min.

2.5. Effect of slow and fast desiccation on RWC and chlorophyll fluorescence parameters

The turgid masses of the apical stem segments were determined by weighing blotted material that had been soaked in 25 ml distilled H_2O for 10 min, with gentle shaking on an orbital shaker. The relative water content (RWC) during drying was estimated as (fresh mass after drying − dry mass)/ (turgid mass − dry mass). Mosses were dried for 72 h at 70°C to obtain their dry mass. RWC was therefore estimated as:

RWC= (FM-DM)/(TM-DM)

FM – Fresh mass (mass at any given time)

TM – Turgid mass

DM – Dried mass (oven dried at 70˚C).

For both *D. subsubulata* and *A. androgynum*, 2 cm apical stem segments were cut. Chlorophyll fluorescence parameters were measured using a Hansatech Instruments FMS 2 modulated fluorometer. Silica gel and sodium chloride (NaCl) were used as desiccants, providing fast and slow drying respectively. For each treatment 10 stem segment for each species were used. Treatments were as follows; First, both *D. subsubulata* and *A. androgynum* were desiccated above a saturated solution of NaCl (giving a RH of c. 75%) for 72 h, with measurements being made after 0, 4, 24, 48 and 72 h. Second, both *D. subsubulata* and *A. androgynum* were desiccated using NaCl for 24 h followed by silica gel for 48 h, and measured 0, 4, 24 and 96 h after the start of the experiment. In addition, *A. androgynum* was desiccated using silica gel for 48 h, and measured after 0, 4, 48 h. *D. subsubulata* was desiccated using NaCl for 24 h followed by silica gel for 144 h, and measurements taken after 0, 4, 24, 48 and 144 h. In all cases, mosses were rapidly rehydrated in liquid water, and measurements taken after 4 and 24 h.

2.6. The effect of ABA treatment on desiccation tolerance

D. subsubulata and *A. androgynum* pre-treated with 100 µM ABA (cis, trans; Sigma) which was dissolved with 0.1% DMSO. The pHs of the ABA solution and the water control (also containing 0.1% DMSO) were adjusted to 5.6 with HCl. Apical stem segments were cut, and 15 were gently shaken in ABA for 10 min, while 10 (the controls) were treated with distilled H_2O. Samples were first vacuum infiltrated in H_2O or 100 µM ABA solution to promote ABA uptake. Stem segments were transferred to Petri dishes with filter paper and left in the growth cabinet (10^0C at 25 µ mol m^{-2} s^{-1}) for 3 d without drying out. *D. subsubulata* was then dried for 1 week over silica gel in a desiccation chamber. *A. androgynum* was dried for 48 h above silica gel. Mosses were rehydrated using distilled H_2O, and chlorophyll fluorescence parameters (F_V/F_M, NPQ, and ETR) were estimated every 15 min for 3 h and 24 h as above using the FMS 2 modulated fluorometer.

2.7. The effect of a combination of ABA and low light treatment on the susceptibility of mosses to high light treatment

Apical stem segments (three replicates of 10) of *D. subsubulata* and *A. androgynum* were pre-treated by gently shaking in 100 µM ABA (cis, trans; Sigma) (dissolved in 0.1% DMSO) or distilled H_2O for 1 h. The pHs of the ABA solution and the water control (also containing 0.1% DMSO) were adjusted to 5.6 with HCl. Samples were first vacuum infiltrated in H_2O or 100 µM ABA solution to promote ABA uptake. ABA treated stem segments were pretreated with low light stress, 400 µmol m^{-2} s^{-1} for 4 h and left overnight at 12ºC 30 µmol m^{-2} s^{-1} and then treated with high light stress, 1200 µmol m^{-2} s^{-1} for 4 h the next day. Control 1 segments were only treated with high light stress, 1200 µmol m^{-2} s^{-1} for 4 h on the second day, and Control 2 stem segments were placed on the laboratory light (c. 5 µmol m^{-2} s^{-1}) for 3 d without high light stress. F_V/F_M and NPQ were measured using a Hansatech FMS 2 modulated fluorometer at specific time intervals throughout the experiment (0, 4, 24, 28, 48, and 52 h).

2.8. Determination of POX activity

Stem segments were homogenized in 0.05 M phosphate buffer, pH 7, and then centrifuged at 4ºC, 10000 *g* for 5 min. The reaction mixture comprised of 1 mM *o*-dianisidine (3,3′-dimethoxybenzidine), 1 mM H_2O_2, and 0.05 mM acetate buffer pH 5. The production of oxidized dianisidine was measured spectrophotometrically (ε_{460} = 1.13 X 10^4 M^{-1} cm^{-1}).

2.9. Effect of slow desiccation on enzyme activity

For both *D. subsubulata* and *A. androgynum* changes in the activity of POX were studied during a drying/wetting cycle. Each treatment (sampling time) contained five replicates each comprising of five 2 cm apical stem segments (*c*. 0.04 g dry mass). The material was initially fully hydrated by gentle shaking on an orbital shaker in distilled H_2O for 1 h, then slow dried by placing stems segment over a saturated solution of $MgCl_2$ (resulting in a relative humidity of 34%) in the growth cabinet for 96 h. At selected time intervals the relative water content (RWC, see below) and POX activity was measured. After grinding as described above, extracts were centrifuged at 4^{o}C in Eppendorf tubes for 5 min at 1000 *g* and supernatants frozen at -24^{o}C until analysis.

2.10. Changes in RWC during slow and fast desiccation

For both *D. subsubulata* and *A. androgynum* 2 cm apical stem segments were cut and placed and used to check for relative water content (RWC) during dehydration and rehydration. Initially, the material was fully hydrated by gentle shaking on an orbital shaker in distilled H_2O for 1 h and weighed to get the turgid mass. Segments of both species were then placed in small (65 mm) Petri dishes (5 replicates of 5 segments). To compare the effect of slow and rapid dehydration, Petri dishes with stem segments were placed for 48 h above a saturated solution of $MgCl_2$ (giving a relative humidity of c. 40%) or silica gel (giving a relative humidity of 0%) in the growth cabinet as described above. Segments were weighed after 4, 8, 24, 28, 32, and 48 h and were then rapidly hydrated by placing in distilled water and weighed again after 4 and 8 h (corresponding to 52 and 56 h from the start of the experiment). Mosses were dried for 48 h at 70°C to obtain their dry mass. RWC was estimated as above.

2.11. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was used to test for the presence of peroxidase isoforms in the two species. Fresh apical stem segments were homogenized using phosphate buffer pH 7 and then the extracts were centrifuged at 3^{o}C for 5 min at 10000 *g*. *A. androgynum* extracts were concentrated by dialysis on solid sucrose overnight, and then reverse dialyzed again with phosphate buffer pH 7 overnight, to remove sucrose from the samples. Extracts were further concentrated by centrifugation in Millipore “microcons” with a molecular cut-off of 10 000 Da according to manufactures recommendations. Extracts from *D. subsubulata* did not need concentrating.

A modification of the method of Laemmli (1970) was used, using native 12% and 6% gels. Peroxidase activity was visualized using acetate buffer (0.25 M, pH 5.0) containing 10% glycerol and 1 mM *o*-dianisidine with 2 mM H_2O_2. To test for superoxide production, Na-phosphate buffer (50 mM, pH 7.4) containing 10% glycerol, 0.1 mM $MgCl_2$, and 1mM $CaCl_2$ was used to wash the gel for 30 min. The gel was then incubated in the dark with the same buffer containing 0.4 mM NADH and 0.5 mM NBT for 30 min. Serrano at al. (1994) method of staining was followed to estimates the molecular sizes of DNA.

3.1 Light response curves

D. subsubulata and *A. androgynum* are adapted to relatively high light intensities, and both species were affected by storage at 10°C, 30 µmol m^{-2} s^{-1} for one week (Fig 5). However, ETR clearly saturated at higher light intensities in *D. subsubulata* than *A. androgynum*. Even at 500 µmoles $m^{-2}s^{-1}$ ETR in *D. subsubulata* was not quite saturated, while in *A. androgynum* ETR appeared to saturate at around 200 µmoles m^{-2} s^{-1}. Similarly, NPQ kept increasing at higher light intensities in *D. subsubulata*, while only increasing a little beyond 200 µmoles m^{-2} s^{-1} in *A. androgynum*. In *D. subsubulata* storage in cool dim conditions reduced ETR and NPQ. The effects were similar in *A. androgynum*, although at lower light intensities (below 200 µmoles m^{-2} s^{-1}) storage increased NPQ.

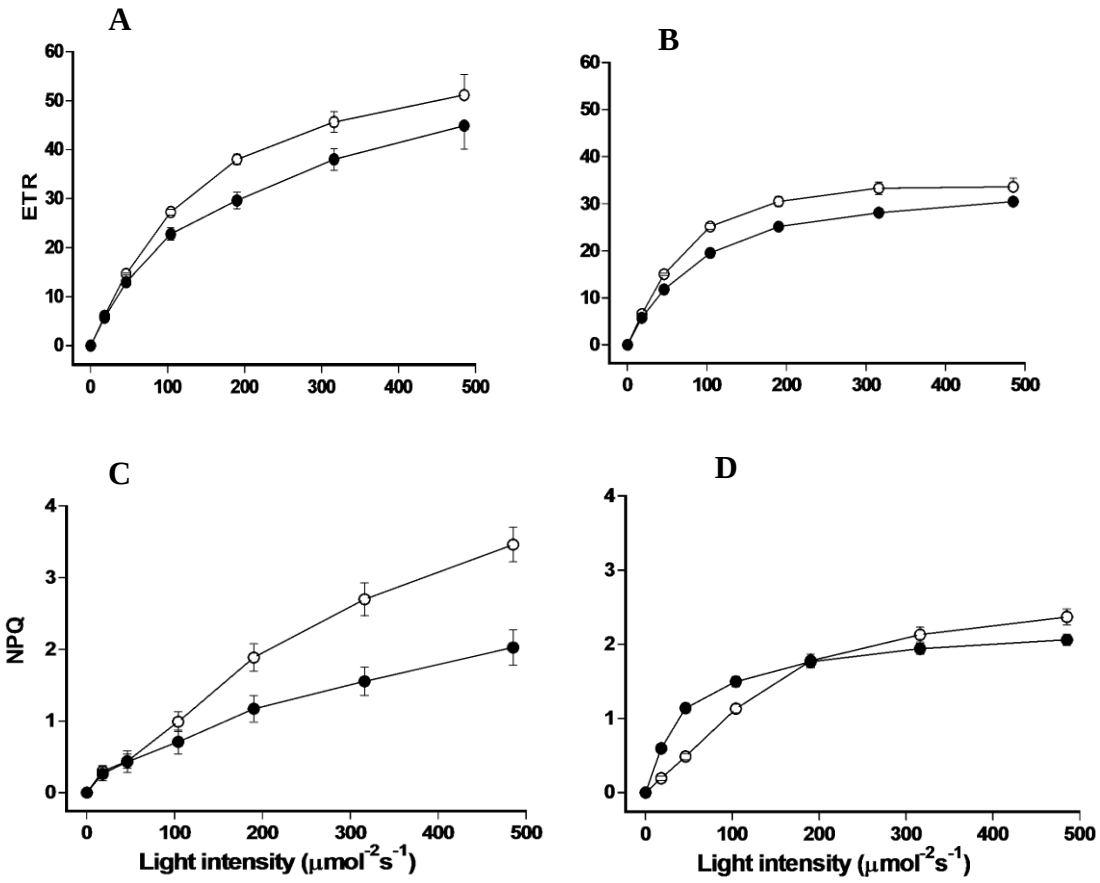


Fig 5: Light response curves of *D. subsubulata* (A, C) and *A. androgynum* (B, D) in freshly collected material and material stored at 10°C, 30 µmol m^{-2} s^{-1} for 7 d. Open circles represent fresh and closed circles represent stored material. Error bars indicates standard error of the mean (mean ± S.E), n=10.

3.2 Recovery from photoinhibition in dim light and the dark

The effect of light during the recovery from photoinhibition was assessed by measuring the chlorophyll fluorescence parameters F_V/F_M, ETR and NPQ in *D. subsubulata* and *A. androgynum* (Fig 6). Mosses were given a high light stress of 1200 µmol m^{-2} s^{-1} for 4 h. *A. androgynum* was more sensitive than *D. subsubulata*. F_V/F_M was reduced to just below 0.6 in *D. subsubulata*, but to c. 0.4 in *A. androgynum*, and ETR was reduced to a greater extent in *D. subsubulata* than in *A. androgynum*. NPQ was reduced much more by high light stress in *A. androgynum* than *D. subsubulata*. In both species, the recovery of F_V/F_M and ETR was similar in the light and the dark for the first 6 h. However, for both species, recovery 20 h after exposure was significantly better if material was allowed to recover in the light. For ETR, transferring material that had been allowed to recover in the dark to the light resulted in rapid recovery to values similar to those of material that the recovered in the light. In *A. androgynum*, while light stress reduced NPQ, values gradually recovered, and were similar in the light and the dark.

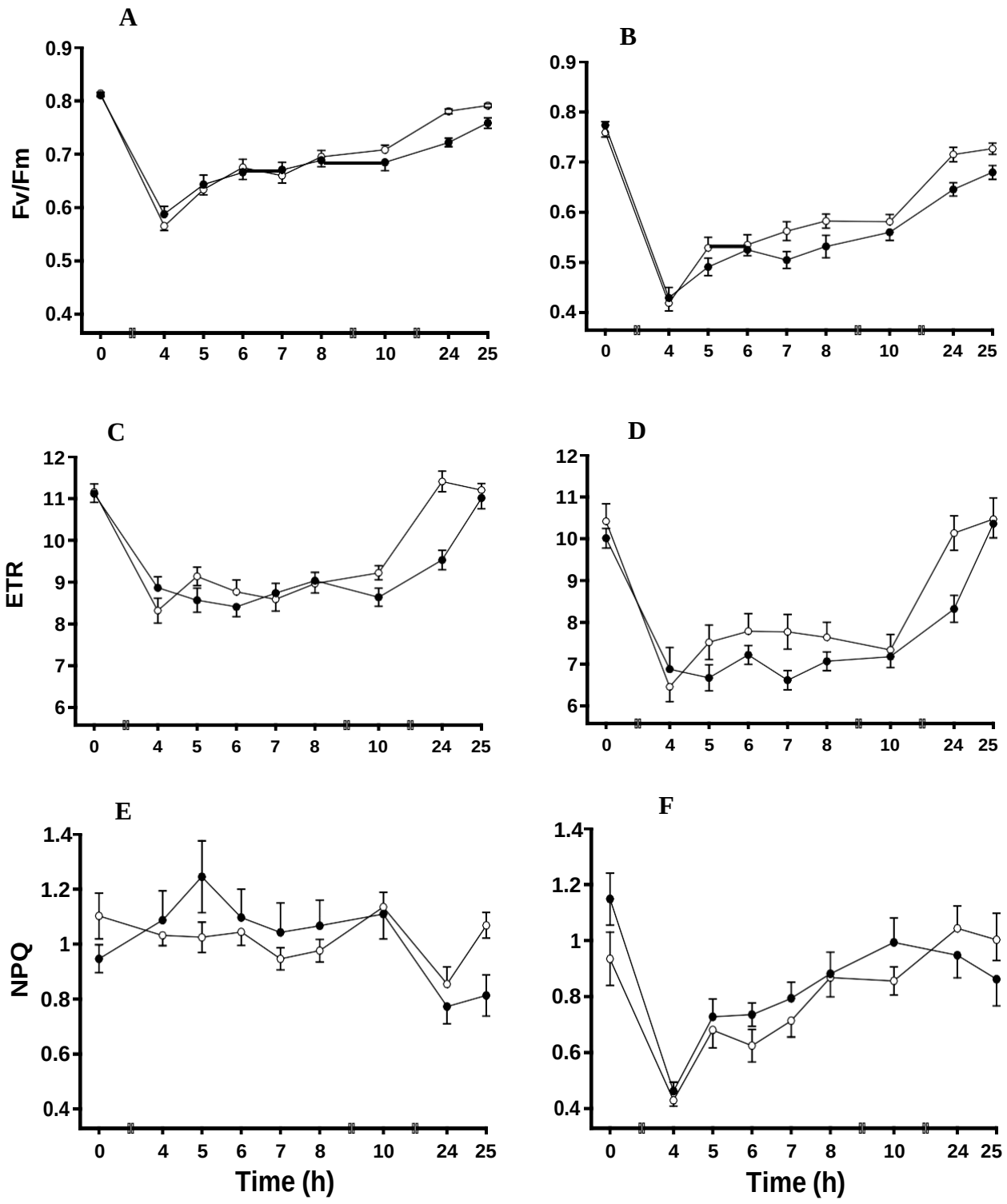


Fig 6: The effect of photoinhibition on *D. subsubulata* (A, C, E) and *A. androgynum* (B, D, F) Mosses were exposed to 1200 µmoles m^{-2} s^{-1} for 4 h. From 24 to 25 h the "dark" recovery segments were transferred to light. "Light" corresponds to laboratory light, around 5 µmoles m^{-2} s^{-1}. Open circles represent *D. subsubulata* and *A. androgynum* recovering in the light, while

closed circles represent material recovering in the dark. Error bars indicates standard error of the mean (mean ± S.E), n=10.

3.3. Effect of slow desiccation over a saturated solution of $CaCl_2$

The effect of slow desiccation on chlorophyll fluorescence parameters was assessed by storing material above a saturated solution of $CaCl_2$. *A. androgynum* was more sensitive to desiccation than *D. subsubulata* (Fig 7). *D. subsubulata* recovered fast following desiccation for 48 h, F_V/F_M, ETR and NPQ initially recovery quickly (within 10 min), ETR reached initial values after 24 h. After desiccation for 1 week, recovery of fluorescence parameters was very slow, and even after 24 h was only c. 30% of the initial values. By contrast, in *D. subsubulata*, recovery was faster, and reached initial values even after desiccation for 1 week.

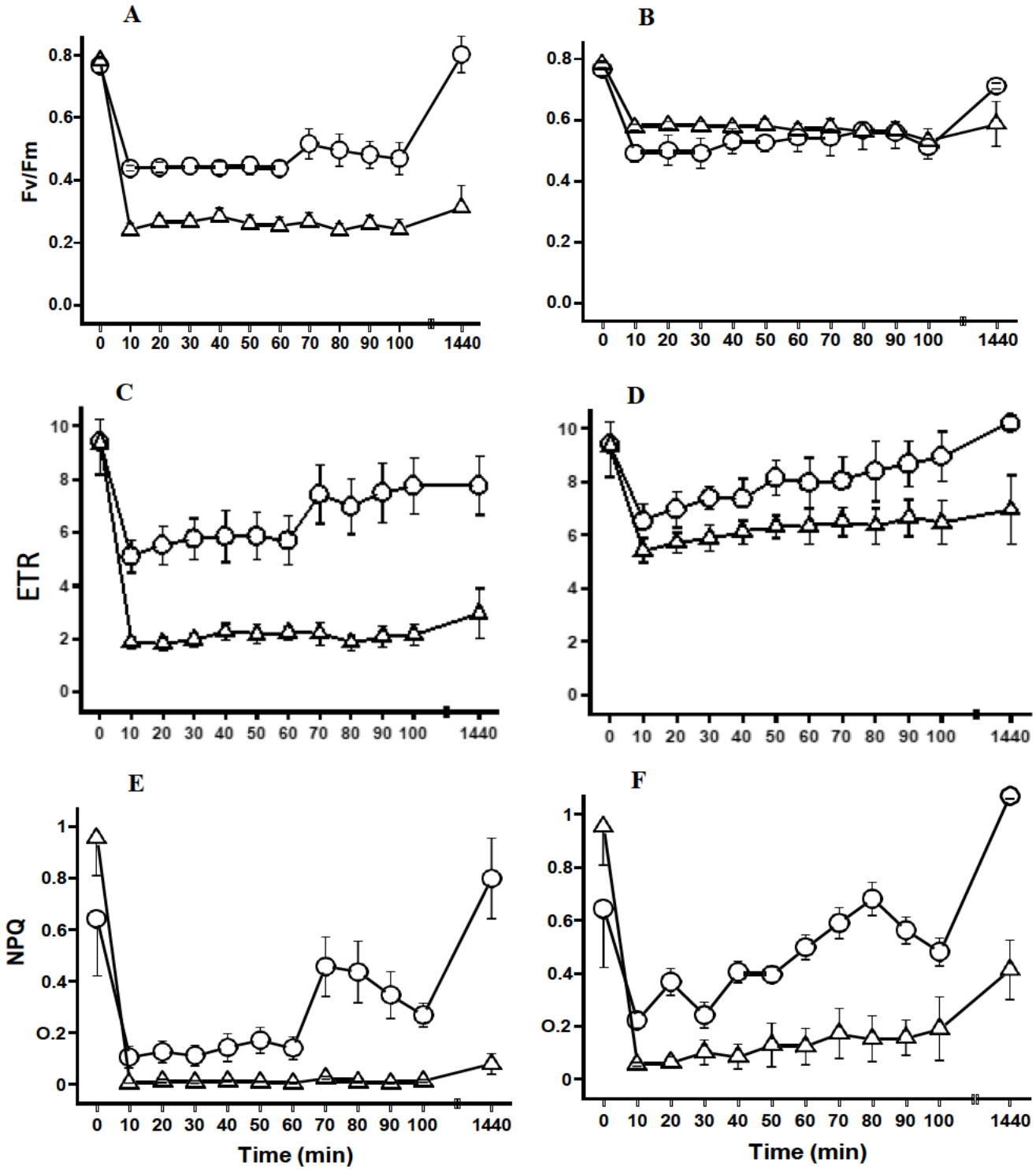


Fig 7: The effect of slow desiccation using calcium chloride ($CaCl_2$) on *D. subsubulata* (open circle) and *A. androgynu*m (open triangles) for 1 week (A, C, E) and 48 h (B, D, F) followed by rapid rehydration. Values at time zero correspond to initial values. Recovery was measured every 10 min for 2 h and the next day, corresponding to 24 h after the start of rehydration. Error bars indicate the standard error of the mean (mean ± S.E), n=10.

3.4. Effect of slow and fast desiccation on RWC

D. subsubulata and *A. androgynum* lost at least 60% and 80% respectively of their water content after 72 h when desiccated above NaCl (Fig 8A). Desiccating *D. subsubulata* and *A. androgynum* above NaCl for 24 h followed by 96 h above silica gel reduced water contents to low values (Fig 8B). When desiccating the mosses above silica gel, *A. androgynum* quickly lost 60% water after 4 h (Fig 8C). *D. subsubulata* only lost 30% of RWC in 4 h of slow drying but lost 70% of its water content after 48 h of fast desiccation (Fig 8C).

3.5. Effect of slow and fast desiccation on photosynthesis

Slow desiccation for 72 h above NaCl, progressively reduced FV/FM, ETR and NPQ in both *D. subsubulata* and *A. androgynum* (Fig 9A, C, and E). Chlorophyll fluorescence parameters rapidly recovered to their original values following rehydration in both species. When desiccated slowly above NaCl, and then placed above silica get, in both *D. subsubulata* and *A. androgynum* FV/FM and ETR recovered rapidly and completely (Fig 9 B, D). While NPQ fully recovered in *D. subsubulata*, in *A. androgynum* NPQ initially increased and then then decreased to low values (Fig 9F). By contrast, following rapid drying for 48 h above silica gel, none of the chlorophyll fluorescence parameters in *A. androgynum* fully recovered (Fig 10A, B, C). In *D. subsubulata* FV/FM fully recovered (Fig 10A). Although the recovery of ETR and NPQ was incomplete for both species, *D. subsubulata* recovered better than *A. androgynum* (Fig 10B, C).

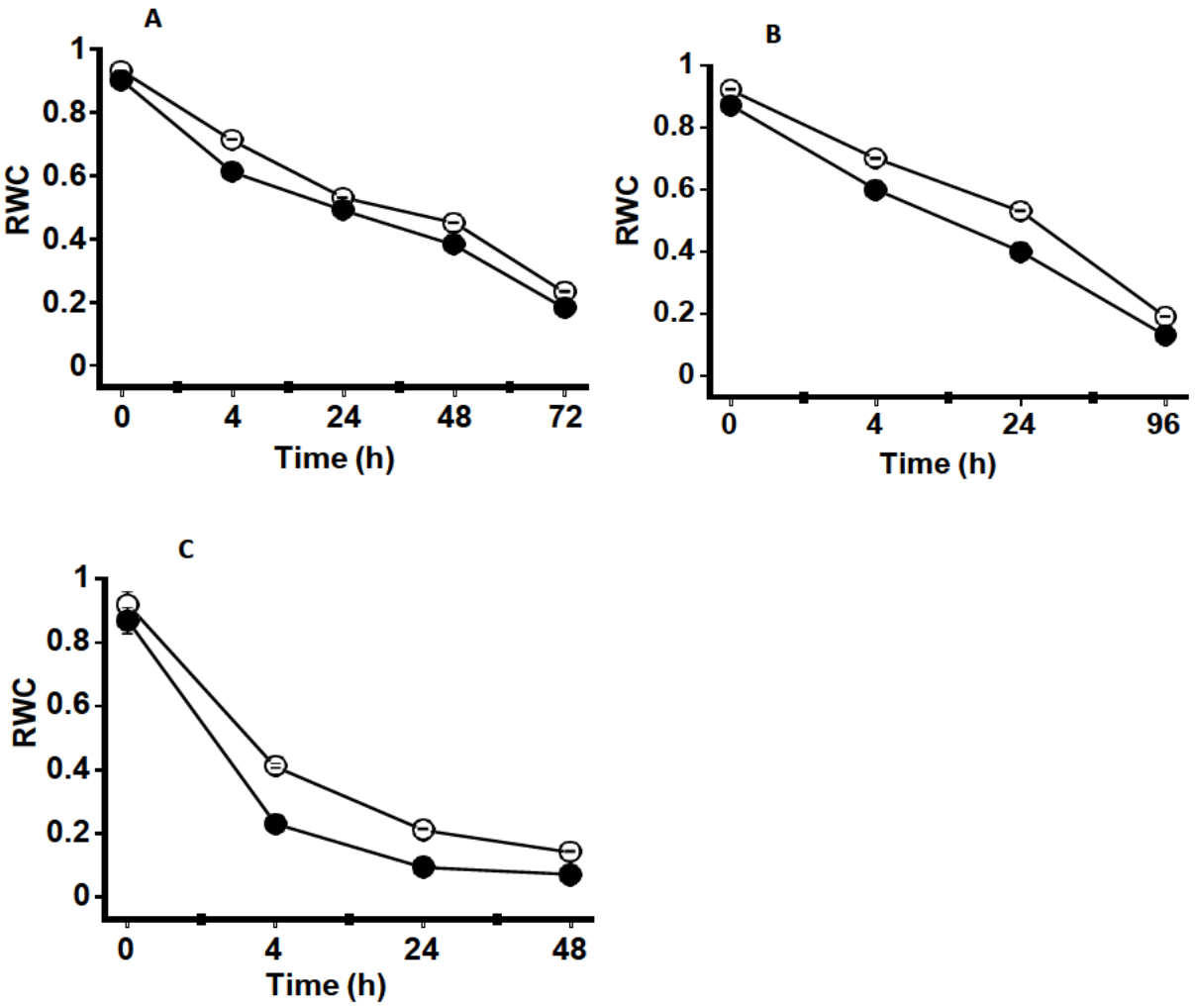


Fig 8: The effect of desiccation on the RWC of *D. subsubulata* and *A. androgynum*. A. The effect of slow desiccation using NaCl on the RWC of *D. subsubulata* (open circle) and *A. androgynum* (closed circle). B. The effect on RWC in *D. subsubulata* and *A. androgynum* desiccated initially over NaCl for 24 h followed by silica gel for 48 h (total time 96 h). C. The effect of fast desiccation using silica gel on the RWC of *A. androgynum* and *D. subsubulata*. Error bars indicate the standard error of the mean (mean ± S.E), n=10.

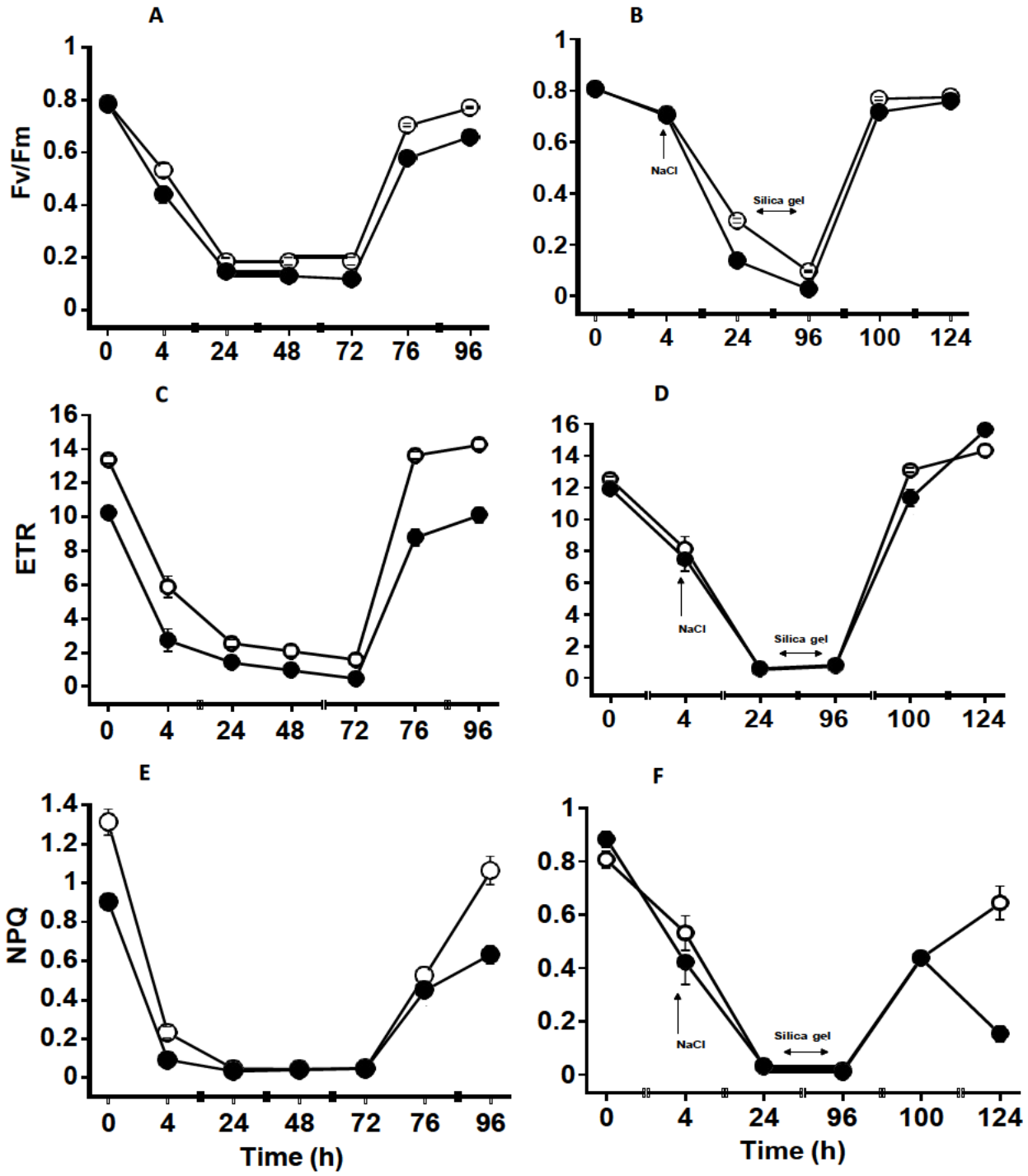


Fig 9: The effect of slow desiccation using sodium chloride (NaCl) on *D. subsubulata* (open circles) and *A. androgynu*m (closed circles) for 72 h (A, C, E) and effect of slow (NaCl) followed by fast desiccation (silica gel) (B, D, F) followed by rapid rehydration. Values at time zero correspond to initial values. Error bars indicate the standard error of the mean (mean ± S.E), n=10.

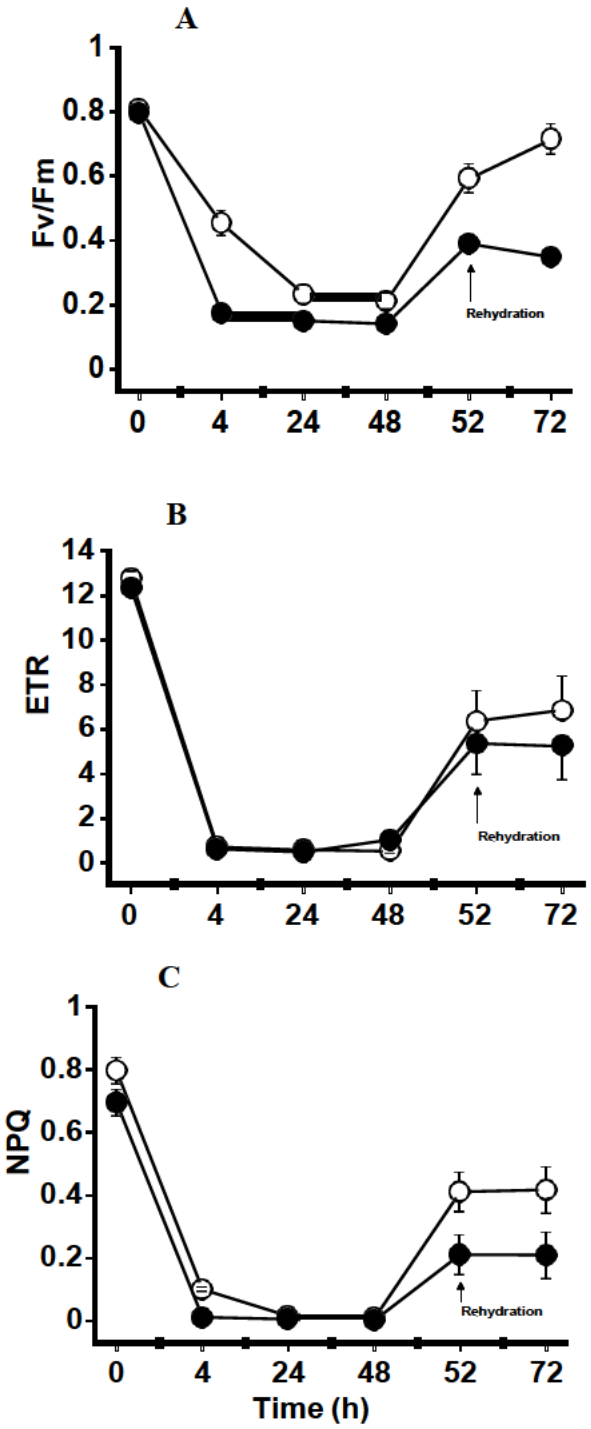


Fig 10: The effect of fast desiccation using silica gel on *D. subsubulata* and *A. androgynu*m for 48h (A, C, E). Recovery for fast desiccation was measured for 4, 24, and 48 h and rehydrated for 52 and 72 h corresponding to 4 and 24 h after the start of rehydration. Values at time zero correspond to initial values. Error bars indicate the standard error of the mean (mean ± S.E), n = 10.

3.6. The effect of ABA pretreatment on desiccation tolerance

D. subsubulata and *A. androgynum* were both pre-treated with 100 µM ABA or distilled water and desiccated using silica gel for 1 week. Chlorophyll fluorescence parameters of both species of moss recovered well following desiccation (Fig 11). Pretreatment with ABA improved the rate of recovery of ETR in both species, but only improved the rate of recovery of F_V/F_M in *D. subsubulata*. Overall, *D. subsubulata* was more desiccation tolerant than *A. androgynum* (Fig 11). NPQ recovered more slowly than the other parameters, and ABA increased the rate of recovery in *A. androgynum* but not *D. subsubulata* (Fig 11 E, F).

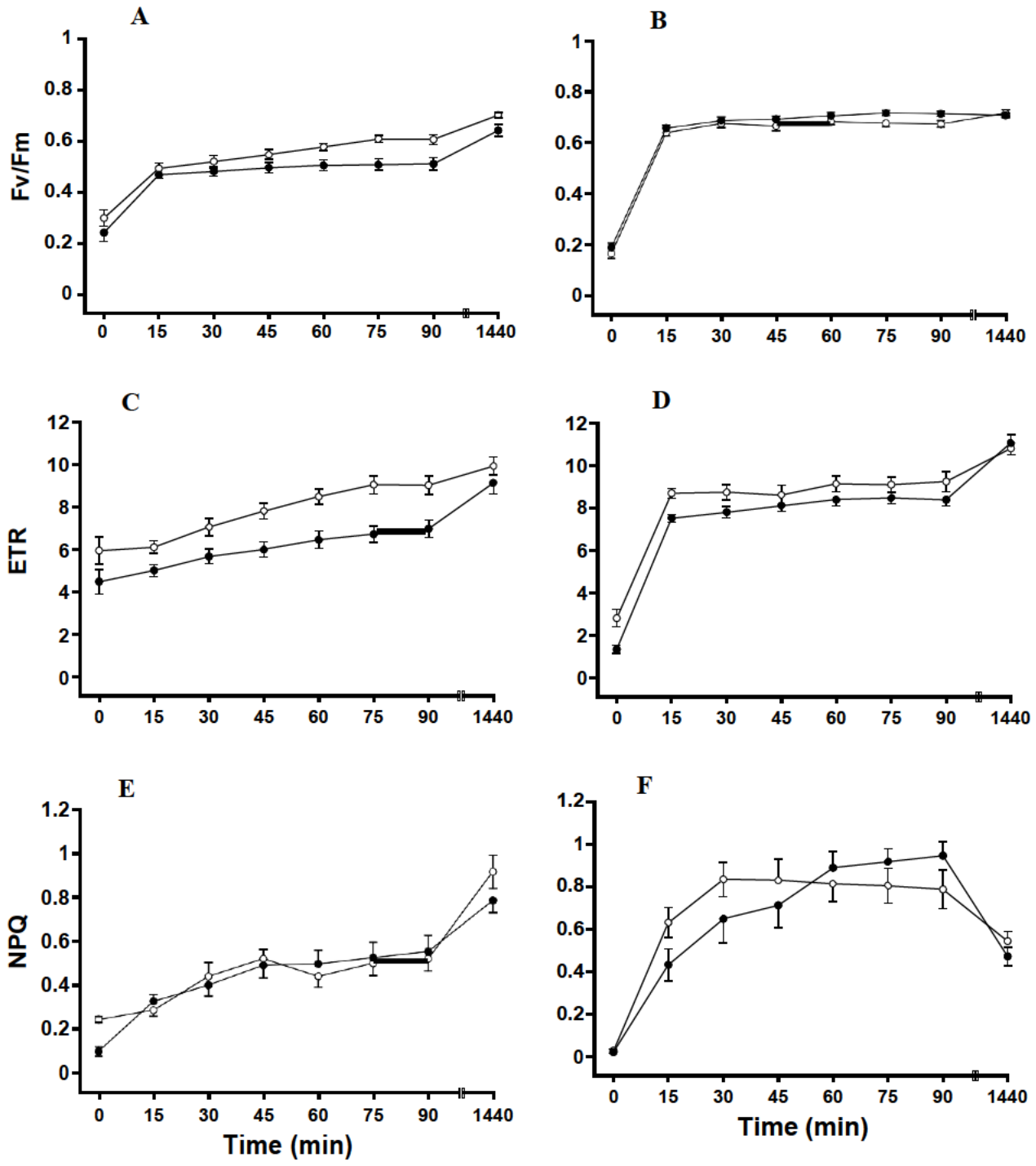


Fig 11: Effects of pretreatment with abscisic acid (ABA) on DT in *D. subsubulata* (A, C, E) and *A. androgynum* (B, D, F). Segments were treater with H_2O or ABA and stored for 3 d in $10^0C \pm 30^0$ C µmol m^{-2} s^{-1}, desiccated using silica gel, and then rapidly rehydrated. Parameters were measured during rehydration. Control material is indicated by closed circles, and ABA treated material by open circles). Error bars indicates standard error of the mean (mean ± S.E), n = 15.

3.7. ABA and light stress

Pretreatment of *D. subsubulata* and *A. androgynum* with low light (400 µmol m^{-2} s^{-1}) and ABA had effect on the sensitivity to subsequent high light treatment (1200 µmol m^{-2} s^{-1}) (Fig 12). ABA, low light pretreatment and high treatment decrease the FV/FM of *D. subsubulata* and *A. androgynum*. ABA pretreatment tended to increase NPQ in both *D. subsubulata* and *A. androgynum*. *D. subsubulata* and *A. androgynum* pretreated with ABA and low light were more sensitive to high light than same species treated with high light.

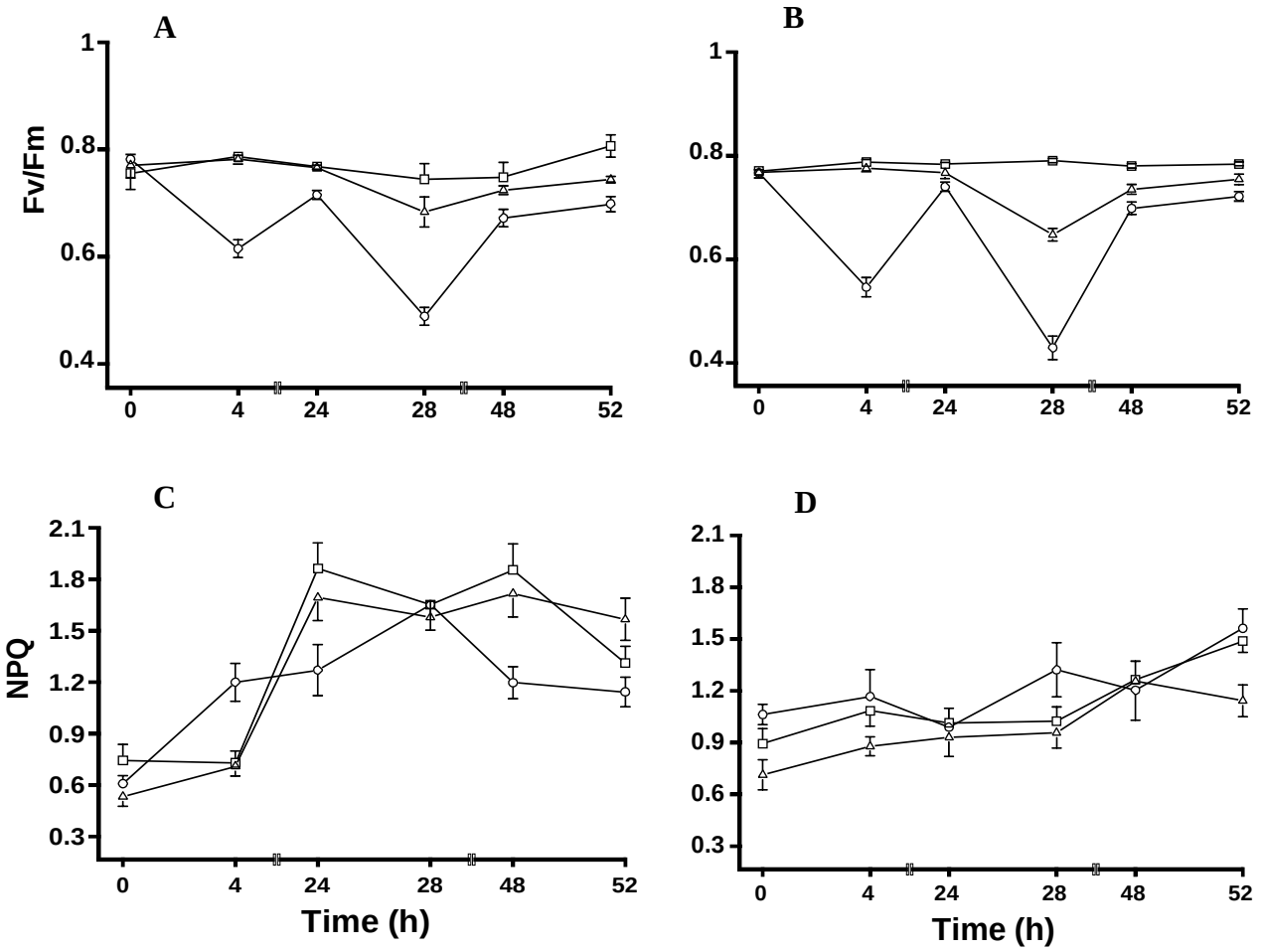


Fig 12: The effect of pretreatment with a combination of light at 400 µmol m^{-2} s^{-1} and ABA on the tolerance of chlorophyll fluorescence parameters to a high light stress of 1200 µmol m^{-2} s^{-1} in *D. subsubulata* (A, C) and *A. androgynum* (B, D). Open circles represent mosses receiving ABA and light at 400 µmol m^{-2} s^{-1} as pre-treatments, and 1200 µmol m^{-2} s^{-1} treatment, open triangles represent mosses receiving only the treatment with 1200 µmol m^{-2} s^{-1} light, squares represent control with neither ABA or light treatment. The light pretreatment (400 µmol m^{-2} s^{-1}) was given from 0 to 4 h, and the light stress (1200 µmol m^{-2} s^{-1}) from 24 to 28 h. Error bars indicate the standard error of the mean (mean ± S.E), n=10.

3.8. The effect of desiccation of the activity of POX

Stem segments of *A. androgynum* and *D. subsubulata* were slowly desiccated over a saturated solution of $MgCl_2$; drying curves are presented in Fig13. POX activity in *A. androgynum* was much lower than that of *D. subsubulata* (Fig 14). Slow desiccation of both species increased POX activity. Apart from the absolute differences in activity, the main difference between the two species was that activity continued to increase from 72 to 96 h in *D. subsubulata* but decreased in *A. androgynum*. Statistical analysis showed that the effect of desiccation on POX activity was significant on *D. subsubulata*, $p<0.05$ but not significant on *A. androgynum*, $p>0.05$ (Tables 1 and 2).

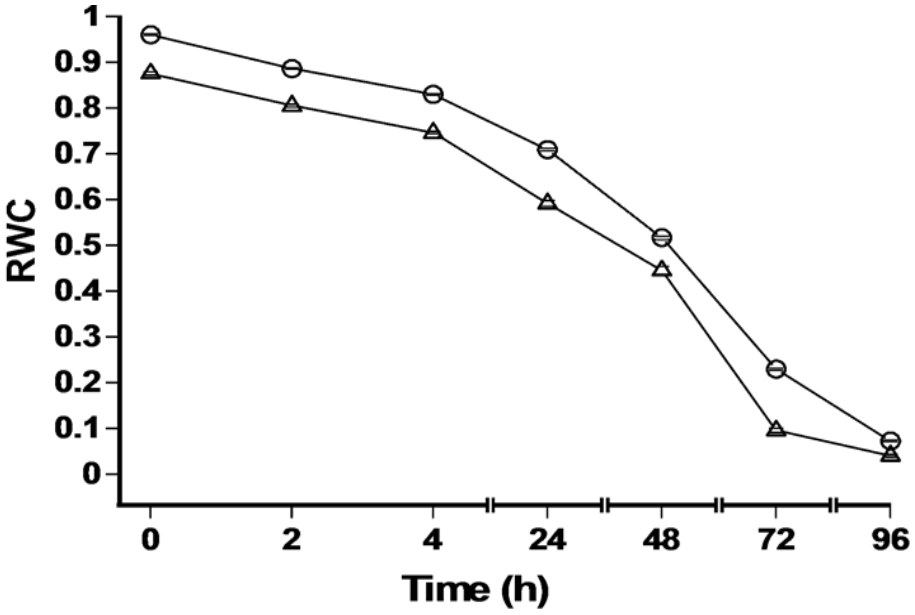


Fig 13: Slow drying curves of *D. subsubulata* and *A. androgynum* desiccated above a saturated solution of $MgCl_2$. *D. subsubulata* is represented by open circles and *A. androgynum* by open triangle. Error bars indicates standard error of the mean (mean ± S.E).

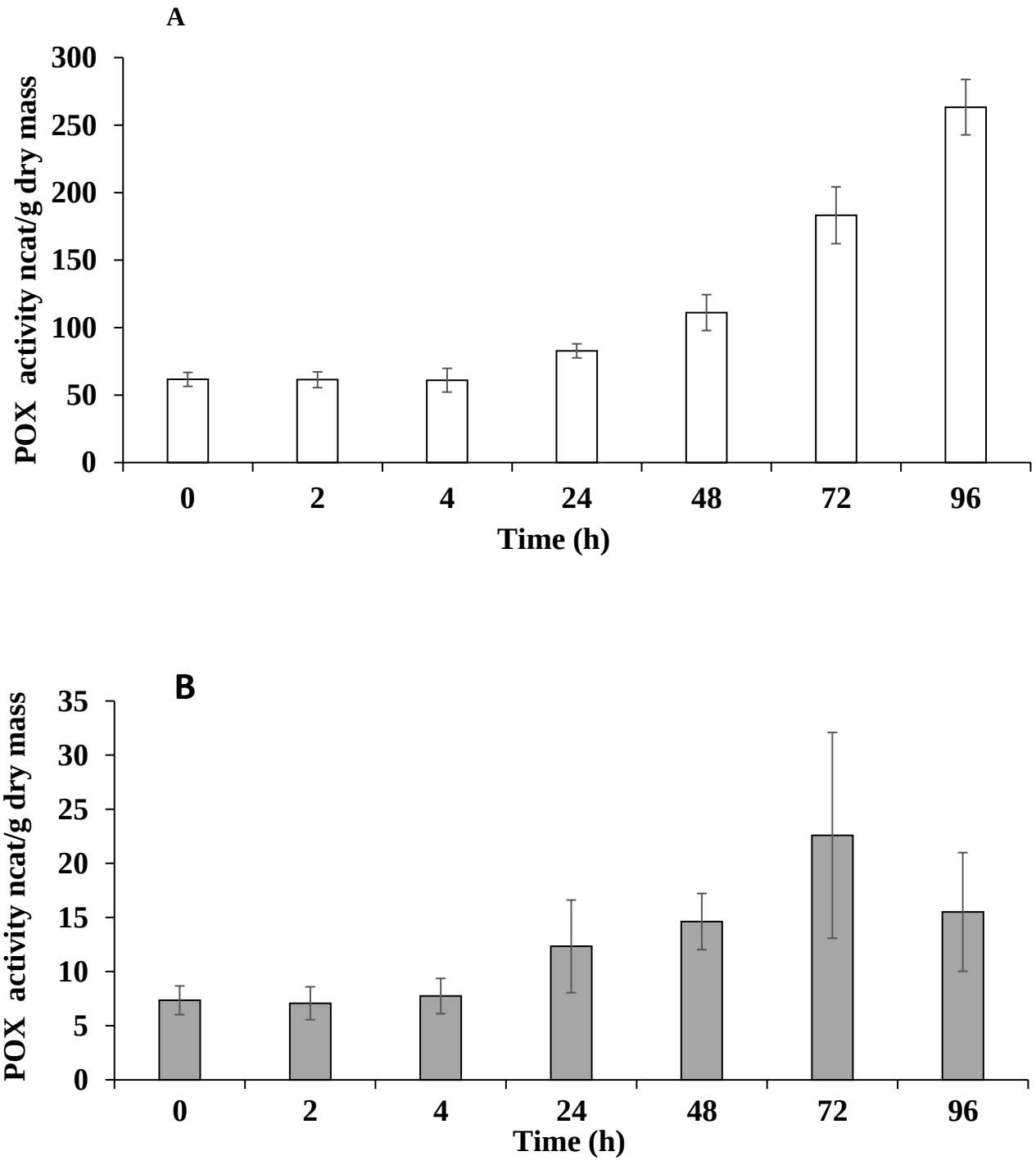


Fig 14: The effect of slow desiccation for 96 h on POX activity in *D. subsubulata* (A) and *A. androgynum* (B). Error bars indicates standard error of the mean (mean ± S.E), n=20.

Table 1: The effect of time on POX activity of *A. androgynum* dried over a saturated solution of $MgCl_2$ for 96 h.

ANOVA	*Atrichum androgynum*					
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	969.1642	6	161.5274	1.485047	0.219423	2.445259
Within Groups	3045.538	28	108.7692			
Total	4014.702	34				

Table 2: The effect of time on POX activity in *D. subsubulata* dried over a saturated solution of $MgCl_2$ for 96 h.

ANOVA	*Dicranella subsubulata*					
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	181351.5	6	30225.25	28.4024	0.00	2.445259
Within Groups	29797.02	28	1064.179			
Total	211148.6	34				

3.8. Analysis of Peroxidase enzyme using PAGE electrophoresis.

Moss extracts were subjected to PAGE electrophoresis followed by visualizations of POX activity using with *o*-dianisidine and H_2O_2. *D. subsubulata* had one POX isoform, with a molecular mass of 57-55 kDa. The electrophoresis was repeated four times, and this isoform was clearly visible in all 4 visualizations (Fig 15 B-E). *A. androgynum* had very low enzyme activity and could not be visualized even after concentration of crude extracts using dialysis on solid sucrose overnight, followed by reverse dialysis to remove sugar. Staining gels of *D. subsubulata* for superoxide production indicated that extracts did not contain measurableamounts of superoxide producing enzymes.

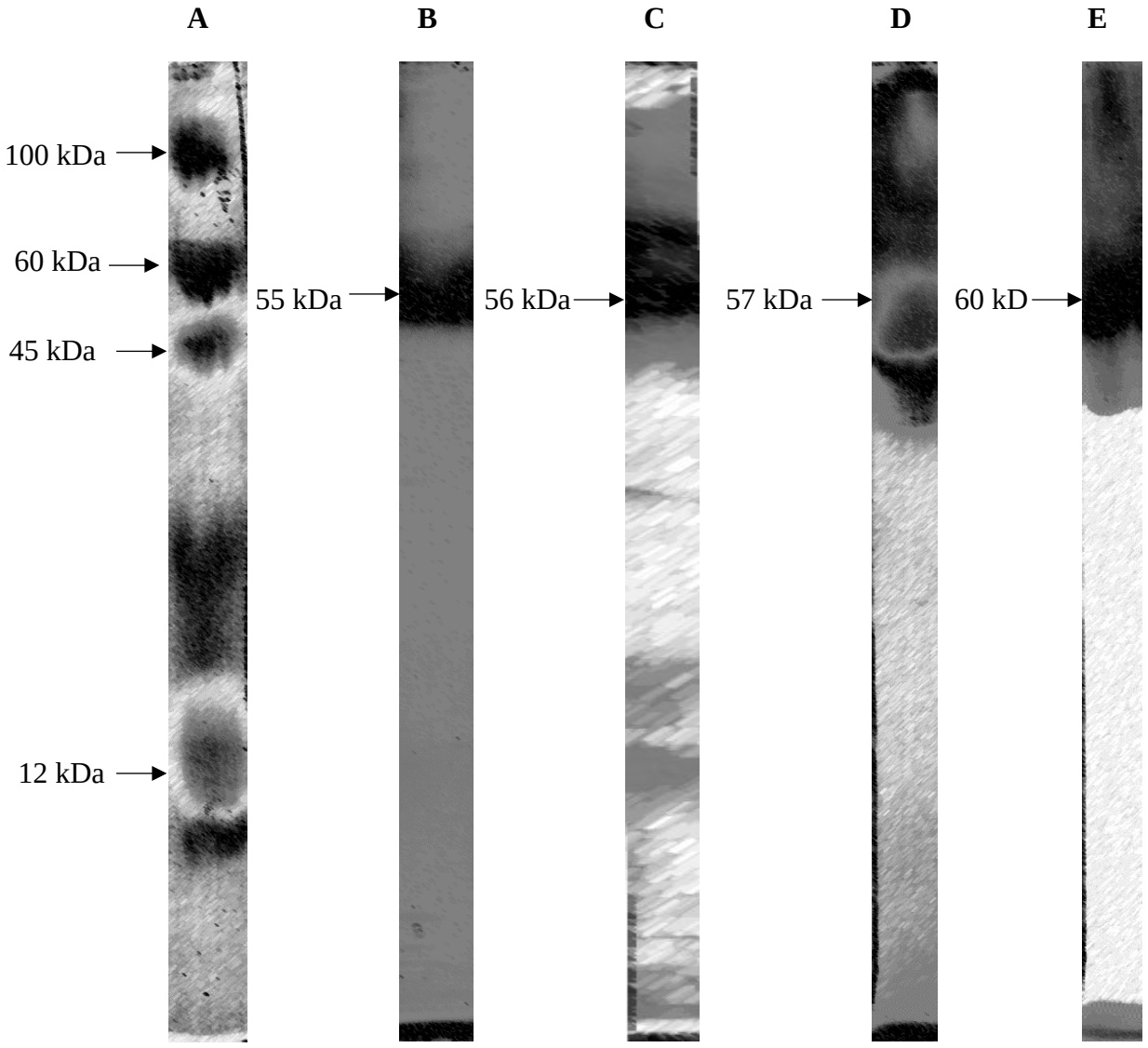


Fig 15: Native PAGE electrophoresis gels of *D. subsubulata* stained for POX activity. The electrophoresis was repeated four times, and this isoform was clearly visible in all visualizations (B, C,D, E). Molecular weight markers are illustrated in lane A.

As discussed in the Introduction, the flora of larger mosses in the Afromontane vegetation around Pietermaritzburg is dominated by two mosses, *Atrichum androgynum*, and *Dicranella subsubulata*. *A. androgynum* tends to grow in wetter, more shaded habitats and is a rather delicate species, while *D. subsubulata* grow in open, drier habitats and is more robust. Rarely, the species grow together, for example at the transition of a shaded indigenous woodland to plantations (Fig 3, 4). We hypothesized that the more robust species has higher stress tolerance, and largely constitutive stress tolerance mechanisms. By contrast, we hypothesized that the more delicate species is less tolerant and may rely more on inducible tolerance mechanisms.

4.1. Response of photosynthesis to light intensity

Results showed that *D. subsubulata* and *A. androgynum* are adapted to shade environments. However, *D. subsubulata* appears to be adapted to higher light levels than *A. androgynum*, reaching A_{MAX} at around 500 μmol m^{-2} s^{-1} (Fig 5A), while *A. androgynum* reached A_{MAX} at around 200 μmol m^{-2} s^{-1} (Fig 3B). Mosses survive in the shade by having specific adaptations to low light intensity (Hájek et al., 2009; Marschall and Proctor, 2004). We expected that species found in more exposure microsites in the exotic plantation forest would have higher photosynthetic capacity than species growing in Afromontane forests. Results were consistent with our expectations, with *D. subsubulata* having a higher maximum ETR than *A. androgynum*. Hájek et al. (2009) reported that the moss genus *Sphagnum*, found in open habitats had lower photosynthetic capacities than the species growing in more shaded environments. As for almost all plants (Osmond et al. 1997), photosynthetic light responses are plastic in the two species studied. Storage in cool dim light reduced maximum rates of ETR (Fig 5). As would be expected from the reduced need for photoprotection, storage reduced NPQ in *D. subsubulata* (Fig 5C) but counterintuitively increased NPQ in *A. androgynum* (Fig 5D). There are no obvious explanations for the result of light intensity in mosses. Results of the present study confirm that *D. subsubulata* has a higher inherent tolerance to desiccation and high light stress than *A. androgynum*.

4.2. Photoinhibition

Both species showed an excellent ability to recover from moderately severe photoinhibition, caused by exposure to 1200 µmoles m^{-2} s^{-1} for 4 h (Fig 6). The recovery is likely due to re-synthesis of the D1 protein which becomes preferentially damaged in moss (Rintamäki and Riitta Salo, 1994). In general, F_V/F_M, ETR and NPQ were all more inhibited in *A. androgynum* than *D. subsubulata*. Consistent with results obtained for lichen photobionts (Solhaug, 2018) photosystems in the two species of mosses tested here need low light to recover from photoinhibition. Although similar for the first 10 h, after 24 h recovery was much better if mosses had been allowed to recover from photoinhibition in low light. Interestingly, transferring mosses that had been kept in the dark to dim light (lab without light on) from 24 to 25 h after the start of the experiment enabled them to recover to similar F_V/F_M and ETR as mosses allowed to recover from photoinhibition in dim light. Reasons for the better recovery of mosses in dim light rather than darkness are unclear, but in higher plants it has been shown that the D1 protein repair cycle is light dependent (Bergo et al., 2003).

4.3. Tolerance to dehydration using $CaCl_2$

Mosses in general are highly desiccation tolerant (Oliver et al. 2020). In initial experiments we tested the ability of D. *subsubulata* and *A. androgynum* to tolerate moderately fast drying for 48 h and 1 week over a saturated solution of $CaCl_2$, corresponding to a relative humidity of c. 35% at 15^{o}C. Both species rapidly recovered after 48 h of desiccation (Fig 7B, D, F), although recovery was slightly faster and more complete in *D. subsubulata* than *A. androgynum*. These mosses can therefore be classified as "fully desiccation-tolerant" (Wood, 2007). However, after desiccation for 1 week, recovery in *D. subsubulata* was much slower than after 48 h, but complete after 24 h of rehydration (Fig 7A, C, E). By contrast, desiccation of *A. androgynum* was probably lethal, with parameters not recovering after rehydration for 24 h. Results clearly showed that *D. subsubulata* was more tolerant to desiccation stress than *A. androgynum*. It is well known that DT in Bryophytes varies greatly between species (Wood, 2007). The differences in DT between *D. subsubulata* and *A. androgynum* here reflect anecdotal observations that *D. subsubulata* grows in generally more exposed habitats than *A. androgynum,* where it is likely to suffer more rapid and profound anddesiccation.

4.4. Effect of slow and fast desiccation on RWC and chlorophyll fluorescence

Results presented here clearly show that *D. subsubulata* is tolerant of both slow and fast drying (Fig 9, 10). *A. androgynum* is tolerant to slow drying (Fig 9A, C, E), and furthermore can survive reaching significant low water contents over silica gel if drying is initially slow (Fig 9B, D, F). However, *A. androgynum* cannot tolerate slow drying followed by storage over silica gel (Fig 10). The implication is that *A. androgynum* requires slow drying to "induce" tolerance mechanisms, while in *D. subsubulata* mechanisms are already constitutively in place. The mechanisms that were induced were not investigated in the present study. However, Mayaba et al. (2001) showed that treating *A. androgynum* with ABA increased both DT and the concentration of soluble sugars. As discussed in the Introduction, sugars server as the cells channel and promotes stabilize membranes, but there is less evidence that sugars play a key role in DT in bryophytes, compared to, for example ferns and higher plants (Zhang et al. 2016). Apart from sugar synthesis, the most likely explanation of the results presented here is that during slow drying *A. androgynum* induces the synthesis of proteins that are responsible for DT. As discussed in the introduction. Proteins induced by desiccation may act directly in a variety ofways to protect mosses during dehydration and rehydration (Oliver et al., 2020).

Therefore, a key finding of the present study is that while *D. subsubulata* can tolerate both rapid and slow drying down to low water contents, *A. androgynum* can survive desiccation down to a low water content (over silica gel), but only if desiccation is initially slow (over NaCl). It cannot survive rapid drying over silica gel only after slow desiccation with NaCl. In other words, what appears to be lethal is not a low RWC, but rather rapid drying. We understand therefore that DT mechanisms are "put in place" during slow drying that enable the moss to survive subsequent low RWCs. This may be less so in *D. subsubulata*, which appears to have more constitutive mechanisms in place. However, even the highly desiccation tolerant moss *Synchytrium* possesses some inducible mechanisms responsible for dessication tolerance during extremely harsh conditions (Stark, 2017). It likely that *D. subsubulata* also has some inducible mechanisms, but probably fewer than *A. androgynum*. In the more shaded natural habitat of *A. androgynum*, there may be a period where the air remains humid after a rainfall event, and mosses are likely to dry slowly. This will enable them to prepare for DT by inducing various mechanisms of DT. The habitat of *D. subsubulata* is more open, and perhaps resembles in some ways the habitats of some lichens, where organisms dry rapidly after rainfall or dewfall events. It seems likely that in such plants a powerful antioxidant

system, and high concentrations of polyols and “DT proteins” such as HSP and LEA proteins must be constitutively present (Gasulla et al., 2021).

4.5. The effect of ABA pretreatment on desiccation tolerance

Desiccation tolerance mechanisms in bryophytes are either inherent (“constitutive”) or can be induced by hardening treatments such as partial desiccation or treatment with the stress hormone ABA (Mayaba et al. 2001; Stark 2017). The moss, *Physcomitrium patens* had been used as a model species to show that ABA plays a major role in inducing stress tolerance in mosses (Oliver et al. 2020). Endogenous ABA in found moss species (Ergün et al. 2002; Werner et al. 1991; Xiao et al. 2017). In the present study, mosses were treated with ABA, and then rapidly dried, to reduce the induction of DT mechanisms during natural drying. ABA treatment significantly improved the ability of the chlorophyll fluorescence parameters F_V/F_M, and ETR of *D. subsubulata* to recover during rehydration (Fig 11A, C). By contrast, the effect of ABA on the ability of *A. androgynum* to recover was much less (Fig 11B, D). ABA had little effect on NPQ in *D. subsubulata* (Fig 11E), but slightly increased NPQ activity of *A. androgynum* during rehydration (Fig 11F). Mayaba et al. (2001) also found that ABA treatment tends to increase NPQ activity. However, increased NPQ did not appear to improve the ability of the mosses to recover from desiccation stress. Taken together, results presented here do not support the original hypothesis that *D. subsubulata* has more constitutive tolerance mechanisms that *A. androgynum*.

Results suggest that *D. subsubulata* is more responsive to ABA than *A. androgynum*. However, for desiccation tolerance, counter to the original hypothesis, results with “hardening” pre-treatments and treatment with ABA showed that *D subsubulata* is as much if not more dependent on inducible tolerance mechanisms as *A. androgynum*. While precise tolerance mechanisms were not investigated here, for desiccation tolerance, induction of the activity of antioxidative enzymes, specifically peroxidases seem likely to be an important component of tolerance. It is worth noting that not all DT mechanisms are inducible by ABA (Blomstedt et al. 2018), and possibly *A. androgynum* possesses more of such mechanisms than *D. subsubulata*. Blomstedt et al. (2018) suggest that in higher plants further research is needed to understand how desiccation tolerance is induced, specifically on the roles of phytohormones such as, strigolactone and other potential xylem-messengers during drying and rehydration. Within the bryophytes, Xiao et al. (2017) found that for *Physcomitrium* the initial drying rate, and not the amount of endogenous ABA, may be critical in the acquisition of desiccation tolerance, also suggesting the presence of non-ABA inducible DT mechanisms

in that species.

4.6. ABA and high light treatment

Attempts to modulate the effects of light stress with a combination of low light and ABA were not successful. Short-term ABA treatment, in combination with a pretreatment with moderate light (400 µmol m^{-2} s^{-1}), did not increase the tolerance of either *D. subsubulata* or *A. androgynum* to photoinhibition (Fig 12). Results were consistent with those of Jia and Lu (2003) who reported that short term ABA treatment increases the sensitivity of maize (*Zea mays*) to photoinhibition. Results presented here suggest that ABA treatment increases the susceptibility of *D. subsubulata* and *A. androgynum* to light stress. An original aim of this thesis was to attempt to modulate the tolerance of the mosses to photoinhibition by using various ABA and moderate light intensities, with the intention to test the hypothesis that *A. androgynum* displays greater plasticity than *D. subsubulata*. Unfortunately, attempts at "hardening" were unsuccessful. While it seems likely that, as for lichens, tolerance to photoinhibition is a plastic character in mosses, more work is needed to establish how tolerance can be modulated to enable our original hypothesis to be tested.

4. 7. Enzyme activity changes in mosses

4.7.1. Peroxidase activity

In general, *D. subsubulata* possesses high peroxide activity and *A. androgynum* has low POX activity (compare the y-axes in Fig 14). The best POX substate for both species was *o*-dianisidine, a commonly used substrate for POX activity (Bania and Mahata 2012). Desiccation induced the level of POX activity in both species (Fig 14). Peroxidase was induced by desiccation even after 4 d (Fig 14). In this experiment, a saturated solution of $MgCl_2$ was used to induce a relatively slow drying of *D. subsubulata* and *A. androgynum* (Fig 13), which would enable any upregulation of POX activity to occur. It seems logical to assume that in both *D. subsubulata* and *A. androgynum* the level of antioxidant enzymes increases or is maintained during dehydration to scavenge desiccation-induced ROS formation. Farrant et al. (2007) reported this as a common feature of desiccant tolerance in higher plants. Liu et al. (2019) studied the effects of various stress on SOD and POX activities in the moss *Hypnum plumaeforme*. One-time or repeated desiccation stress and cross-stresses (low temperature followed by desiccation stress) increased both SOD and POX activities. *Dicranum scoparium*, closely related to *Dicranella* species, also had high peroxidase activity that increased significantly during desiccation (Onele et al., 2018). Interesting, the effect of desiccation on POX activity in *A. androgynum* was not statistically significant (Table 1), while in *D. subsubulata* the increase in activity was significant (Table 2). Taken together, results on

peroxidase activity suggested that both species display an inducible tolerance mechanism, and counter to our original hypothesis, the inducibility of the mechanism was greater not less in *D. subsubulata* than *A. androgynum*. However, future work should focus on other antioxidant enzymes not studied here e.g., SOD (Mayaba and Beckett, 2003).

4.7.2. Gel electrophoresis analysis

A. androgynum has too low peroxidase activity, and the enzyme could not be visualized using gel electrophoresis, despite using sugar dialysis to concentrate samples, thus results could not be presented. This was not the case in *D. subsubulata* peroxidase; perhaps surprisingly activity occurred largely as a single isoform with a molecular mass of 55 to 60 kDa (Fig 15 B-D). Most plants appear to possess multiple POX isoforms, and each isoform presumably has a slightly different role in plants (Cosio and Dunand, 2009). Higher plant class III peroxidases are involved in both ROS production and ROS scavenging, important components for stress tolerance (Almagro et al., 2009). *Dicranum scoparium* displays multiple isoforms (Onele et al., 2019). Possibly *D. subsubulata* possesses multiple isoforms with the same molecular mass. This could be investigated in future using isoelectric focussing or more molecular biological techniques. Interestingly, the *D. subsubulata* POX did not appear to produce superoxide radicals (data not shown), unlike, for example, the POX of the liverwort *Dumortiera hirsuta* which often grows in Afromontane forests in wetter microhabitats (Li et al. 2010). POX from *D. subsubulata* therefore seem to be more involved in ROS scavenging than ROS production.

Chapter 5: General Conclusion and Recommendations

5.1. General conclusion

Desiccation and high light are probably the main abiotic stresses that limit the distribution of the mosses *D. subsubulata* and *A. androgynum* in the KwaZulu Afromontane. Both species can recovery from severe desiccation, and both are clearly DT. As would anecdotally be suggested by looking at the plants in the field, results presented here suggest that *D. subsubulata* is generally more stress-tolerant than *A. androgynum*. Intuitively, mosses and lichens grow in particularly stressful habitats will be regularly subjected to sudden stressful events (e.g., rapid desiccation). There is a general view that such mosses and lichens are therefore likely to depend on constitutive rather than inducible tolerance mechanisms (Stark 2017; Gasulla et al. 2021). Therefore, as outlined in the Introduction, the main hypothesis tested in this thesis was that *D. subsubulata* depends more on constitutive stress tolerance mechanisms than *A. androgynum*. While some of the results reported here support this hypothesis, evidence was also found that significant stress tolerance can also be induced in *D. subsubulata*. In support of the hypothesis that *D. subsubulata* depends more on constitutive tolerance mechanisms was the finding that this species can readily survive rapid desiccation and storage over silica gel. By contrast, *A. androgynum* can only survive storage over silica gel if desiccation in slow, presumably to induce some tolerance mechanisms (Fig 9). However, at variance with this hypothesis was the observation that while treatment with ABA clearly increased DT in *D. subsubulata* it had very little effect on the tolerance of *A. androgynum* (Fig 11). Furthermore, while slow desiccation significantly increases POX activity in *D. subsubulata*, in *A. androgynum* desiccation tends to increase POX activity, but the increase is not significant (Fig 11). As discussed in more detail above, the much lower effect of ABA in *A. androgynum* than *D. subsubulata* may simply indicate that other hormones are involved in stress signalling in *A. androgynum*. Nevertheless, it seems clear that even highly DT mosses such as *D. subsubulata* do not rely exclusively on constitutive tolerance mechanisms, but in addition, possess some inducible mechanisms.

Unfortunately, it could not be established from the experiments on high light stress whether *D. subsubulata* possesses more constitutive tolerance mechanisms that *A. androgynum*. While clearly showing that *D. subsubulata* is more tolerant to photoinhibition than *A. androgynum* (Fig 6), our moderate light and ABA "hardening" treatment did not increase tolerance to photoinhibition in either species. More work using a variety of different (possibly less stressful) hardening treatments is needed to find out whether tolerance to high light stress in these two

species can be modulated, and whether modulation is greater in *A. androgynum* than *D. subsubulata*.

5.2. Future recommendations

As outlined above, in future studies it would be desirable to test ways of modulating tolerance to high light stress in these two species. Furthermore, desiccation tolerance of *D. subsubulata* is poorly understood, but future work could focus on characterizing the peroxidase genes present using the genome sequences available for closely related species *D. scoparium*. Other tolerance mechanisms worth studying in more detail are the induction of potentially protective sugars during desiccation. Furthermore, the induction of antioxidant enzymes during desiccation in addition to POX such as SOD could be tested, and other proteins associated with DT in other organisms such as HSP and LEA proteins.

A long-term gain would be to use knowledge on DT mechanisms in bryophytes to improve the stress tolerance of crop plants. Bryophytes were the first land plants, and possess high DT, thus genetic transformation using Bryophyte genes could be beneficial. To give examples from other organisms, genetic engineering or modification was successful in tomatoes using an anti-freeze gene from the Arctic fish to promote tolerance to frost damage (Le Gall et al., 2003). Tobacco has been genetically modified to produce artemisinin drugs that have anti-malarial properties (Farhi et al., 2011). In the Bryophytes, *P. patens* has been proposed to be a good model species for genetic engineering, and indeed the entire genome has been sequenced (Cove, 2005). However, simply to confine research to this species may "miss" tolerance mechanisms found in other bryophytes. Therefore, it seems highly desirable that in addition to *P. patens*, other bryophytes continued to be studied for stress tolerance mechanisms. The world is predicted to face drastic climate change, and genetic engineering may be needed to ensure security of food production in the future.

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