UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

ROLES OF ROS SCAVENGING ENZYMES AND ABA IN DESICCATION TOLERANCE IN FERNS

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 January 2017 to December 2018. The research was financially supported by the National Research Foundation (South Africa).

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.

Signed: Mkhize K.G.W

Signed: Beckett R.P

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Declaration 1: Plagiarism

I, Kwanele Goodman Wandile Mkhize, student number: 213501630 declare that:

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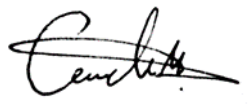

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Declaration 2: Publications

CONFERENCE CONTRIBUTION

1. South African Association of Botanists (SAAB) annual congress, January 2018, University of Pretoria. Oral presentation on the Roles of ROS scavenging enzymes in desiccation tolerance in ferns. Authors: Mkhize, K.G.W., Beckett, R.P.
2. South African Association of Botanists (SAAB) annual congress, January 2019, University of Johannesburg. Oral presentation on the The effect of long-term dehydration on desiccation tolerance mechanism of *Loxogramme abyssinica* and *Crepidomanes inopinatum.* Authors: Mkhize, K.G.W., Beckett, R.P.

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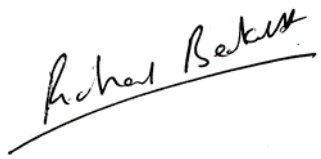

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Abstract

Roles of ROS scavenging enzymes and ABA in desiccation tolerance in ferns

The fern species *Loxogramme abyssinica* and *Crepidomanes inopinatum* occur widely in KwaZulu Natal Afromontane forests. Although both species are suspected to be desiccation tolerant (DT), little is known about their mechanisms of tolerance. The present study was undertaken to characterize DT in both ferns, specifically the roles of the reactive oxygen species (ROS) scavenging enzymes superoxide dismutase (SOD) and peroxidase (POX), sugar afccumulation, and abscisic acid. Broadly speaking, DT mechanisms are either constitutive (always present) or induced in response to stress. *C. inopinatum* dries rapidly because it is a "filmy" fern, lacking a cuticle; in contrast *L. abyssinica* possesses a cuticle, and therefore dries slowly. It was predicted that the fast-drying *C. inopinatum* would rely mainly on constitutive mechanisms, while the slow-drying *L. abyssinica* would depend on inducible mechanisms. Plant were collected from the field, transported to the laboratory and then subjected to desiccation. Two methods of desiccation were used. The first one was a relatively mild, slow and short term (48 h) desiccation over calcium acetate, and the second was a harsher, rapid and long term (1 week) desiccation over silica gel. Measurement of chlorophyll fluorescence parameters showed that both species displayed rapid recovery during rehydration after slow or fast desiccation, confirming that both species are genuinely poikilohydric. POX activity remained constant in both species during slow desiccation and subsequent rehydration, suggesting that ROS scavenging by POX is a constitutive DT mechanism. However, the absolute POX activity of *C. inopinatum* was much higher than in *L. abyssinica*. Rapid long term desiccation reduced POX activity in both species, but the activity recovered during rehydration. In both species, slow desiccation increased SOD activity, and activity declined to original values during rehydration. Slow and fast drying increased the concentrations of soluble sugars in both species, and concentrations rapidly declined to initial values during rehydration. ABA pretreatment had little effect on DT in either species, although tolerance was slightly increased in *L. abyssinica*. Results of this study suggested that both species depend largely on inducible DT mechanisms. Counter to the original hypothesis, inducible mechanisms occur even in filmy ferns that desiccate rapidly. The results from this project will contribute to our understanding of how ferns can survive in stressful environments in South Africa, and potentially could help improve abiotic stress tolerance in crop plants.

Keywords: Desiccation tolerance, *Loxogramme abyssinica* and *Crepidomanes inopinatum* Reactive oxygen species (ROS), Peroxidase (POX), Superoxide dismutase (SOD), Soluble sugars, Abscisic acid (ABA), Rapid and Slow desiccation.

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Secondly, I would like to thank NRF (South Africa) for funding me on this project and also the University of KwaZulu-Natal for providing me with all the necessary facilities for the research, I'm truly grateful.

Finally yet importantly, I am grateful for the support, words of encouragement and prayers from my family and friends. Particularly, my friends from UKZN have played a huge role towards the success of this project. All the motivation, help with proof reading and stats and the love they showed me, gave me hope and strength to perform at my best.

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List of Abbreviations

ROS	Reactive oxygen species
POX	Peroxidase
Asc-pox	Ascorbate peroxidase
SOD	Superoxide dismutase
ABA	Abscisic acid
GSH	Glutathione
ASC GSH	Ascorbate glutathione
DT	Desiccation tolerance
IDT	Inducible desiccation tolerance
CDT	Constitutive desiccation tolerance
ETR	Electron transport rate
F_V/F_M	Maximum quantum efficiency of photosystem II
NPQ	Non-photochemical quenching
Fo	Minimum fluorescence
Fm	Maximum fluorescence
PS II	Photosystem II
Chl	Chlorophyll
DMP	2, 6-dimethoxyphenol
DMSO	Dimethyl sulfoxide
Min	Minute(s)
H	Hours
ml	Millilitre(s)

mM	Millimolar(s)
Native PAGE	Non-denaturing gel electrophoresis
nm	Nanometer(s)
PAR	Photosynthetically active radiation
RH	Relative humidity
Rpm	Revolutions min^{-1}
DRP	Desiccation related proteins
LEA	Late embryogenesis abundant proteins
RFO	Raffinose
CGA	Chlorogenic acid
CA	Caffeic acid
GR	Glutathione reductase
PPO	Polyphenol oxidase

Chapter 1: Literature Review

1.1. The pteridophytes

The continued existence of pteridophytes could be viewed as remarkable, given that they first evolved more than 400 million years ago (mya). The first definite pteridophyte was *Rhynia gwynne-vaughnii* (Edwards., 1979), the earliest spore bearing vascular plant. Pteridophytes *sensu lato* are vascular plants (i.e. with xylem and phloem) that reproduces via spores. Collectively, they belong to the former and now invalid taxon "pteridophyta". The term is still quite useful however, and will be used here to informally refer to ferns, horsetails, and the lycophytes (clubmosses, spikemosses, and quillworts). These are not a monophyletic group because ferns and horsetails are more closely related to seed plants than to the lycophytes (Wikipedia contributors, 2018).

Ferns are believed to have dominated the Carboniferous landscape before the age of dinosaurs. Because of the drastic change in climate conditions during the Permian and early Triassic periods, the earth became warmer and drier, thereby favouring seed plants domination and resulted in decrease in spore bearing plants (pteridophytes) which mostly relied on water for dispersal of spores (Boyer, 2018). At the end of the Permian period, around 251 million years ago, most of the pteridophytes became extinct, including the scale trees and arborescent horsetails (Boyer, 2018). In fact, over geological time, the pteridophytes have survived more than four mass extinction events, and to do so has required that they be highly tolerant to abiotic stresses, in particular, UV and desiccation. Today, pteridophytes can be found occupying a great variety of niches, including growing as epiphytes on rocks and trees. Epiphytes are highly susceptible to desiccation, and therefore many epiphytic pteridophytes can survive almost complete desiccation, and then resume metabolic activity when water becomes available again (Dubuisson et al., 2013).

1.2 Occurrence of DT in the pteridophytes

An estimate in 2012 suggested that about 1000 pteridophytes have been identified as desiccation tolerant plants (Gechev et al., 2012), a large increase from just a few decades ago when estimates were only about 60-70 species (Reynolds and Bewley, 1993b; Gaff, 1989). Table 1 is an edited list of worldwide distribution of desiccation tolerant ferns. This list clearly

shows that new species of poikilohydric ferns continue to be discovered. The flora of southern Africa comprises many DT species, including *Mohria caffrorum*, *Crepidomanes inopinatum*, most of the Pteridaceae including *Cheilanthes depauperata, C. dinteri, C. marlothii* and also some of *Selaginella sp* and *Asplenium sp*. Interestingly more than 31 DT species are native to southern Africa.

Table 1: Worldwide distribution of desiccation tolerant ferns along with it native location (in red). Newly introduced species are written in bold. Distribution of these species is taken from mainly two websites: The Encyclopedia of Life (EOL) and Global Biodiversity Information Facility (GBIF). Correct nomenclature was confirmed from GBIF and The Plant List website. The abbreviated country locations are taken from Sustainable Sources. All these sites are included in the reference list.

Family	Species	Native location & Worldwide distribution	References
Anemiaceae	*Anemia tomentosa*	AR, MX, BR, PY, UY, VE	Hietz, 2010
	Anemia villosa	VE, BO, CO, GY, SR, GF, EC, PE, BR	Hietz, 2010
	Anemia mexicana	MX, US	Kessler and Siorak, 2007
	Anemia rotundifolia	BR, BO	Kessler and Siorak, 2007
	Mohria caffrorum	Southern Africa	Gaff, 1977, Hietz, 2010
Aspleniaceae	*Asplenium aethiopicum* (Burm) B.	Hawaii, US, Eastern & Southern Africa, NE, NG, IN	Gaff, 1977; Hietz, 2010
	Asplenium ceterach	Europe (all around)	Hietz, 2010; Proctor, 2009; Fahmy et al., 2006
	Asplenium friesiorum	Southern Africa, KE, CG, BR	Hietz, 2010
	Asplenium mannii	Eastern & Southern Africa, AO, GN, SL, TZ	Hietz, 2010
	Asplenium praegracile	KE, MG, TZ, UG	Hietz, 2010
	Asplenium sandersonii Hook	Eastern & Southern Africa, ST, GQ, CM, NG, CG, GA	Gaff, 1977; Hietz, 2010

	Asplenium theciferum	Southern Africa, AO, BI, CM, GQ, ET, KE, RW, TZ, UG	Hietz, 2010
	Asplenium uhligii	ZW, UG, KE, TZ, CM, ET, MW, CG, UG, MG	Hietz, 2010
	Asplenium rutifolium (Willd.) Kze.	Southern Africa, KE, TZ, YE, UG, MU, RE	Gaff, 1977
	Asplenium cordatum	Southern Africa, KE, TZ, ET, SO, ST, AO, RE	Gaff, 1977
	Asplenium rutamuraria	Europe, East Asia, and Eastern North America	Proctor, 2009
	Asplenium septentrionale	Western North America, Europe, Asia	Proctor, 2009
	Asplenium trichomanes	Europe, Asia, North & Southern Africa, TR, IR, YE	Proctor, 2009
Hymenophylaceae	*Hymenophyllum capillare*	Southern Africa, CM, CG, GH, KE, RE, UG, ST, BI, RW	Hietz, 2010
	Hymenophyllum kuhnii	Southern Africa, TZ, KE, CG, UG, KM, NG, SL, ST, AO, LR	Hietz, 2010
	Hymenophyllum splendidum	BO, CO, UG, KE, TZ, ST, CM, GA, CG, RW, BI	Hietz, 2010
	Hymenophyllum tunbrigense	US, MX, AU, CL, CR, DE, IE, IT, JM, KE, MG, NZ, ZA, FR, BE, ES	Hietz, 2010; Proctor, 2003
	Hymenophyllum wilsonii	FO, FR, IE, NO, PT, ES, US	Bravo et al., 2016
	Hymenophyllum dentatum	CL, AR	Hietz and Briones, 1998
	Hymenophyllum polyanthos	PR, VI, CR, Caribbean	Cea et al., 2014. Proctor, 2012

	Hymenophyllum caudiculatum	BR	Cea et al., 2014
	Hymenophyllum fucoides	PR, MX, BR, CO, EC	Proctor, 2012
	Hymenophyllum sanguinolentum	NZ, Three kings, Stewart and cook island	Proctor, 2012
	Trichomanes bucinatum	MX	Hietz, 2010
	Trichomanes pyxidiferum	KE, CG, RW, BI, ZM, MZ, ZW, CU	Hietz, 2010
	Trichomanes ramitrichum	KE, ZW, CG, RW, BI, UG, TZ, ZM, MZ	Hietz, 2010
	Trichomanes rigidum	**PR**	Hietz, 2010
	Didymoglossum erosum (Willd.) J.P. R	ZW, ZA, MZ, CG, AO, CM, NG, GH, LR, SL, SC, UG, KE, TZ, GN, CF, GA, BI, RW	Hietz, 2010
	Vandenboschia radicans (Sw.)	PR, CU, BO, BR, EC, GT, MX, NI, KE, MG, CN, JP	Hietz, 2010
	Abrodictyum rigidum (Sw.) E&D	PR, ZW, CG, CM, CF, GN, KE, ZA, MG, SC	Hietz, 2010
	Crepidomanes inopinatum	ZA, SZ, ZW, RW, BI, RE, MU	Dubuisson et al., 2014
	Crepidomanes melanotrichum	Central to Eastern & Southern Africa	Hietz, 2010
	Crepidomanes Chevalieri (Christ)	Tropical Africa, Bioko	Hietz, 2010
	Hymenoglossum cruentum.	South America	Bavestrello et al., 2016
Polypodiaceae	*Ctenopteris heterophylla*	NZ, Three kings, North & South Stewart, AU	Hietz, 2010
	Loxogramme abyssinica	Central to Eastern & Southern Africa	Hietz, 2010

Loxogramme lanceolate	UG, LR, GH, CM, CG, AO, Bioko	Hietz, 2010
Melpomene flabelliformis	MX, CR, CO, VE, EC, PE, BR, BO, Southern Africa, TZ, RW	Hietz, 2010
Melpomene peruviana	BR, MX, AR, BO	Hietz, 2010
Polypodium virginianum	Eastern North America, CO, CA	Hietz, 2010
Polypodium vulgrare	IE, North Africa, FR	Hietz, 2010
Polypodium cambricum	Southern & western Europe, SY, LB, IL, PS, LY, TN, DZ, MA	Proctor, 2009
Polypodium interjectum	CZ	Proctor, 2009
Polypodium remotum	Mesoamerica, Caribbean, CU, DO, HT, PR, MX, GT, NI	Hietz and Briones, 1998
Pleopeltis angusta	Mesoamerica	Hietz, 2010
Pleopeltis crassinervata	Mesoamerica	Hietz, 2010
Pleopeltis furfuracea	MX, GT, HN, SV, NI, CR, PA	Hietz, 2010
Pleopeltis hirsutissima	BR, AR, PY, UY	Hietz, 2010
Pleopeltis macrocarpa	PR, MX, HN, Southern Africa, CR, PA, NI, CO, VE, EC, PE, BO	Hietz, 2010
Pleopeltis mexicana	MX, GT, HN, SV, NI	Hietz, 2010
Pleopeltis plebeia	MX, GT, HN, SV, NI, CR, PA	Hietz, 2010
Pleopeltis polypodioides	Florida, PR, MX, BZ, HN,GT, SV, NI, CR, VE, BS, TT, JM, CU, AI	Hietz, 2010; Gaff, 1977; Layton et al., 2010
Pleopeltis squalida	US, BO, PY, UY, BR, AR,	Hietz, 2010

	Microgramma piloselloides	EC, PE, NI, Caribbean, PR, VI, MX, BR, CO	Kessler and Siorak, 2007
	Pecluma eurybasis	BO, PR, VI, HT, CR, CO, EC, GY, CU	Kessler and Siorak, 2007
Dryopteridaceae	*Elaphoglossum acrostichoides*	Southern Africa, KM, UG, KE, TZ, CM, GA, LR, CG, AO, RW	Hietz, 2010
	Elaphoglossum petiolatum	PR, DM, MS, GP, MQ, CM, GD, LC, VC, KM	Hietz, 2010
	Elaphoglossum spathulatum	PR, Southern Africa, TZ, MU, KE, LK,	Hietz, 2010
Pteridaceae	*Actiniopteris radiata*	Southern Africa, UG, KE, TZ, DJ, ML, NE, TO, CM, KE, ET	Gaff, 1977; Hietz, 2010
	Actiniopteris semiflabellata	RE, IN, AW, YE, SD, EG, MR, NA, UG, KE, SO, DJ, CG	Hietz, 2010
	Actiniopteris dimorpha	Southern Africa, BI, RE, UG, KE, TZ, ET, SD, SO, KM, CG	Gaff, 1977
	Actiniopteris australis	East & Southern Africa, IN, AW	Fahmy et al., 2006
	Adiantum hispidulum	ZA, KE, TZ, MZ, MG, MW, CN, Asia	Hietz, 2010
	Adiantum incisum	Southern Africa, GH, NG, TG, UG, KE, TZ, SO, MG, RW, CM	Hietz, 2010
	Adiantum nigrum	Northern & Southern Africa, Western & Central Asia	Proctor, 2009

Adiantum latifolium	Europe, North America, PR, VI, Caribbean, Central to South America	Muhamad, 2017; Lubaina et al., 2016
Haplopteris volkensii	ZW, MW, MZ, KE, TZ, ET, UG, CG, SD	Hietz, 2010
Vittaria isoetifolia	Southern Africa, TZ, KE, RE, MG, KM, SC	Gaff, 1977
Pellaea boivinii	Southern Africa, KM, MU, KE, TZ, LK, IN	Gaff, 1977
Pellaea calomelanos	Eastern & Southern Africa	Gaff, 1977
Hemionitis arifolia	IN, LK, ID, MY, PH, TW	Kessler and Siorak, 2007
Hemionitis palmata	PR, VI, MX, BZ, GT, HN, SV, NI, CO, CR, EC, PE, BO, BR, GP, MS	Kessler and Siorak, 2007
Cheilanthes depauperata	ZA	Gaff, 1977
Cheilanthes dinteri	ZA, NA, AO, tropical east Africa	Gaff, 1977
Cheilanthes eckloniana	Southern Africa	Gaff, 1977
Cheilanthes hirta	Southern Africa, KE, RE, UG, TZ, ET	Gaff, 1977
Cheilanthes inaequalis	Eastern & Southern Africa, GN, CM, CG, NG	Gaff, 1977
Cheilanthes marlothii	ZA, NA, AO	Gaff, 1977
Cheilanthes multifida	Southern Africa	Gaff, 1977
Cheilanthes parviloba	ZA, NA, ZW	Gaff, 1977
Cheilanthes myriophylla	Central American to South Argentina	Kessler and Siorak, 2007
Cheilanthes notholaenoides	MX, GT, CR, HN, CO, BZ, PA, PE, AR, JM, EC, US	Kessler and Siorak, 2007
Cheilanthes quadripinnata	Eastern & Southern Africa	Gaff, 1977

	Cheilanthes viridis	Eastern & Southern Africa, CG	Gaff, 1977
	Cheilanthes catanensis (Cosent.) *H.P.Fuchs*	East - Tropical Africa, MX, GT, HN, CR, ZW, CO, OM, GN, CG	Fahmy et al., 2006
	Cheilanthes pteridioides	Western & Eastern Europe, Middle East, North & Eastern Africa	Fahmy et al., 2006
	Cheilanthes coriacea	PT, IT, ES, BV, FR, YU, AL, BG, TR, CY, LB, SY, IQ, IR, IL, JO, PS	Fahmy et al., 2006
	Aleuritopteris farinose	SO, YE, OM, SA, ET, TZ, DJ, SJ, EG, TD, IR	Gaff, 1977
Isoetaceae	*Isoetes australis*	West Australia	Hietz, 2010
Schizaeaceae	*Schizaea pusilla*	CA, US, EC, PE, PM	Hietz, 2010
Woodsiaceae	*Woodsia ilvensis.*	CA, US, CN,	Hietz, 2010
Selaginellacea	*Selaginella caffrorum* (Milde) H.	Eastern & Southern Africa	Gaff, 1977
	Selaginella digitate	MG	Gaff, 1977
	Selaginella dregei (C. Pres) H.	Eastern & Southern Africa	Gaff, 1977
	Selaginella echinata	MG	Gaff, 1977
	Selaginella imbricate	Eastern & Southern Africa	Gaff, 1977
	Selaginella nivea	Southern Africa	Gaff, 1977
	Selaginella lepidophylla	MX, US, CR, SV	Dinakar and Bartels, 2013
	Selaginella tamariscina	CN, IN, JP, TW, RU, KP, KR, PH, TH, VN	Dinakar and Bartels, 2013
	Selaginella sellowii	CN, AR, PY, BO, BR, CU, VE, EC, PE, CO, UY	Dinakar and Bartels, 2013
	Selaginella bryopteris	IN, NP	Oliveira and Moraes, 2015

	Selaginella helvetica	Central and southeast Europe, Asia	Kessler and Siorak, 2007
	Selaginella trisulcata	BO, BR, CO, EC, PE	Kessler and Siorak, 2007
Adiantaceae	*Doryopteris concolor* (Langsd. and Fisch) K.	Southern Africa, South America & Caribbean	Gaff, 1977
	Doryopteris kitchingii (Bak.) B.	MG	Gaff, 1977
	Doryopteris collina	BR, TT, GY, SR, PY, BO	Moraes et al., 2014
Tectariaceae	*Arthropteris orientalis* (Gmel.) P.	Central, Eastern & Southern Africa	Gaff, 1977
Gleicheniaceae	***Dicranopteris linearis***	Eastern & Southern Africa, CN, TW, JP, AU,	Kavitha and Murugan, 2017

DT ferns are mainly temperate and tropical (epiphytic) terrestrial species (Pittermann et al., 2013; Watkins et al., 2007a,b; Cea at al., 2014). Many DT ferns are found on substrates such as exposed rocks and tree barks. Locally (around Pietermaritzburg) *Selaginella dregei* (Cumberland) occurs on rocky outcrops (extremely exposed), and while *Crepidomanes inopinatum* occurs in (relatively) wet Afromontane forest, the microhabitat of the fern is rocky boulders i.e. is quite xeric. *Loxogramme abyssinica* is commonly found growing together with *C. inopinatum* and can be epiphytic or lithophytic. *Asplenium* spp. are widely known for their extreme DT mechanisms and they are adapted to rocky xeroseres, although most of them occur adjacent to swamps, damps or streams (Klopper and Crouch, 2012). The hygrophilous epiphytes lack xerophytic traits and humus-collecting strategies, making them very much restricted to humid environments (along streams and water falls in forests) (Dubuisson et al., 2013). Even this brief overview indicates that different species are adapted to different microenvironments, so possibly they tend to display different tolerant mechanisms.

There are two main types of desiccation tolerant pteridophytes. The first type belongs to the Hymenophyllaceae or “filmy ferns”. The leaves of these ferns are one cell thick, and they possess only a very rudimentary cuticle. The second type possess a well-developed cuticle and occur in a variety of families. Filmy ferns desiccate rapidly causing metabolism to cease within a few minutes. In this respect, it has been argued that the evolution of filmy ferns is a derived tendency towards a ‘bryophyte-like’ strategy (Dubuisson et al., 2013). By contrast,

pteridophytes with a cuticle will remain metabolically active for many hours even if conditions become rapidly hot and dry. Intuitively, filmy ferns could be expected to rely mainly on constitutive protection mechanisms. In those species with a cuticle however, when a water deficit is sensed before it become severe, the plant may have time to put in place inducible protective mechanisms (Inducible desiccation tolerance – IDT). Constitutive protection mechanisms (Constitutive desiccation tolerance – CDT) require constant levels of gene expression, transcription and translation. The problem with constitutive mechanisms is that they are active even when they are not required, thereby wasting energy that could otherwise be used for growth and development (Stark, 2017). Unlike constitutive mechanisms, inducible mechanisms use less energy but require time to be induced; if drying is too rapid, cellular damage can occur, usually during rehydration (Stark, 2017). Therefore, IDT species require slow drying rate to induce protective mechanisms (Greenwood, 2017). Due to such inducible responses to desiccation, Hilker et al. (2016) termed this strategy a "basic cost-saving strategy" (Stark, 2017).

Another way of classifying DT pteridophytes or DT plants in general is based on whether chlorophylls are retained during desiccation. The first strategy is termed "homoiochlorophyllous". These plants retain their chlorophyll and thylakoid structure upon desiccation. The second strategy is termed "poikilochlorophyllous". Here, chlorophyll breakdown occurs during desiccation, and chlorophylls are resynthesized upon rehydration (Bravo et al., 2016; Oliveira and Moraes, 2015) e.g. in *Pleopeltis pleopeltifolia* (Voytena et al., 2014). Kessler and Siorak (2007) described species of *Anemia* and *Bolivia* that can roll up, become brownish in colour (corresponding to loss of chlorophyll) during dehydration, and then return to their normal state upon rehydration. Poikilochlorophyllous ferns are believed to exhibit an extremely derived form of DT. Superficially, plants appears dead and unable to recover. The advantage of being poikilochlorophyllous is that when chlorophylls are broken down, energy capture will be reduced, reducing the danger of excessive ROS production (Gechev et al., 2012). By contrast, in homoiochlorophyllous photosynthesis is reversibly inactivated, allowing the plant to quickly recover upon rehydration (Gechev et al., 2012; Garces et al., 2018). Most pteridophytes are homiochlorophyllous, and the precise advantages homiochlorophylly confers remain unclear. However, Oliver et al. (2000) have pointed out that homiochlorophyllous DT plants tend to be adapted to drying and wetting cycles that extend from hours to days or a few weeks, while poikilochlorophyllous modified desiccation-tolerant plants are adapted to cycles lasting from weeks to months.

1.3. Morphological and anatomical mechanisms of DT in the pteridophytes

The most visible consequence of water loss is the folding of fronds which in turn helps prevent mechanical damage. The presence of arabinose-rich pectins and proteins makes it possible for these plants to fold up upon desiccation and unfold upon rehydration (Lopez-Pozo et al., 2018). This process also aids in prevention of photooxidative damage by reducing the leaf area exposed to extreme light (Lopez-Pozo et al., 2018). DT pteridophytes typically have reduced amounts of primary xylem. Their water conducting cells are arranged so that they resemble the tightly packed tracheid of conifers (Pittermann et al., 2013). The pit membranes found in ferns are porous and occur along the tracheid walls which are diaphanous and more porous, reducing resistance to water flow (Pittermann et al., 2013).

Several ferns have special leaf anatomy that helps them cope with the particular stresses found in a particular environment (Ribeiro et al., 2007). Leaf structural characteristics include a general reduction of leaf surface area, increased thickness of epidermal cell walls, increased thickness of cuticle, high trichome density and stomata located on the abaxial surface. These characters will reduce water loss. *Anemia tomentosa* and *Anemia villosa* (Schizaeaceae) which are found in vegetation islands on rocky outcrops in Rio de Janeiro also have "stegmata", made of silica, occurring as intracellular crystals, which aids in maintenance of rigidity when turgor is lost (Ribeiro et al., 2007). DT in fern gametophytes has received little attention, but interestingly, Pittermann et al. (2013) showed that the gametophytes of pteridophytes with complex three-dimensional thalli are more desiccation tolerant than the ones with normal thalli.

1.4. Physiological and biochemical mechanisms of DT in the pteridophytes

Most of our knowledge of the physiology and biochemistry of DT mechanisms comes from studies on seeds, resurrection angiosperms and bryophytes (Black and Pritchard, 2002; Jenks and Wood 2007). Comparison of changes in the transcriptome of a range of DT organisms shows that important adaptations include active ROS scavenging systems, synthesis of sugars, upregulation of LEA and heat shock proteins, maintenance of the cytoskeleton and activation of the cytochrome P450 to detoxify harmful molecules intracellularly (Costa et al., 2017). As will be outlined below, most pteridophytes appear to share the classic "universal" mechanisms of desiccation tolerance.

1.4.1. DT mechanisms based on avoiding ROS formation

One of the most harmful effects of desiccation on plant tissue is the formation of ROS (Smirnoff, 1993). ROS are produced by plants as part of normal metabolism, and play very important roles in intracellular signalling for process such as growth, development, response to biotic and abiotic stresses, and programmed cell death (Serres and Mittler, 2006). However, abiotic stress can greatly stimulate their production, and when they occur in excess they can be very toxic and cause intracellular destruction (Ntuli, 2012). This can occur particularly in the chloroplasts when the light absorbed contains more energy than can be used in CO_2 fixation, for example when water deficit restricts fixation. Furthermore, during the rehydration of poikilohydric organisms carbon fixation can take longer to recover than photophosphorylation, and under these conditions ROS formation can occur in the photosynthetic apparatus. ROS production can be partly prevented by dissipating excess light energy through the xanthophyll cycle as heat by "non-photochemical quenching" (NPQ), reducing the energy reaching the photosynthetic reaction centres (Mafole et al., 2017). To carry out NPQ, plants probably use a variety of reactions, mostly involving carotenoids. For example, β-carotene can quench $^1O_2$. Xanthophylls, the oxygenated derivatives of carotenes, can also quench $^1O_2$, e.g., lutein and neoxanthin. However, the main contributor to NPQ is the xanthophyll cycle (Frank et al., 1999; Demmig-Adams, 2006). In this cycle, solar radiation is dissipated as heat while violaxanthin undergoes de-epoxidation to antheraxanthin and then zeaxanthin, partly preventing the formation of $^1O_2$. With the development of portable chlorophyll fluorescence devices, NPQ can be readily measured in the field. While the relationship between desiccation tolerance and NPQ has not been rigorously investigated in pteridophytes, desiccation tolerant bryophytes displayed a greater capability of using NPQ than sensitive species (Deletoro et al., 1998a,b).

1.4.2. DT mechanisms based on antioxidant enzymes

Once formed, ROS can be scavenged by both enzymatic and low-molecular weight, non-enzymatic antioxidants. ROS scavenging enzymes include superoxide dismutase (SOD), catalase, class I and class III peroxidases (POX) and auxiliary enzymes, such as mono- and dehydroascorbate reductases and glutathione reductase (GR), which regenerate the electron donors. Catalases break down H_2O_2 very rapidly, but are less effective than peroxidases at removing H_2O_2 present in low concentrations (Golan et al., 2013), because of their lower affinity for H_2O_2. Peroxidases use a variety of substrates to break down H_2O_2 to H_2O e.g. ascorbate which is converted into mono dehydroascorbate (MDA) in the process (Choudhury

et al., 2013). The genome of most plants typically contain a large number of peroxidase isoforms (Dunand et al., 2003). An interplay between low molecular weight and enzymatic antioxidants to scavenge ROS in plants was suggested by Foyer and Halliwell (1976) and has not been challenged since. These authors proposed an ascorbate/glutathione cycle ("Halliwell/Foyer/Asada cycle") for the scavenging of H_2O_2 produced from $O_2^{\bullet -}$ by SOD, in which ascorbate and glutathione play pivotal roles as reductants (Figure 1).

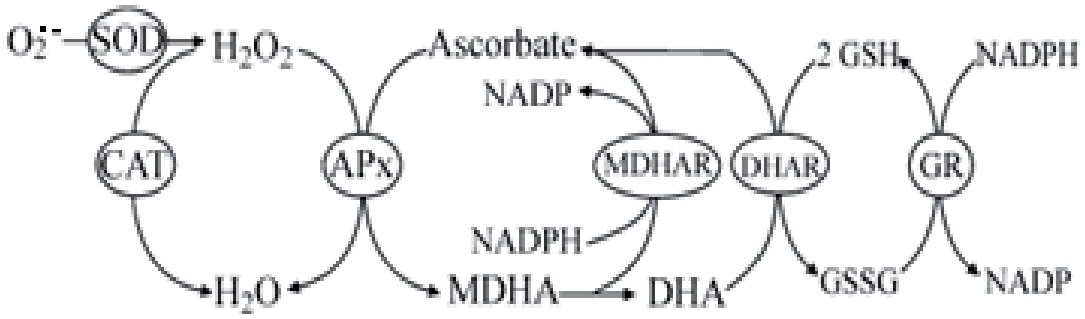

Figure 1. The ascorbate-glutathione cycle (AsA-GSH) (ROS scavenging pathway) found in stress tolerance plants, in chloroplasts, cytosol, mitochondria, apoplast, and peroxisomes (Foyer and Halliwell, 1976).

One of the most comprehensive studies on ROS scavenging enzymes in ferns was that of Kavitha and Murugan (2017) with *Dicranopteris linearis.* The activity of ascorbate peroxidase (APX) increased during desiccation and dehydroascorbate (DHA) was induced after desiccation for 6 d, but decreased thereafter due to the regeneration of ascorbate by the enzymes DHAR and MDHAR. Catalase was also significantly induced (7 fold) after desiccation for 10 d. The activity of POX and SOD also increased in desiccated fronds compared to the control. On the other hand, GR only started to increase after 4 d and progressively increased until 10 d. GR accumulation was associated with the regeneration of the reduced form of glutathione (from oxidised glutathione) to aid in scavenging of excessive amounts of hydrogen peroxide.

1.4.3. DT mechanisms based on non-enzymatic antioxidants

Although they probably play a smaller role than the enzymatic antioxidants, non-enzymatic antioxidants assist in keeping the ROS levels at acceptable levels (Caverzan et al., 2016). Probably the most important non-enzymatic antioxidant is ascorbate which can completely remove $O_2^{\cdot -}$, OH·, and $^1O_2$, and also (as discussed in section 1.4.2) convert hydrogen peroxide into water in a reaction catalysed by ascorbate peroxidase. Ascorbate can also regenerate

tocopherol from the tocopheroxyl radical. Tocopherol scavenges the peroxyl radical found in lipid bilayers and also protects the chloroplasts from photo-oxidative damage (Caverzan et al., 2016). GSH also maintains the redox balance in all cellular compartments. In response to abiotic stresses, plants increase the activity of GSH biosynthetic enzymes and levels (Apel and Hirt, 2004). Once oxidized, GSH can be regenerated by the enzyme glutathione reductase (GR) as discussed in section 1.4.2 (Terzi and Kadioglu, 2006). Ascorbate and glutathione were found present in *Dicranopteris linearis* and together in Asc-GSH redox chain, they can scavenge excess amounts of hydrogen peroxide (Kavitha and Murugan, 2017).

Interestingly, Kavitha and Murugan (2016) found that *Dicranopteris linearis* produces carotenoids during desiccation as a protectant against oxidative stress thereby decreasing ROS formed during photo-oxidative stress. Carotenoids have three main functions in plants. Firstly, they transfer the light absorbed at wavelengths of 400 to 550 nm to the Chl – chlorophylls. Secondly, they are involved in reducing free radicals formed naturally during photosynthesis. Thirdly they stabilize the thylakoid membrane and the associated photosystems (Ahmad et al., 2009; Gill and Tuteja, 2010).

A wide range of plant flavonoids and phenols are also known to have antioxidant properties (Smirnoff, 1993). They can potentially contribute to the overall antioxidant protection (Rice-Evans et al., 1996), but also have other important roles. For example, flavonoids and phenols can act as "sun screen" pigments, shading the desiccated photosynthetic apparatus, and this will help avoid ROS formation. The brown colour associated with poikilochlorophylly outlined in section 1.2, is probably connected to accumulation of phenolics synthesised by the phenyl propanoid pathway (Lubaina et al., 2016). Accumulation of phenolics in ferns is said to be an important adaptation to the terrestrial environment (Ribeiro et al., 2007). PPO (polyphenol oxidase, a Cu-containing enzyme) together with peroxidase are responsible in the oxidation of phenolics through oxygen-dependent oxidation of monophenols or *o*- diphenols to *o*-quinones (Živković et al., 2010). This enzyme is not only involved in tissue browning, but also in lignification and the defence mechanism against biotic stresses like insects and plant pathogens (Živković et al., 2010). Phenolic acids can also act as a signalling molecule and are mainly stored in the vacuole or in the apoplast (Živković et al., 2010). Just like ascorbate, the reduced forms of phytophenolics are also regarded as strong antioxidant agents (Živković et al., 2010). Živković et al. (2010) showed decreased levels of chlorogenic (CGA) and caffeic acid (CA) phenolics in *Asplenium ceterach* during short term dehydration, which suggests that they may have a role in the antioxidant defence system of the plant.

Taken together, from the above it is clear that avoidance of ROS production and ROS scavenging are important components of the overall mechanism of stress tolerance in plants. However, and it would be simplistic to assume that in desiccation tolerant ferns scavenging mechanisms will invariably be maintained at high levels or induced in response to stress, and other mechanisms are undoubtedly involved.

1.4.4. DT mechanisms based on sugars

Sugars also play an important role in the desiccation tolerance mechanisms of plants. Sugars function as osmoprotectants of biological membranes (Gechev et al., 2012). They are also known to promote cytoplasm vitrification, which will help preserve subcellular structures (Oliveira and Moraes, 2015). Sucrose is often found in desiccation tolerant plants in high concentrations. Apart from stabilizing drying cells and intracellular glass formation, sucrose can also stabilize many enzymes in the dry state (Toldi et al., 2009). The accumulation of sugars, specifically sucrose, tends to occur at a late stage of desiccation (Toldi et al., 2009). An intracellular glass is a viscous undercooled cytoplasmic liquid that is made of proteins and sugars and aids in prevention of cellular membranes and deleterious effects of radical reactions (Buitink and Leprince, 2004; Koster, 1991; Wolkers, 1998). Raffinoses (RFO's) are also one of the most abundant sugars in DT species. RFO's can also act as storage carbohydrates, and are mobilized during drought as sources of energy and for sucrose synthesis (Gechev et al., 2012). However, it is important to know which sugars are present in a particular DT plant and at what quantity, because different sugars play different roles in DT. For instance, accumulation of the C8 sugar, 2-octulose was found to be high in hydrated leaves of the DT higher plants *Craterostigma plantagineum* and *Craterostigma wilmsii* but not in the desiccated state, when it was replaced by increase in concentration of sucrose (Dinakar and Bartels, 2013). Changes in the types of sugars also occurs in *Selaginella lepidophylla* which maintains higher levels of trehalose than sucrose and glucose upon desiccation, yet in hydrated state, sugar alcohols such as sorbitol, xylitol, arabitol, erythritol, myo-inositol, and mannitol accumulate (Dinakar and Bartels, 2013).

1.4.5. DT mechanisms based on LEA and other proteins

The accumulation of specific proteins during desiccation has long been considered to form an important part of DT mechanisms in plants. Commonly, interest has been focused on LEA (late embryogenesis abundant) proteins. LEA proteins are involved in the formation and maintenance of the intracellular glass (Ntuli, 2012). This is a result of induction of particular

LEA proteins by sucrose upon dehydration, resulting in an α-helical conformation, which forms an intracellular glass (Ntuli, 2012), although interestingly, they are largely unstructured in a dilute solution (Goyal et al., 2005). Furthermore, LEA proteins maintain hydrogen bonding within and between a variety of macromolecules (Voytena et al., 2014*).* These proteins may increase the water holding capacity and/or minimize water loss of cells by acting as a hydration buffer. They can do this by seizing ions, directly protecting other proteins or membranes, and renaturing unfolded proteins (Oliveira and Moraes, 2015; Toldi et al., 2009). Layton et al. (2010) postulated that LEA D-11 (also known as dehydrin) is also involved in maintenance of cytosolic desiccation tolerance and cell wall mechanical failure. A desiccation-tolerant fern *Polypodium polypodioides* was found to express 31-kDa putative dehydrin polypeptide during desiccation but not upon rehydration (Layton et al., 2010). In plants, about six different classes of LEA proteins that have been identified and sequenced. All typically show highest expression during desiccation (Toldi et al., 2009). Generally, they are found in the cytosol, chloroplasts, mitochondria and/or nuclei (Dinakar and Bartels, 2013).

1.5. Role of ABA in DT

Abscisic acid (ABA) has been found to be the dominant plant hormone involved in stress signalling in many DT organisms (Alpert, 2000). While ABA-induced stomatal closure is regarded as one of the first response to desiccation stress, ABA signalling is also involved in the upregulation of genes that are crucial for desiccation tolerance. Endogenous changes of ABA levels upon dehydration seem to be species specific. The importance of ABA signalling has been noted in *Selaginella tamariscina* upon desiccation, resulting in a stagy increase in ABA content and induction of ABA-responsive genes such as ELIP, OSTI and many LEA genes (Liu et al., 2008). By contrast, in the majority of species, including some like *H. caudiculatum* and *P. virginianum*, endogenous ABA decreases and is maintained at low concentration upon dehydration and rehydration cycles (Garces et al., 2018). Exogenous ABA application to a variety of plants has often been reported to induce desiccation related proteins which are involved in DT (Hellwege, 1994; Stark, 2017).

1.6. DT in pteridophytes – summary

Table 2 is a list of pteridophytes with major (not all) mechanisms involved in DT. This summary suggests that pteridophytes share the classic “universal” mechanisms of desiccation tolerance.

Table 2: The mechanisms involved in desiccation tolerance in pteridophytes

Fern species	Major DT mechanism	Reference
Selaginella tamariscina	Upregulation of sugar metabolism, stress response proteins and proteins related to photosynthesis.	Gechev et al., 2012
	Increase in superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione reductase (GR) activities during dehydration.	Wang et al., 2010
Anemia villosa	Presence of phenolic compounds and starch grains	Ribeiro et al, 2007
Selaginella sellowii	Increase in disaccharides of glucose and trehalose during stress	Oliveira and Moraes, 2015
Hymenophyllum dentatum	Accumulation of changes in cell wall polysaccharides (pectin and arabinan) and proteins (LEA proteins) during desiccation.	Bravo et al., 2016
Mohria caffrorum	Protection of the cell wall during desiccation using arabinogalactan proteins (AGPs)	Moore et al., 2013
Polypodium polypodioides	Expression of 31-kDa putative dehydrin polypeptide during desiccation	Layton et al., 2010
Dicranopteris linearis	Upregulation of ascorbate peroxidase, superoxide dismutase, catalase, peroxidase and glutathione reductase activities upon desiccation	Kavitha and Murugan, 2017
Selaginella lepidophylla	Accumulation of purine nucleoside containing the purine-base hypoxanthine, sugar D-ribose and the choline phosphate (lipid metabolite) during desiccation. Furthermore, phenolics (caffeate), flavonols (apigenin and naringenin), and phenylpropanoids (coniferyl alcohol) are present in desiccated tissues.	Dinakar and Bartels, 2013

	Other sugar accumulate includes sucrose, mono- and polysaccharides, and sugar alcohols (sorbitol, xylitol). In desiccated tissues concentrations of trehalose, sucrose and glucose increase.	 Adams et al., 1990
Asplenium ceterach L	High increase in phenolics content of CGA, an ester of caffeic and quinic acid. Induction of Class III peroxidases and polyphenol oxidase activity upon dehydration.	Živković et al., 2010
Polypodium virginianum	Induction of dehydrin-type proteins during dehydration. Reduction of drying rate by application of abscisic acid (ABA)	Reynolds and Bewley, 1993a Reynolds and Bewley, 1993a
Hymenophyllum caudiculatum *Hymenophyllum dentatum*	Accumulation of sucrose in hydrated and desiccated fronds. Two 2-cys peroxyredoxin proteins highly accumulated in hydrated and rehydrated fronds. One copper/zinc superoxide dismutase variant 1 was more abundant in desiccated fronds. Induction of sucrose in desiccated fronds. Two 2-cys peroxyrredoxin proteins mostly accumulated in desiccated fronds. while one pyridoxal biosynthesis protein accumulated in desiccated fronds	Cea et al., 2014
Adiantum latifolium	Increase in phenols, specifically ferulate, sinapic acid and pholorogucinol in desiccated fronds. Also, phenylalanine ammonia lyase (PAL) activity increased during desiccation	Lubaina et al, 2016
Anemia tomentosa, Anemia villosa & Doryopteris collina	Accumulation of sucrose, glucose and fructose	Moraes et al, 2014

1.7. Introduction to the present study

An increase in research of DT in pteridophytes, results in more and more species being described. Mechanisms of DT in pteridophytes can be very usefully studied, and they are excellent model systems for studying stress tolerance, and the information derived may be useful in improving stress tolerance in crop species. While enzymatic antioxidants are the most widely studied mechanism of DT, and no doubt play crucial roles, it is becoming clear that many other mechanisms exist.

As discussed in section 1.2, *Crepidomanes inopinatum* is a Hymenophyllaceae filmy fern that typically grows as an epiphyte in the understory of the Afromontane biome of southern Africa. In this species, the leaves are one cell thick, and lack stomata. *Loxogramme abyssinica*, is also an epiphytic fern and usually grows in the same microhabitats as *Crepidomanes inopinatum*, but by contrast possesses a well-developed cuticle. These two species are often found growing together on the same boulder in the understory of Afromontane forests. As epiphytic species, both are often subjected to rapid water shortages, even in fairly low light habitats (Stark, 2017).

The aim of the work presented here was to use *C. inopinatum* and *L. abyssinica* as model species to compare the balance between constitutive and inducible DT mechanisms. We predicted that the rapidly drying *C. inopinatum* would possess mainly constitutive mechanisms, while *L. abyssinica* would rely more on inducible mechanisms. Specifically, the mechanisms tested were the induction of ROS scavenging enzymes and sugar synthesis. The role of ABA in stress signalling was also studied.

Chapter 2: Material and Methods

2.1. Plant Material

Crepidomanes inopinatum (Pic.Serm.) J.P.Roux and *Loxogramme abyssinica* (Baker) M.G. Price 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) before each experiment. Material was cleaned and rehydrated, and then stored at 12°C under dim light for several days in a growth chamber fully hydrated to remove any residual effects of field stress.

Fig 2: The collection site of model species of *C. inopinatum* and *L. abyssinica* at Ferncliffe Nature Reserve in Pietermaritzurg. The pictures represents a site map (A) and entrance board of the site (B).

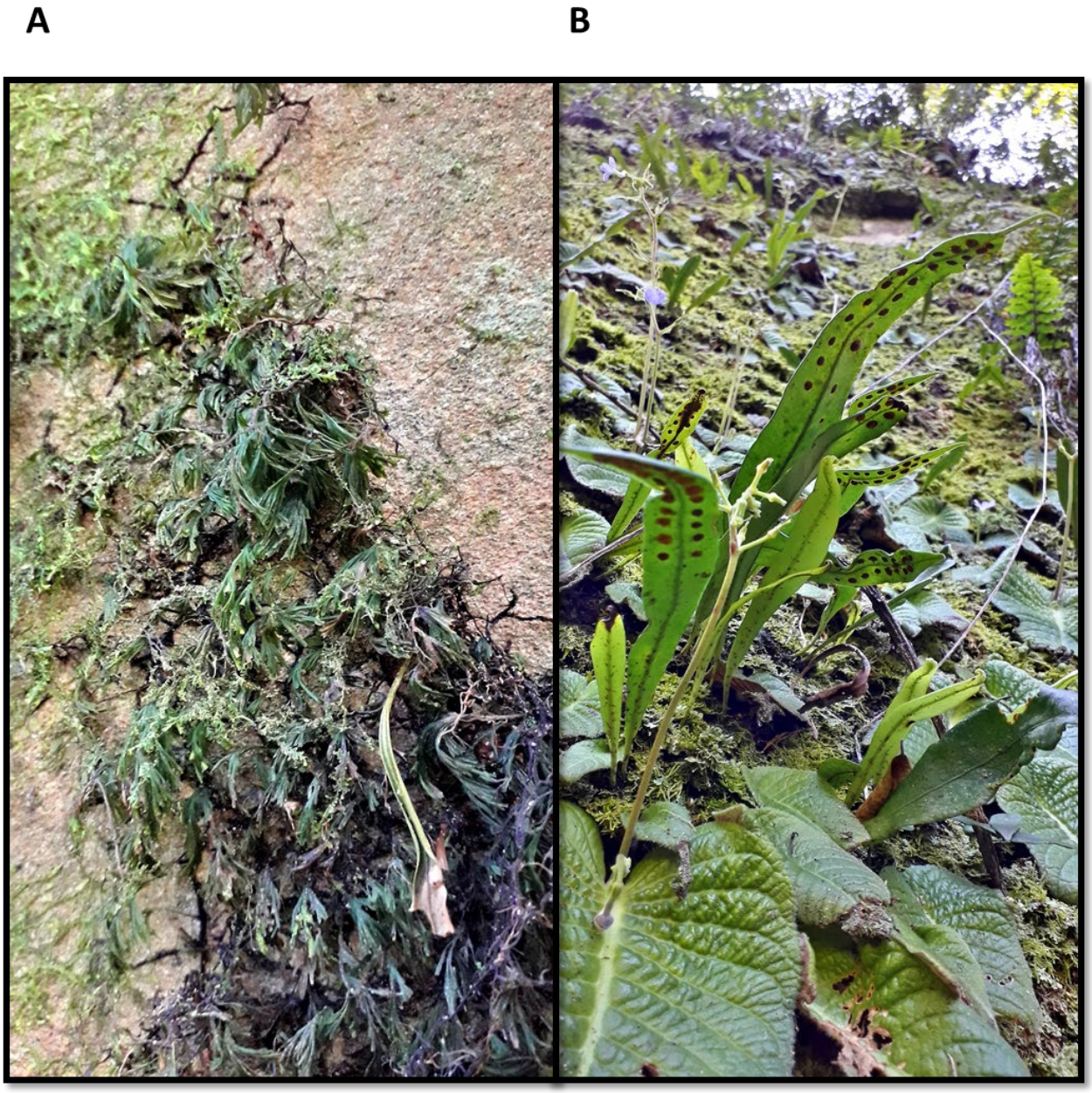

Fig 3: The model species of *C. inopinatum* (A) and *L. abyssinica* (B) found growing together in the understory of an Afromontane forest of Ferncliffe Nature Reserve.

Fig 4: The boulders of the Afromontane forest in Ferncliffe Nature Reserve where the model species (*C. inoptinatum* and *L. abyssinica*) were collected. The pictures represent the actual boarder (A) and the scrapping collection of the model species (B).

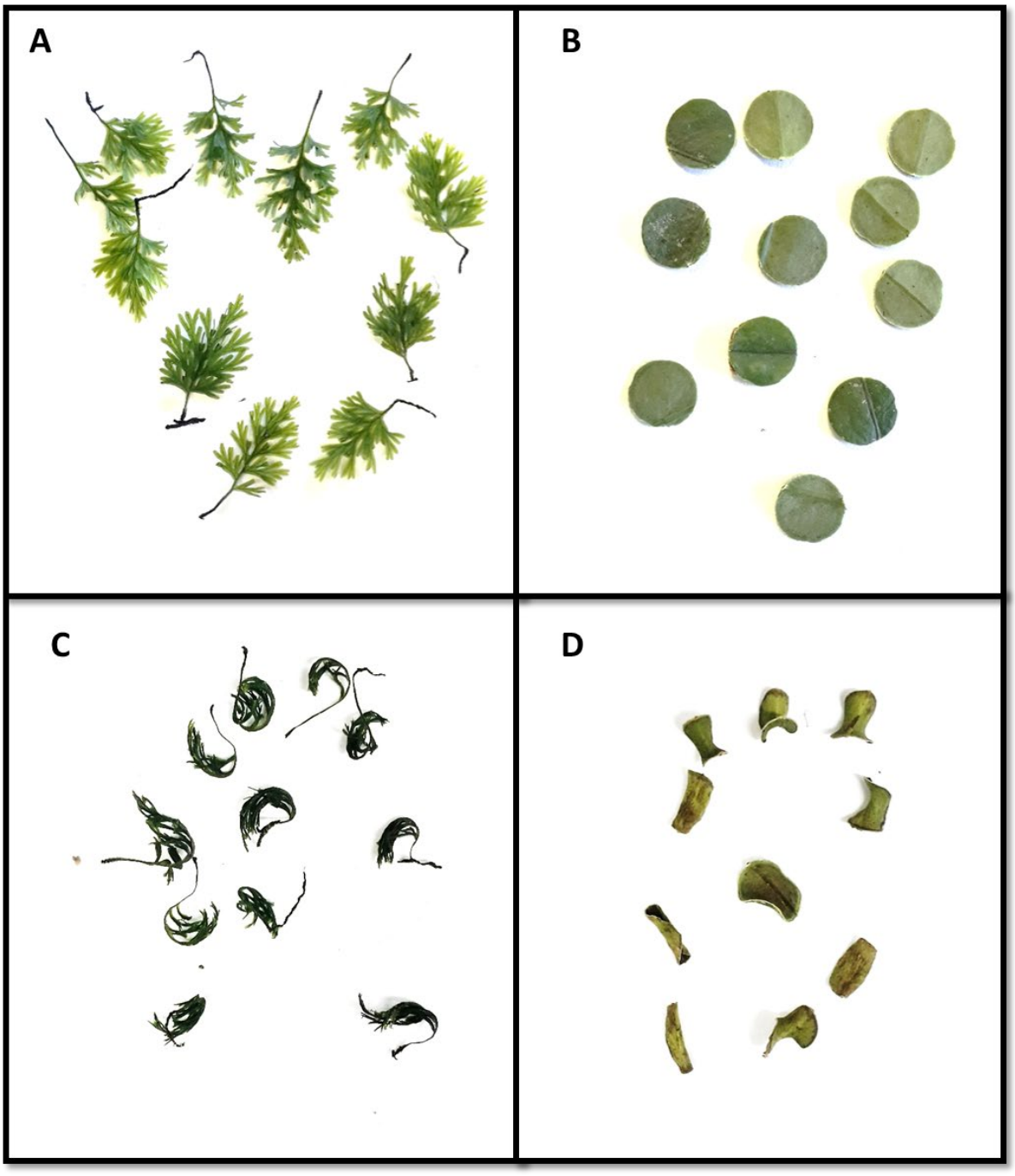

Fig 5: The fronds of the model species of *C. inopinatum* at hydrated state (A) and dried state (C), and also disks of *L. abyssinica* in the hydrated (B) and dried (D) state.

2.2. Effect of pH on peroxidase activity

2.2.1 The effect of pH on metabolism of o-*dianisidine*

The substrate *o*-dianisidine was used to determine the activity of peroxidases at different pH. Class III peroxidase reacts with H_2O_2 to give a product that has an optimum absorption at a wavelength of 440 nm using *o*-dianisidine as a substrate. A range of pH's was generated using a citrate-phosphate buffer. Assay conditions were 25 µL of enzyme/sample extract, 1 mM *o*-dianisidine and 1 mM H_2O_2. The extinction coefficient of *o*-dianisidine at 440 nm is 11.3 mM^{-1} cm^{-1}.

2.2.2 The effect of pH on metabolism of ascorbic acid

To measure the ascorbic (Class I) peroxidases, samples of 50 µL were incubated in quartz cuvettes containing citrate-phosphate buffer with a range of pH's and 1 mM EDTA, 5 mM ascorbate and 1 mM H_2O_2. Oxidation of ascorbic acid was followed spectrophotometrically (A_{280} = 2.8 mM^{-1} cm^{-1}).

2.2.3 Optimal pH for metabolism of ABTS

To measure oxidation of ABTS, samples of 100 µL were incubated in quartz cuvettes containing citrate-phosphate buffer with a range of different pH's and 10 mM ABTS for 15 min at 30˚C. Oxidation of ABTS was followed spectrophotometrically (A_{405} = 36.8 mM^{-1} cm^{-1}).

2.2.4 Optimal pH for metabolism of DMP

To measure oxidation of 2,6-dimethoxyphenol (DMP), samples of 25 µL were incubated in quartz cuvettes containing 1 mM DMP, McIlvaine buffer with a range of pH's, made of 0.1 M citric acid and 0.2 M Na_2HPO_4 and 1 mM H_2O_2. The oxidation of DMP was followed spectrophotometrically (A_{477} = 49.6 mM^{-1} cm^{-1}) at 25˚C.

2.3. Peroxidase characterization

2.3.1 The effect of substrate concentration on peroxidase activity

Enzyme extracts of 25 µL were incubated with a range of concentrations of *o*-dianisidine with 1 mM H_2O_2 in sodium acetate buffer pH 5. Concentrations used were 0.25, 0.5, 0.75, 1, 1.5, and 2 mM.

2.3.2. Effect of hydrogen peroxide concentration on peroxidase activity

Enzyme extracts of 20 µL were incubated with 1 mM *o*-dianisidine and a range of concentrations of H_2O_2 (0, 0.1, 1, 2 and 5 mM) in sodium acetate buffer pH 5.

2.4. Light response curves

Light-response curves of NPQ and ETR were generated using the Hansatech Instruments (King's Lynn, UK) FMS 2 modulated fluorometer by exposing samples to 0 – 87 µmol m^{-2} s^{-1}. Ferns were clamped in standard Hansatech leaf-clips and were dark-adapted for 10 min before the start of light curve measurements to allow determination of fluorescence levels F_o (Minimum fluorescence) and F_M (Maximum fluorescence), required for the calculation of NPQ. The actinic light was then switched on and fluorescence parameters measured in 15 replicates of each species at a range of light intensities. ETR and NPQ were calculated as follows: relative electron transfer rate (ETR) = ΦPSII x 0.5 x PFD, NPQ (estimated as the Stern-Volmer quotient) = $(F_M - F_{M'})/F_{M'}$. The resulting values of ETR and NPQ were then used to calculate QE (Quantum efficiency) and A_{MAX} (Maximum saturation rate) using the "Photosynth Assistant" software.

2.5. Effect of slow and rapid desiccation on relative water content

Fronds of *C. inopinatum* (30 fronds of similar size) and discs of *L. abyssinica* (30 x 1 cm discs) were cut and used to check for relative water content (RWC) during dehydration and rehydration. Fronds of both species were placed in small 65 mm petri dishes (6 replicates of 5 segments) and weighed fully hydrated (turgid mass). To compare the effect of slow and rapid dehydration, petri dishes with fronds were placed for 48 h above a saturated solution of calcium acetate ($(CH_3.COO)_2Ca$), giving a relative humidity of 20% and 1 week in a desiccator suspended above silica gel, giving a relative humidity of 0%, at 10°C under dim light respectively. Fronds were weighed after 0, 4, 8, 24, 28, 32, and 48 h for the material above calcium acetate and those above silica gel. At the end of the experiment, ferns were dried overnight at 70°C to obtain their dry mass. For rehydration, samples (not oven dried samples) were placed moist/hydrated in a growth chamber at 10°C under dim light for 8 h (both slowly and rapidly desiccated material), at specific time intervals (52 and 56 h) respectively. The formula below was used to calculate the RWC.

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

FM – Fresh mass (mass at any given time)

TM – Turgid mass

DM – Dried mass (oven dried at 70˚C)

2.6. Chlorophyll fluorescence during drying and wetting cycles

Chlorophyll fluorescence was measured using a Hansatech Instruments FMS 2 modulated fluorometer. Ferns were clamped in standard Hansatech leaf-clips. To take a measurement, each replicate was pretreated in darkness for a minimum of 10 min, and F_o and F_M were recorded. The actinic light (50 μmol m^{-2} s^{-1}) was then switched on, and F_o and $F_{M'}$ measured after several minutes (when F_o became stable). Fluorescence parameters were calculated as described in section 2.4. For slowly dried material, chlorophyll fluorescence was measured after 0, 4, 8, 24, 28, 32, and 48 h of dehydration above calcium acetate, and up to 8 h of rehydration (48, 52 and 56 h after the start of the experiment). For rapidly dried material, measurements were taken after 0 and 168 h (1 week desiccation) and up to 56 h of rehydration (172, 176, 192, 196, 200, 216, 220, and 224 h after the start of the experiment).

2.7. Ion leakage/conductivity

Ion leakage is a measure of the effect of desiccation stress resulting in ion leakage upon rehydration. A Mettler Toledo conductivity meter was used to measure the amount of ion leakage. Replicates of 5 x 0.05 g of *C. inopinatum* and *L. abyssinica* fronds were desiccated over silica gel for 1 week. Fronds were hydrated shaking the plants for 30 min in 10 ml distilled water in test tubes. Initial measurements were recorded after 30 min, followed by boiling at 100˚C in 5 ml distilled water for 15 min (with a marble on top, to limit evaporation). Plant material was then removed, and 5 ml of distilled water was then added making 10 ml. The mixture was stirred, and the final conductivity was recorded. An average of 2 blanks was used to correct for ions in the treatment solution. The proportion of ions lost during stress was calculated as:

$$\text{Conductivity} = Cv/(Cv + Cf)$$

Cv = Conductivity from each sample
Cf = Final/total electrical conductivity

2.8. Effect of slow and fast desiccation and rehydration on enzyme activity

Ferns were collected as discussed in section 2.1, and 360 fronds and discs selected for *C. inopinatum* and *L. abyssinica* respectively. Half the material was placed in a desiccator suspended above a saturated solution of $(CH_3.COO)_2Ca$ as described above. The remaining material was kept fully hydrated by storage on moist filter paper. After 0, 4, 8, 24, 28, 32 and 48 h of desiccation and 8 h of rehydration (52, 56 h), five replicates of 5 fronds and discs were ground in 5 ml of 50 mM phosphate buffer (pH 7) for 5 min from the desiccated, rehydrated and moist treatments. After grinding, sample extracts were centrifuged at 4˚C in Eppendorf tubes for 5 min at 10000 *g*. The supernatants were frozen at -24˚C until analysis. This was repeated for fronds of both species placed in a desiccator suspended above silica gel for 1 week (168 h) and 56 h of rehydration (as described in section 2.6) and another set rehydrated for 1 week (168 h).

2.8.1. Superoxide dismutase assay

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) by SOD enzyme as described by Giannopolitis and Ries (1977). The SOD assay was only carried out for material slowly dried for 48 h above calcium acetate and for up to 8 h after rehydration (exactly as described in section 2.8). Each replicate comprised five fronds of each species. Replicates were homogenized using 5 ml of 50 mM phosphate buffer (pH 7), and then centrifuged at 10000 *g* for 5 min. The reaction mixture contained 50 mM sodium carbonate,13 mM L-methionine, 63 µM NBT, 1.3 µM riboflavin and 100 µl of sample making up a final volume of 3.0 ml. The mixtures were illuminated with a fluorescent lamp in glass test tubes, aligned in a tube rack, which was covered with aluminium foil. For standardized reaction, the lamp was placed at >30 cm from the tubes for 30 min. The absorbance was recorded at 560 nm using a spectrophotometer. Activity was expressed as units mg^{-1} dry mass and calculated as the inhibition of photochemical reduction of NBT.

The equation used is as follows:

$$\left(\frac{[(V/v) - 1]\,(Dilution\ factor)}{mass}\right) \div 1000$$

- Where *V* and *v* represent the rate of the assay reaction in absence and in presence of SOD, respectively.

- Dilution factor = 30

2.8.2. Peroxidase activity

Peroxidase activity were measured by following the oxidation of 1 mM *o*-dianisidine in the presence of 1 mM H_2O_2 and 50 µL of sample in sodium acetate buffer pH 5 making up a volume of 1 ml (ε_{440} = 11.3 mM^{-1} cm^{-1}). Ferns were subjected to 48 h slow desiccation above calcium acetate, followed by 8 h of rehydration. Measurements were taken at specific time intervals (0, 4, 8, 24, 28, 32 and 48 h) of desiccation and up to 8 h of rehydration (52, 56 h). The absorbance was recorded at 440 nm wavelength using a spectrophotometer. This was also repeated for fronds of both species rapidly dried by placing them in a desiccator suspended above silica gel for 1 week (168 h) and 168 h of rehydration. Measurements were taken at specific time intervals of desiccation (0, 168 h) and rehydration (172, 176, 192, 196, 200, 216, 220 and 224, another set was measured at 192, 216, 240, 264, 288 and 360 h - 1 week, once a day).

2.9. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out to investigate peroxidase isoforms and the presence of superoxide producing enzymes in the two species. Fresh fronds were ground using a phosphate buffer pH 7 and then the extract centrifuged at 5000 *g*. Sample 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.

A modified method of Laemmeli (1970) was used, using native 12% and 6% gels. Peroxidase activity was visualized using a Na-acetate buffer (0.25 M, pH 5.0) containing 10% glycerol, 1 mM *o*-dianisidine and 10 µL of sample with 2 mM H_2O_2. For superoxide production, Na-phosphate buffer (50 mM, pH 7.4) containing 10% glycerol, 10 µL of sample, 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. Molecular mass markers were run at the same time and stained with Coomassie Blue.

2.10. Effect of desiccation and rehydration on soluble sugars

To measure soluble sugars produced during desiccation, the dinitrosalicylic colorimetric method was used (Miller, 1959). Five replicates of 5 fronds and discs were ground in 80%

ethanol after 0, 4, 8, 24, 28, 32 and 48 h of desiccation above calcium acetate and 8 h of rehydration (52, 56 h) and then centrifuged in Eppendorf tubes for 5 min at 13200 rpm. Supernatant (3 ml) was mixed with 3 ml of dinitrosalicylic acid reagent (DNS Reagent) solution in test tubes and heated up in boiling water for 5-15 min. Rochelle salt solution (1 ml of 40% potassium sodium tartrate) was added in all tubes immediately after boiling to stabilize the colour of the mixture. After cooling to room temperature in an ice bath, the absorbance was recorded at 575 nm using a spectrophotometer. Sugar concentrations were measured by reference to a standard curve constructed using glucose (0–500 μg ml^{-1}). This was repeated for fronds of both species placed in a desiccator suspended above silica gel for 1 week (168 h) and up to 5 d of rehydration (192, 216, 240, 264 and 288 h).

2.11. ABA Treatment

The effect of ABA on DT was measured using 100 μM ABA (cis, trans; Sigma) which was dissolved with 0.1% DMSO. The pH of the ABA solution and the water control (also 0.1% DMSO) were adjusted to 5.6 with HCl. Samples were first vacuum infiltrated in H_2O or 100 μM ABA solution, and then six replicates of 5 x 1 cm *L. abyssinica* disks and 5 *C. inopinatum* fronds of same size were shaken for 1 h in the same solutions. The effect of ABA treatment was assessed by measuring the chlorophyll fluorescence parameters as described in section 2.4. Measurements were taken at specific time intervals (0, 1, 2, 3, 4, 8, 24, 28, 32, 48 and 168 h (1 week)) upon rapid desiccation over silica gel and (0, 0:30, 1, 1:30, 2, 4, 8, 24, 28, 32 and 48 h) upon rehydration.

2.12. Data analysis

IBM SPSS Statistics 25 was mainly used in the analysis of results presented here. Tests of two-way ANOVA, Multiple comparison and Interactions were conducted. Graphs were plotted using Fig. P.

3.1. The effect of substrate pH on peroxidase activity

Crepidomanes inopinatum and *Loxogramme abyssinica* exhibit different pH optima for different substrates. *O*-dianisidine metabolism by Class III peroxidases has a pH optimum of pH 5 in both species (Figure 6A). The optimum activity of Class I (ascorbate) peroxidase was at pH 8 in both species (Figure 6B). The optimum activity of ABTS peroxidase was at pH 3.5 and pH 3 respectively (Figure 6C). The optimum activity of DMP peroxidase was at pH 4 and pH 5 respectively (Figure 6D).

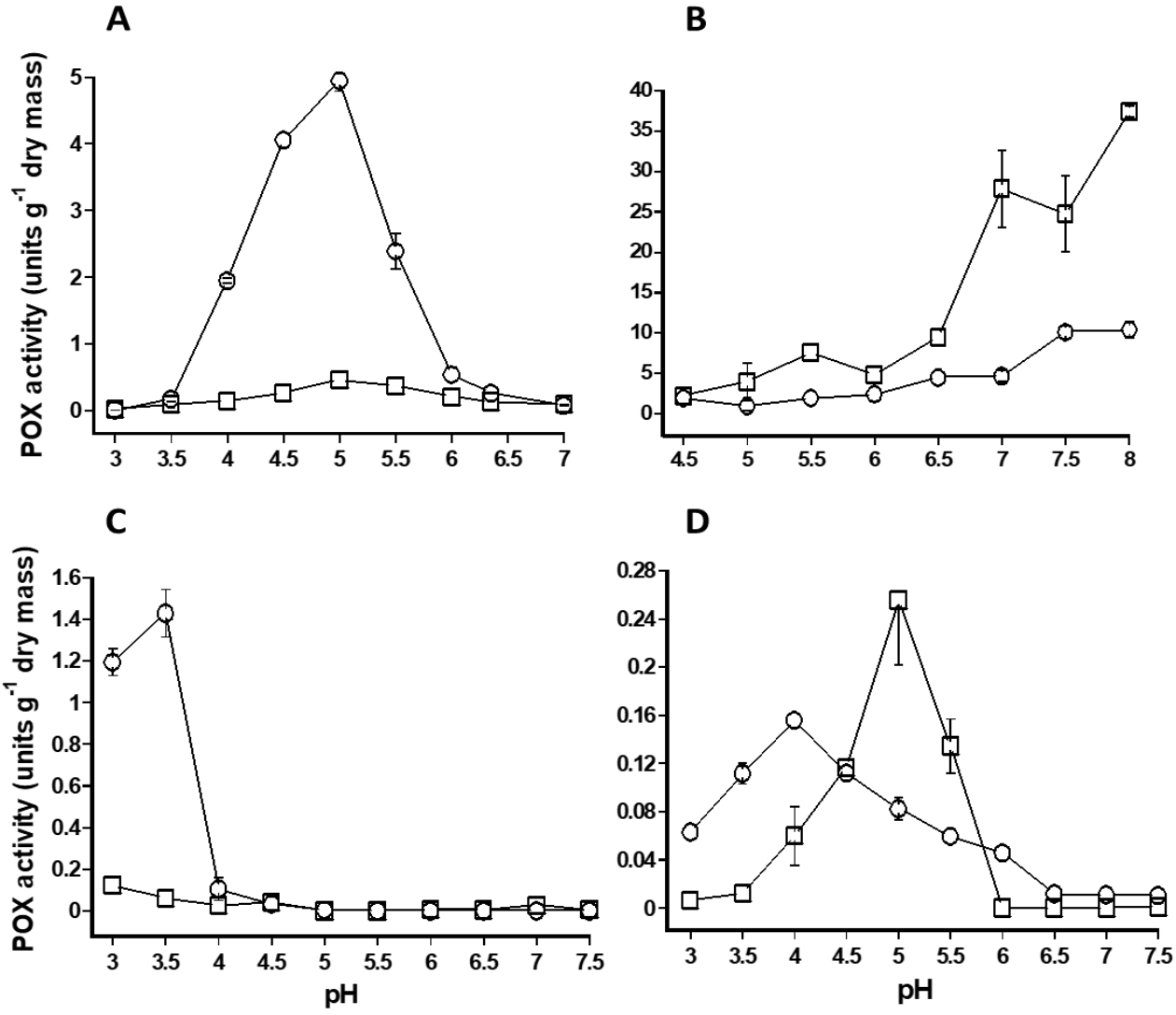

Fig 6: The effect of pH on Class III (*o*-dianisidine) (A) and Class I (ascorbate) (B), ABTS (C) and DMP (D) peroxidase activities in *C. inopinatum* (open circles) and *L. abyssinica* (open squares). Error bars indicates standard error of the mean (mean ± S.E).

3.2. The effect of substrate and hydrogen peroxide concentration on enzyme activity.

The optimum H_2O_2 concentration was 5 mM in *C. inopinatum* and 2 mM in *L. abyssinica* (Figure 7A). The highest concentrations of *o*-dianisidine (substrate) that gave optimal rates of product formation were 3 mM for *C. inopinatum* and 1.5 mM for *L. abyssinica* (Figure 7B). At hydrogen peroxide concentrations above 5 mM, the peroxidase activity starts to decrease gradually in both species. Estimate of kinetic parameters indicated higher K_m (0.96 ± 0.67 mM) and V_{max} (17.23 ± 4.72 units g^{-1} dry mass) values for *C. inopinatum* than *L. abyssinica* where K_m was 0.61 ± 0.56 mM and V_{max} was 1.6 ± 0.05 units g^{-1} dry mass, although the estimates of K_m were rather variable, and difference between the species but not significant.

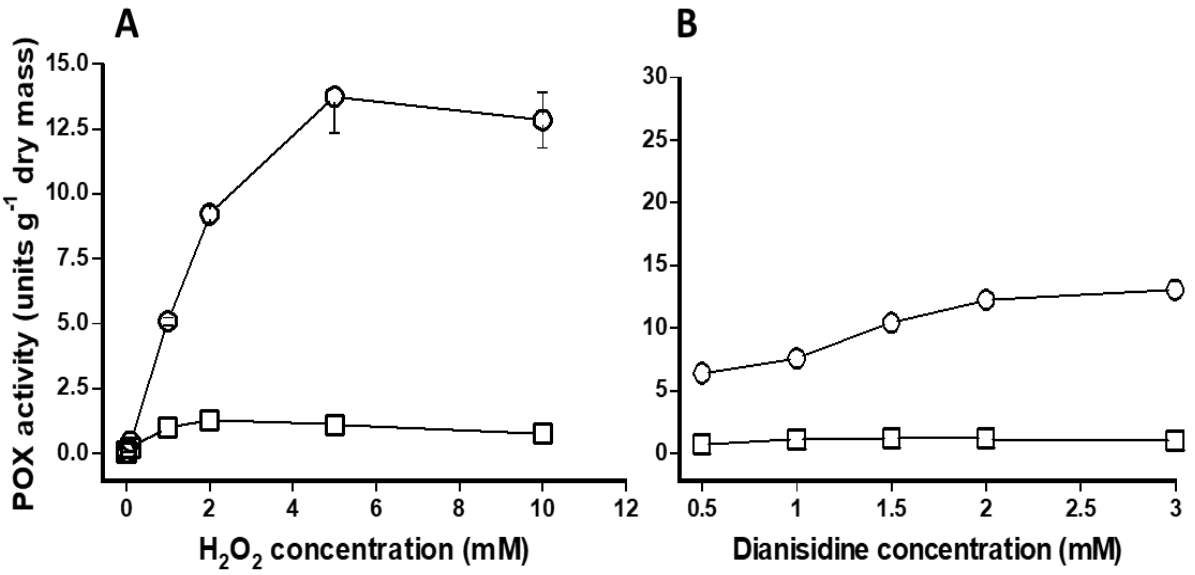

Fig 7: The effect of hydrogen peroxide (H_2O_2) (A) and substrate concentration (*o*-dianisidine) (B) on Class III peroxidase activity in *C. inopinatum* (open circles) and *L. abyssinica* (open squares). Error bars indicates standard error of the mean (mean ± S.E).

3.3. Effect of freezing storage

Unfortunately, it was not possible to store dry material of *C. inopinatum* at -22˚C, as storage dramatically reduced viability (Figure 8). Storage of *L. abyssinica* in the same way caused no damage. Unlike *C. inopinatum*, the metabolism of *L. abyssinica* was maintained at high levels after one week of freezing.

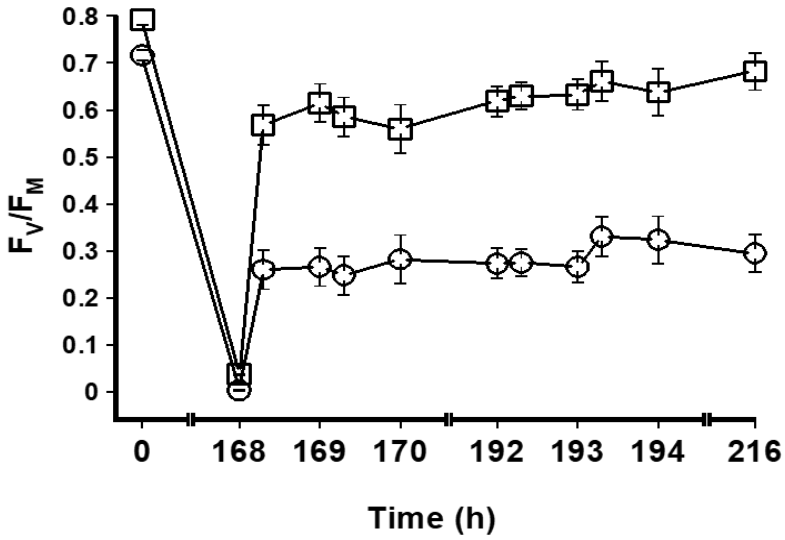

Figure 8: The effect of rapid desiccation on F_V/F_M in *Crepidomanes inopinatum* (open circles) and *Loxogramme abyssinica* (open squares). Both ferns were air dried for 3 days and frozen for one week at -22°C. Error bars indicates standard error of the mean (mean ± S.E).

3.4. Light response curves

Both poikilohydric species are adapted to low light intensity and saturated at light intensity of about 72 µmol m^2 s^{-1} (Figure 9). Plots of ETR as function of light intensity in *C. inopinatum* showed that this species has an A_{MAX} of 5.03 and a QE of 0.299, and NPQ stopped increasing

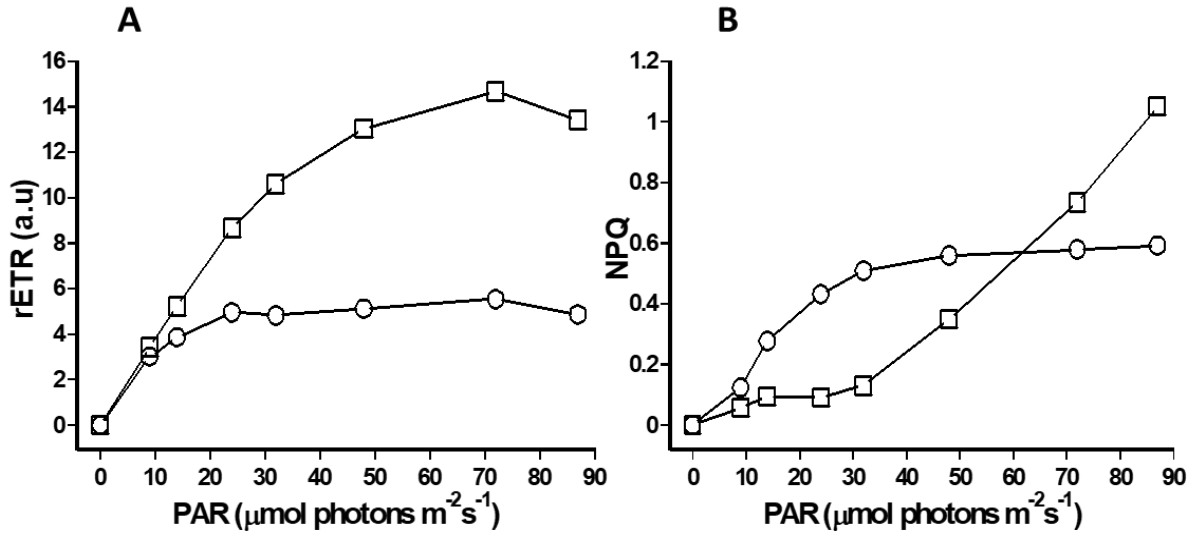

Fig 9: The effect of light intensity on ETR (A) and NPQ (B) in *C. inopinatum* (open circles) and *L. abyssinica* (open squares). Error bars indicates standard error of the mean (mean ± S.E).

when the light intensity exceeded about 30 µmol m^2 s^{-1}. On the other hand, A_{MAX} in *L. abyssinica* was c. 14.5 and QE 0.377; however, NPQ did not saturate.

3.5. Effect of desiccation

3.5.1 RWC

When ferns were placed above calcium acetate, the RWC of the filmy fern *C. inopinatum* fell by c. 80% within 4 h, while *L. abyssinica* dried much more slowly, taking 24 h to lose a similar amount of water (Figure 10A). Both species recovered their initial RWC within 4 h. Above silica gel, *L. abyssinica* dried more slowly than *C. inopinatum* (Figure 10B). Interestingly, both species rehydrated at the same rate suggesting that in the dry state the cuticle of *L. abyssinica* presented no barrier to water uptake.

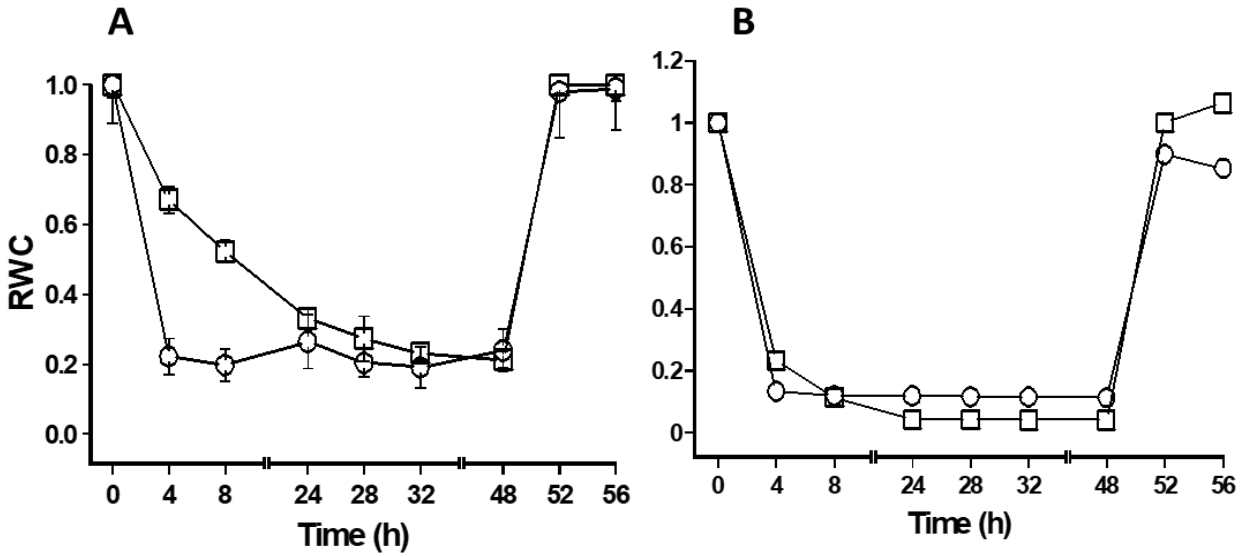

Fig 10: The relative water content (RWC) of *C. inopinatum* (open circle) and *L. abyssinica* (open square) under desiccation stress of 48 h and 8 h of rehydration above calcium acetate (A) and above silica gel (B). Error bars indicates standard error of the mean (mean ± S.E).

3.5.2 Deacclimation effect of storage

Storing *C. inopinatum* under cool, dim conditions significantly increased the sensitivity of the fronds to rapid desiccation over silica gel, followed by rehydration (Figure 11A). F_V/F_M in freshly collected material (open circles) was reduced more than in stored fronds following storage for 1 week over silica gel. However, during rehydration while F_V/F_M in freshly collected fronds recovered to initial values, stored fronds failed to recover completely (open diamonds). No deacclimation occurred in *L. abyssinica* (Figure 11B).

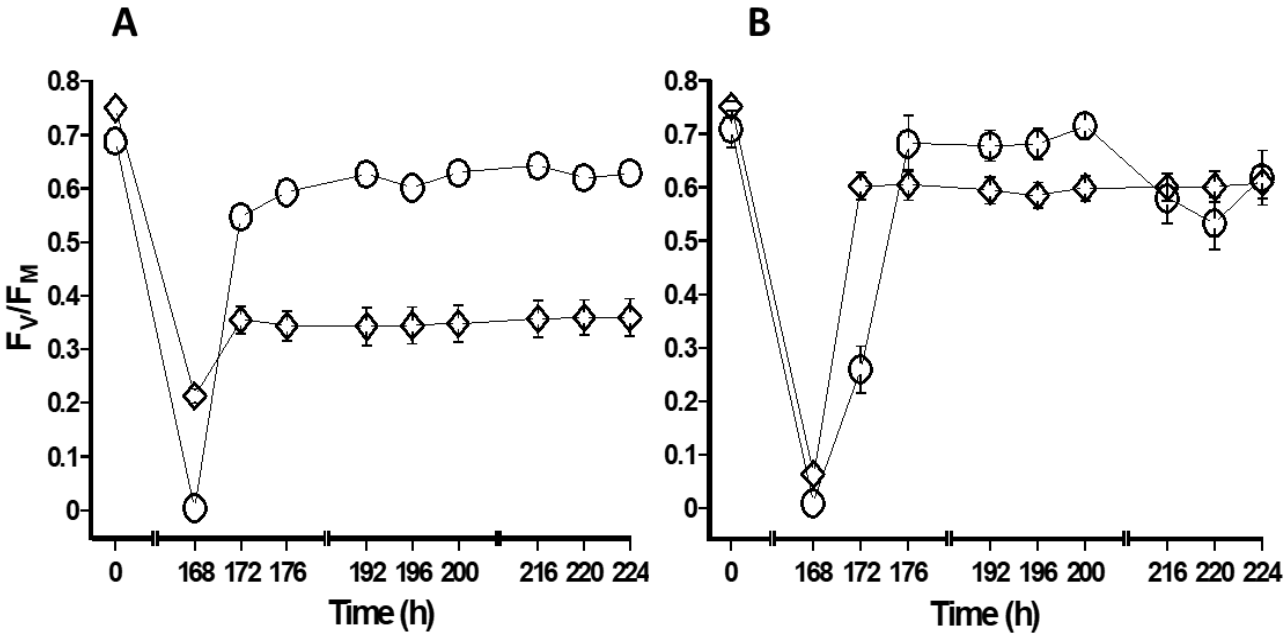

Fig 11: The effect of deacclimation time in *C. inopinatum* (A) and *L. abyssinica* (B), stored under controlled dim light for 24 h (open circles) and more than 168 h (open diamonds). Material was then desiccated for 1 week and rehydrated for 56 h. Error bars indicates standard error of the mean (mean ± S.E).

3.5.3 Effect of desiccation on chlorophyll fluorescence

The chlorophyll fluorescence parameters of F_V/F_M, ETR and NPQ declined much more rapidly above calcium acetate in *C. inopinatum* than in *L. abyssinica* (Figure 12A, C, E). Both species recovered their initial values of F_V/F_M and ETR within 4 h of rehydration. After rehydration the NPQs of both species were higher than the initial values. Long term desiccation above silica gel greater reduced chlorophyll fluorescence parameters in both species, but parameters rapidly and fully recovered during rehydration, and were stable for days following rehydration (Figure 12B, D, F).

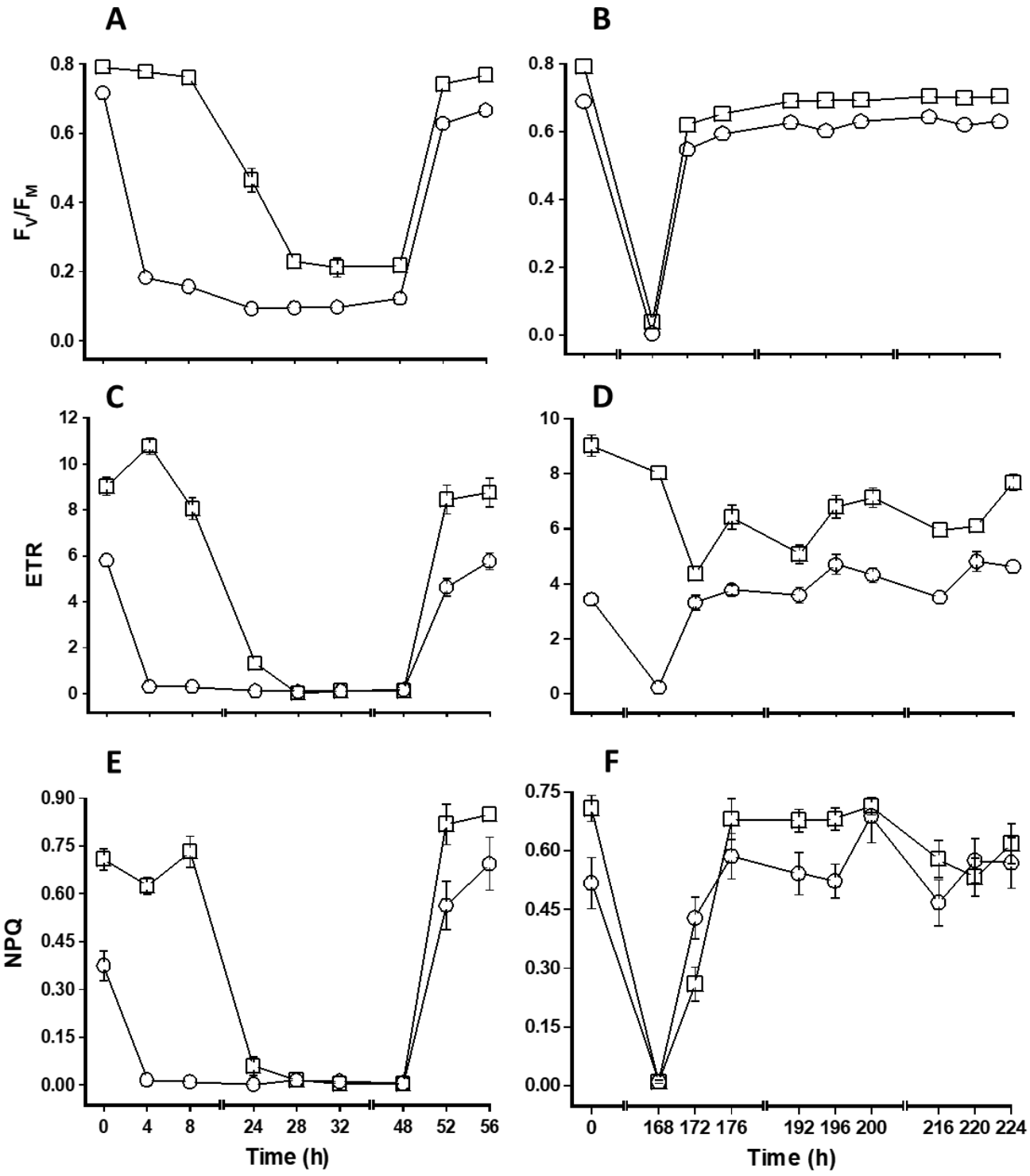

Fig 12: The effect of desiccation stress on different chlorophyll fluorescence parameters in *C. inopinatum* (open circles) and *L. abyssinica* (open squares) measured for 48 h slow desiccation and 8 h rehydration as Fv/Fm (A), ETR (C) and NPQ (E) and also 1 week rapid desiccation and 56 h rehydration, measured as Fv/Fm (B), ETR (D) and NPQ (F). Error bars indicates standard error of the mean (mean ± S.E).

3.5.4 Effect of desiccation on Ion leakage / conductivity

Desiccation of both species for one week above silica gel followed by sudden rehydration caused very little ion leakage in both species (Table 3). Nevertheless, *C. inopinatum* resulted in a significant increased ion leakage following 1-week desiccation, compared to *L. abysinica.*

Table 3: Ion leakage of *Crepidomanes inopinatum* and *Loxogramme abyssinica* after one week of desiccation with silica gel (0% RH). Material was firstly air dried for 3 days and stored frozen, then rehydrated prior to experiment/silica drying. Results are presented as percentage mean ± SD.

Treatment	Ion leakage (%)
Plants desiccated over silica gel for 1 week	
C. inopinatum	3.97 ± 0.92
L. abysinica	7.91 ± 0.89
Plants kept moist	
C. inopinatum	1.66 ± 0.21
L. abyssinica	6.20 ± 2.48

3.6. Effect of desiccation and rehydration on enzyme activity

3.6.1 Superoxide dismutase

The activity of ROS scavenging enzymes was much higher in *C. inopinatum* than *L. abyssinica*, with SOD activity being c. double. In *C. inopinatum* SOD activity rapidly increased during dehydration, remained at similar values for 48 h and then sharply dropped during rehydration (Figure 13A). Activity in moist material remained at similar values. In *L. abyssinica* SOD activity in dehydrated fronds increased but declined rapidly after 24 h and during rehydration (Figure 13B). Activity in the moist controls initially rose slightly, and the declined.

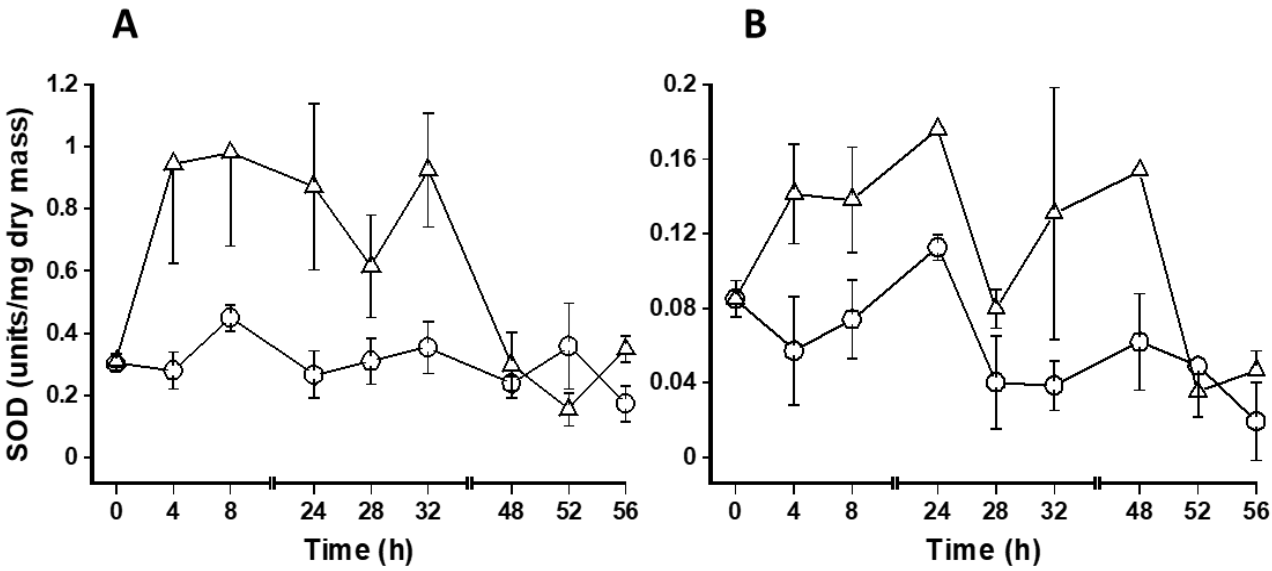

Fig 13: The effect of superoxide dismutase (SOD) in desiccation tolerance of *C. inopinatum* (A) and *L. abyssinica* (B) upon 48 h dehydration and 8 h (48-56 h) rehydration. The open circles represents the control, and open triangle represents the treatment. Error bars indicates standard error of the mean (mean ± S.E).

3.6.2 Peroxidase activity

C. inopinatum resulted in *c.* ten times higher POX activity, than that of *L. abyssinica.* In both species, slow dehydration for 48 h over calcium acetate has little effect on POX activity (Figure 14A, B). Desiccation gradually reduced activity in treated *C. inopinatum*, from 28 h to 52 h, with activity increasing after 52 h upon rehydration. Unlike *C. inopinatum, L. abyssinica* resulted in no effective change of POX activity upon wetting and drying cycle.

The effect of a more severe, prolonged desiccation (above silica gel) on POX activity was also studied (Figure 14C, D, E, F). Upon 1 week desiccation and 56 h rehydration, desiccation in both species reduced POX activity (Figure 14C, D). The second set of experiment of prolonged desiccation slightly increased POX activity in *C. inopinatum* after 1 week desiccation and was reduced to initial values after (Figure 14C). Activity in both species gradually rose upon rehydration and required a minimum of 1 week for full tolerance / recovery (Figure 14E, F).

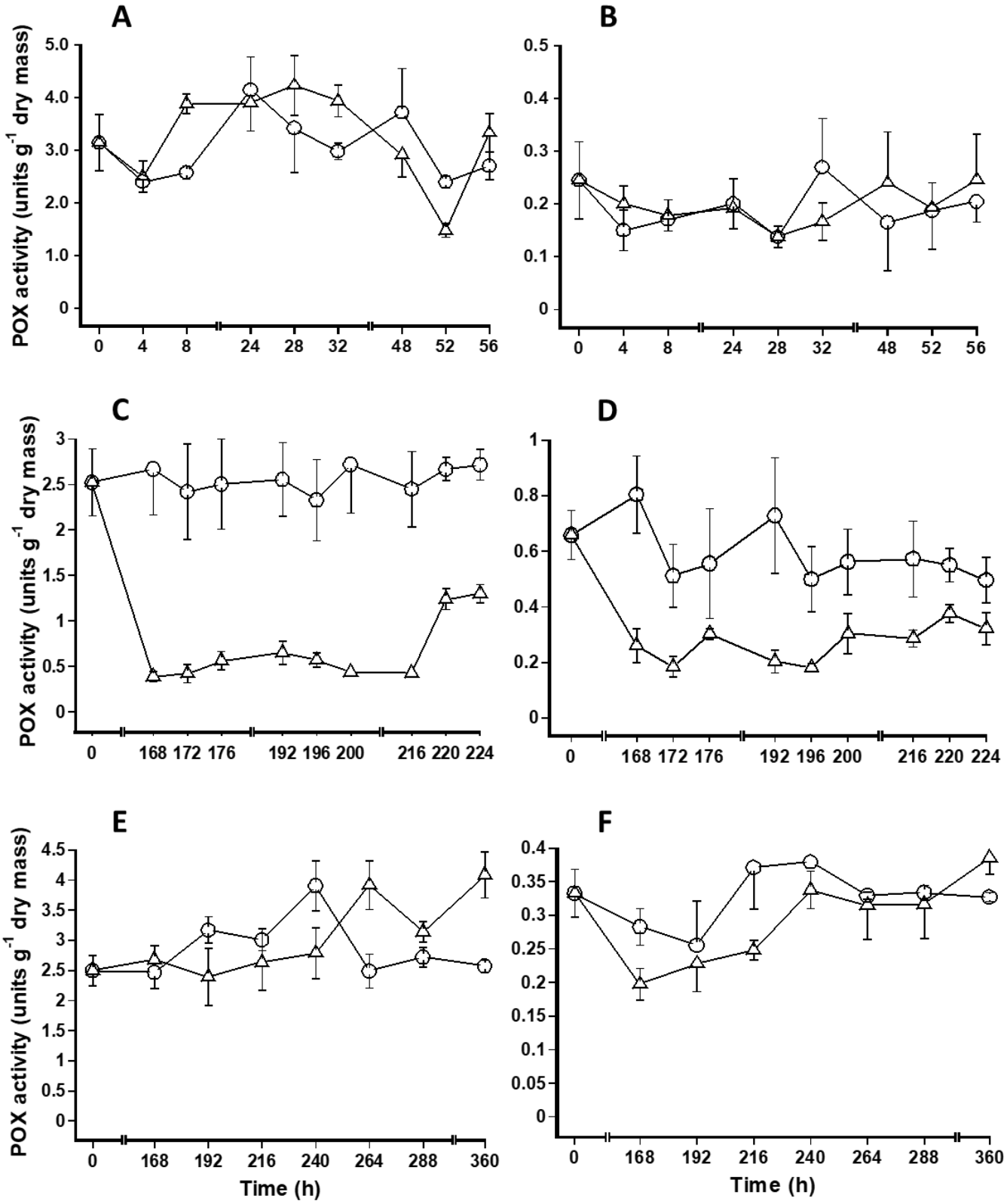

Fig 14: The effect of slow desiccation for 48 h and rehydration for 8 h on POX activity in *C. inopinatum* (A) and *L. abyssinica* (B) and upon 1 week rapid desiccation and 56 h rehydration (C, D) followed by 1 week extended rehydration (E, F) respectively. The open circles represents the control, and open triangle represents the treatment. Error bars indicates standard error of the mean (mean ± S.E).

3.7. PAGE analysis of peroxidase isoforms

Fern extracts were subjected to PAGE electrophoresis followed by visualization of POX activity with *o*-dianisidine and H_2O_2. *C. inopinatum* had two main isoforms of POX, one with a molecular mass of around 60 kDa, and another much less active form of around 140 kDa that was difficult to visualize (Figure 15D), while *L. abyssinica* possessed a single isoform of around 40 kDa (Figures 15B). Incubating gels in a mixture of NADH and NBT showed that *C. inopinatum* produced two enzymes capable of $O_2^{\cdot-}$ production, with molecular masses around 23 kDa and 10 kDa (Figure 15C), while *L. abyssinica* produced one isoform of around 20 kDa (Figure 15A). Thus, the molecular masses of the enzymes that produced $O_2^{\cdot-}$ did not correspond to those of the POX isoforms.

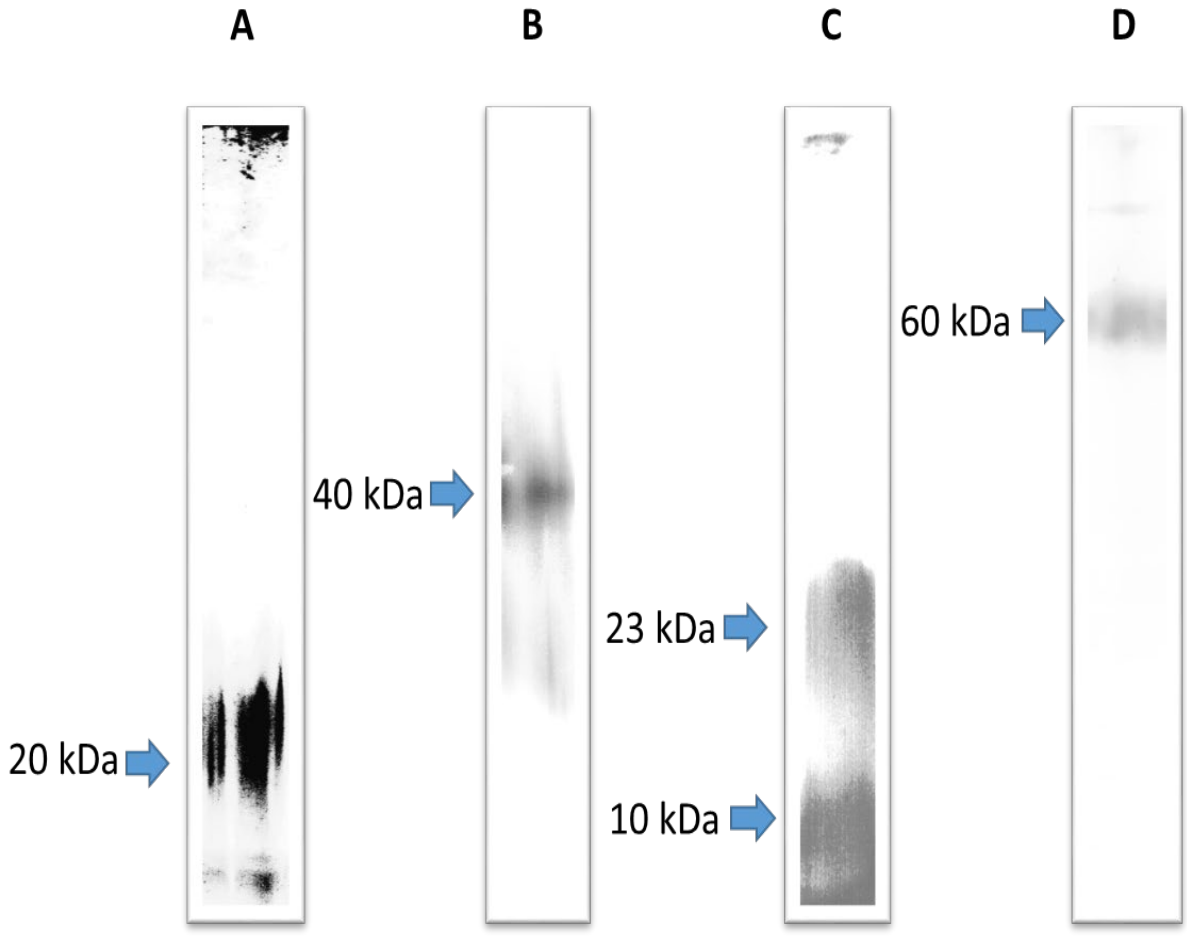

Fig 15: The 6% Native PAGE electrophoresis gels of *L. abyssinica* stained for superoxide production (A) and POX (B) and 12% gels of *C. inopinatum* stained for superoxide production (C) and POX (D).

3.8. Effect of desiccation and rehydration on soluble sugars

In general, in freshly collected material, the concentrations of soluble sugars were approximately twice as high in *C. inopinatum* compared with *L. abyssinica* (Figure 16). During slow drying over calcium acetate, the concentration of soluble sugars rapidly increased in *C. inopinatum* to more than double the initial values, while in *L. abyssinica* concentrations increased more slowly, reaching values three times higher than the initial values (Figure 16A, B). The concentrations of sugars in the controls, which were maintained moist, did not change. In the second experiment, initial concentrations of soluble sugars were approximately half those of the first experiment, but again were approximately twice as high in *C. inopinatum* compare with *L. abyssinica* (Figure 16C, D). Rapid drying increased the concentration of sugars in both species, but concentrations rapidly returned to pre-stress values upon rehydration in both species (Figure 16C, D).

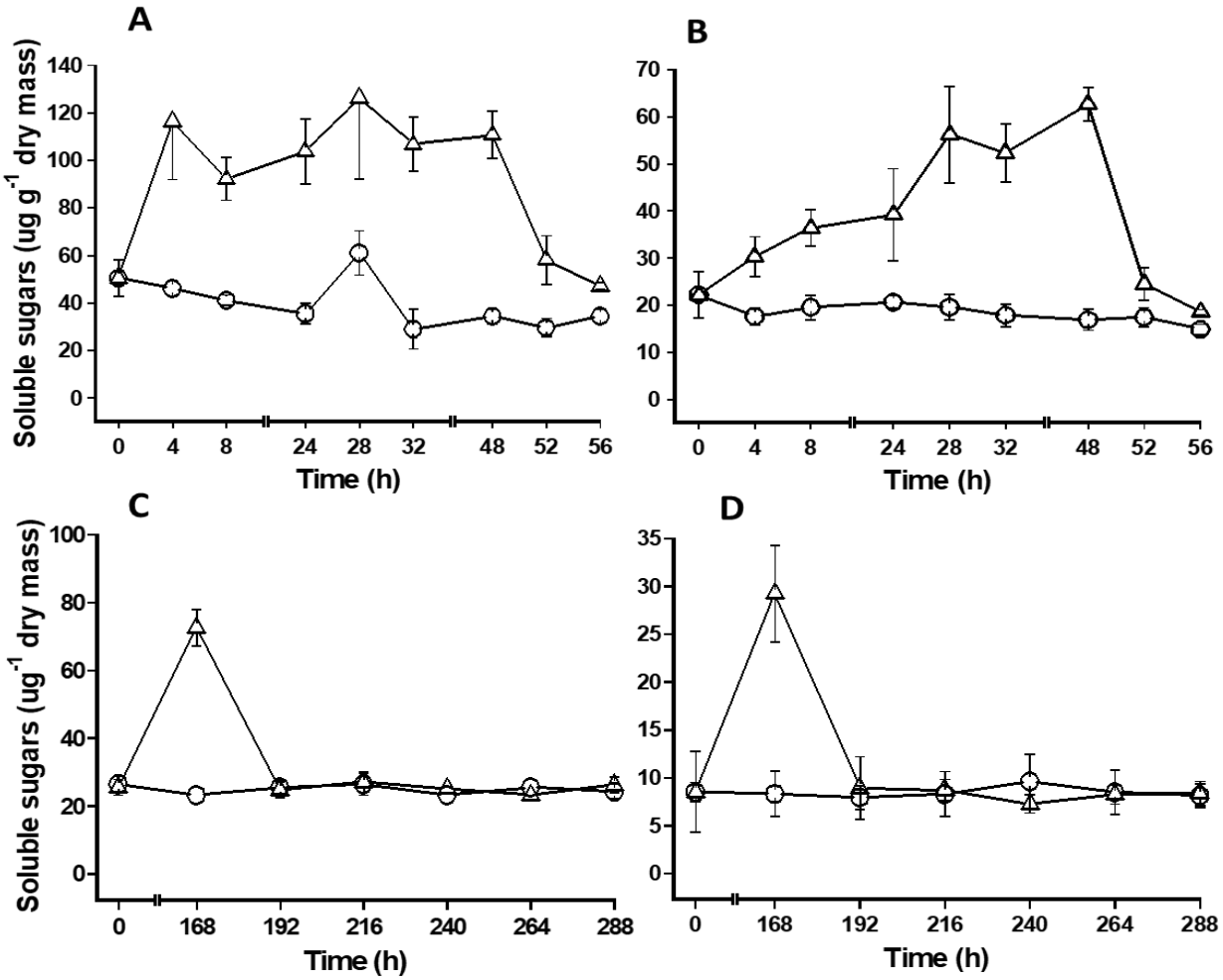

Fig 16: The effect of soluble sugars on desiccation tolerance *C. inopinatum* (A) and *L. abyssinica* (B) upon 48 h dehydration and 8 h rehydration and also subjected to 1 week desiccation and 6 days rehydration (C, D) respectively. The open circles represents the control, and open triangle represents the treatment. Error bars indicates standard error of the mean (mean ± S.E).

3.9. Effect of ABA treatment on the tolerance of photosynthesis to desiccation

ABA treatment slightly decreased the desiccation tolerance of *C. inopinatum* (Figure 17A, C, E). During desiccation, all parameters declined rapidly, and ABA treatment had little effect on their values. F_V/F_M and ETR, but not NPQ, recovered rapidly during rehydration, but then gradually declined (Figure 17B, D, F). Values of F_V/F_M and ETR were significantly lower in ABA treated fronds during rehydration. NPQ only started to increase after rehydration for 8 h, and then only in control fronds. By contrast, ABA treatment slightly improved the tolerance of *L. abyssinica* to desiccation (Figure 18). Compared with *C. inopinatum*, which has no cuticle, F_V/F_M and ETR declined much more slowly during desiccation due to the presence of a cuticle in *L. abyssinica* (Figure 18A, C). During rehydration, F_V/F_M and ETR rapidly recovered close to initial values and, unlike *C. inopinatum*, showed no indication of declining (Figure 18B, D). NPQ declined in a similar way to F_V/F_M and ETR during desiccation but had only recovered to values c. 25% of initial values after rehydration for 8 h. ABA treated fronds of *L. abyssinica* always performed better than the controls.

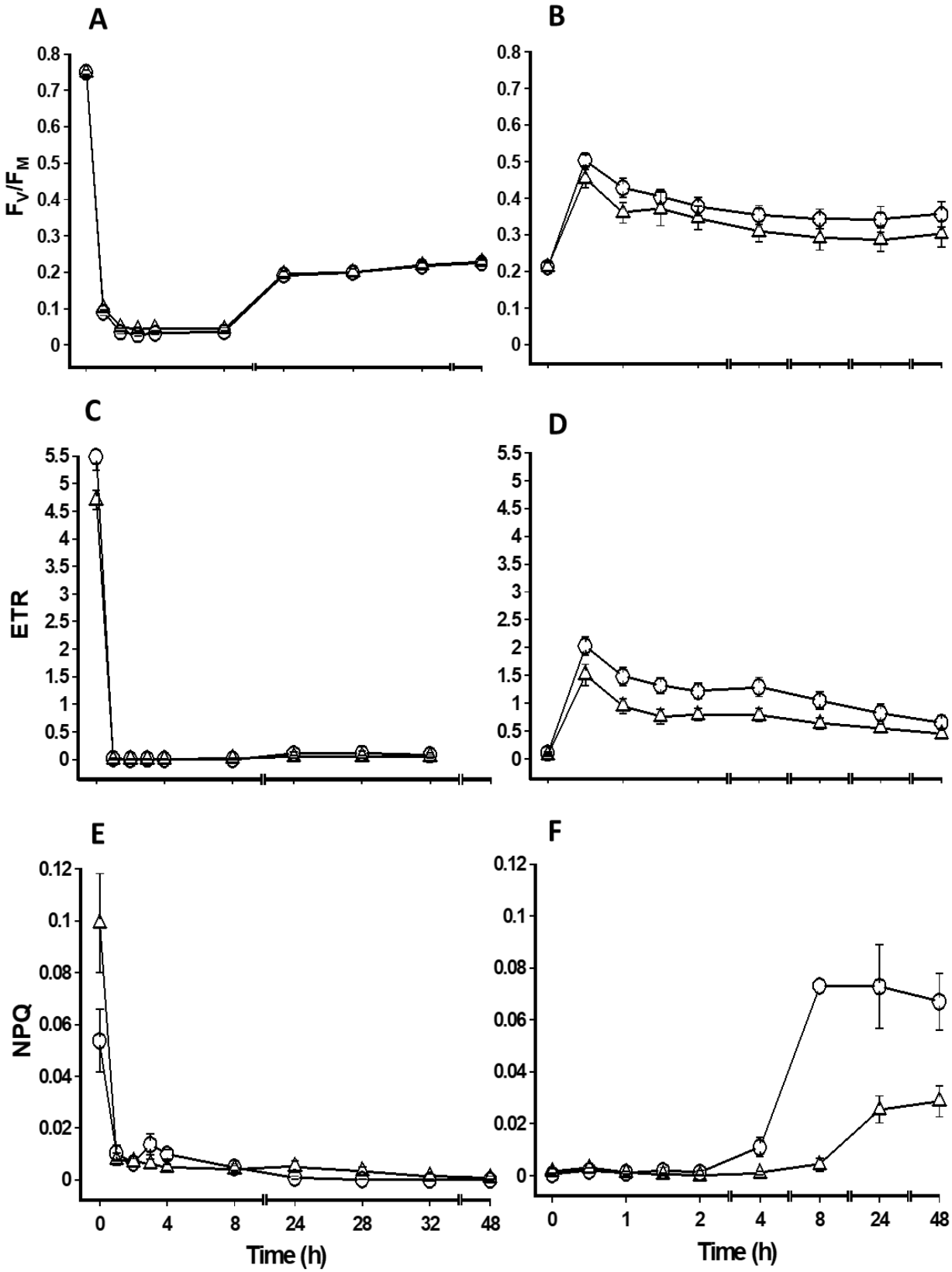

Fig 17: The effect of abscisic acid (ABA) treatment on chlorophyll fluorescence parameters in *C. inopinatum* upon 168 h (one week) desiccation on silica gel measured as Fv/Fm (A), ETR (C) and NPQ (E) and 48 h rehydration (B, D, F) respectively. Open circles represent controls and open triangles represent treatments. The rehydration time 0, is the measurement after 1 week of desiccation. Error bars indicates standard error of the mean (mean ± S.E).

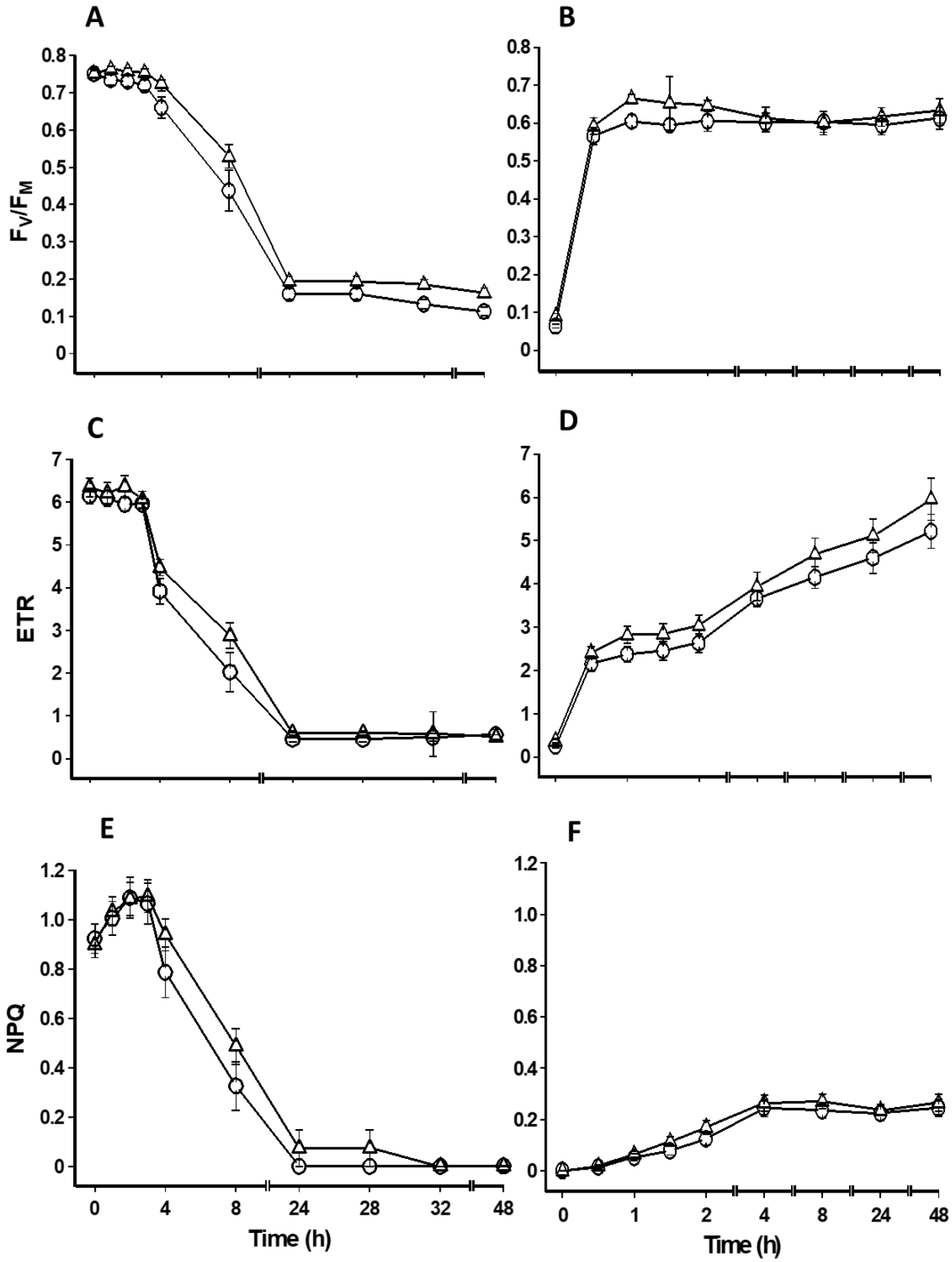

Fig 18: The effect of abscisic acid (ABA) treatment on chlorophyll fluorescence parameters in *L. abyssinica* upon 168 h (one week) desiccation on silica gel measured as Fv/Fm (A), ETR (C) and NPQ (E) and 8 h rehydration (B, D, F) respectively. Open circles represent controls and open triangles represent treatments. The rehydration time 0, is the measurement after 1 week of desiccation. Error bars indicates standard error of the mean (mean ± S.E).

Results presented here confirmed that under controlled conditions, *C. inopinatum* dries much more quickly than *L. abyssinica* (Figure 10A, B). As discussed in the Introduction, we predicted that the rapidly-drying filmy fern may rely primarily on constitutive DT tolerance mechanisms, while the species with a cuticle may possess inducible tolerance mechanisms. However, while both these ferns partially exhibit constitutive mechanisms (e.g. POX activity is maintained under moderate desiccation stress (Figure 14A, B)), in both species, DT appears to be strongly inducible even under rapid desiccation. In both species, slow desiccation increases the activity of the ROS scavenging enzyme SOD (Figure 13A, B) and the concentrations of soluble sugars (Figure 16). DT may even be more strongly inducible in *C. inopinatum*, as storing this species for c. 1 week under cool, dim conditions apparently causes deacclimation, while no loss of DT was observed in *L. abyssinica* (Figure 11). While ABA seems to play no, or only a minor role in inducing DT (Figures 17 and 18), taken together, results suggest both species should be classified as predominantly "IDT". Therefore, it should not be assumed that rapidly drying DT plants necessarily rely on constitutive DT mechanisms; rather each species needs to be investigated individually.

4.1. Effect of freezing storage

Initial experiments tested whether it was possible to collect material and store it so that experiments could be conducted later. Material of *L. abyssinica* fully recovered metabolism following one week of freezing (Figure 8). Similar results have been obtained with the related genus *Pleopeltis* (data not shown). By contrast, freezing caused some damage to *C. inopinatum* with F_V/F_M unable to recover to initial values (Figure 8). The reasons for the sensitivity of this species to freezing are uncertain. While it is tempting to assume that they are related to the absence of a cuticle in this species, many species of lichens and bryophytes, which also lack a cuticle, have been conveniently stored in this way.

4.2. Light response curves

Results from this experiment indicates that *L. abyssinica* and *C. inopinatum* are both shade adapted to Afromontane forest. In both species, A_{MAX} is achieved at a light intensity around 80 μmol photons m^{-2} s^{-1} (Figure 9). These results are consistent with those obtained for *Hymenophyllum cruentum*. In this species A_{MAX} was reached at c. 100 μmol photons m^{-2} s^{-1}; this fern also grows in shaded habitats (Parra et al., 2015).

4.3. Deacclimation of freshly collected material

The rapid recovery of water content and metabolism following 1 week rapid desiccation over silica gel in *C. inopinatum* and *L. abyssinica* indicates that both species are highly tolerant to desiccation (Figure 10; Figure 11). However, it is clear that the way material is stored after collection can have a profound influence on estimates of DT. Stark et al. (2017) emphasized that to distinguish CDT from IDT it is important to fully deacclimate plants material. Results presented in Figure 11 show that "de-acclimation" can occur in *C. inopinatum*; material stored under cool, moist conditions can clearly become much more sensitive to desiccation. A similar trend of de-hardening following storage has been observed in the moss *P. patens* (Greenwood, 2017). By contrast, no deacclimation occurred in *L. abyssinica* under the same conditions (Figure 11).

4.4. Both species of ferns are poikilohydric

It was clear from the effects of slow and rapid drying on chlorophyll fluorescence parameters that both species are genuinely poikilohydric (Figures 12). Very strong DT mechanisms protect cellular metabolism during desiccation and rehydration. Material tested here displayed rapid recovery of chlorophyll fluorescence parameters after desiccation for one week and 56 h rehydration (Figures 12B, D, F). Similar to *P. pleopeltifolia* (Voytena et al., 2014), in *C. inopinatum* and *L. abyssinica* "frond curling on drying might prevent the light–chlorophyll interaction, causing shutdown of ETR and, in turn, minimisation of ROS production".

The very minimal ion leakage that occurs upon rehydration in both species (>3%, Table 3) confirms that these ferns are genuinely poikilohydric. Ion leakage was mimimal, even though the material of *C. inopinatum* had been frozen, which probably caused some damage to the photosynthetic apparatus (Figure 8). Clearly, there are mechanisms that maintain membrane integrity in the dry state, as has been reported in other poikilohydric organisms (Kruger, 1998). Although minimal ion leakage was observed, signs of deleterious effects were visible, such as occasional incomplete recovery of chlorophyll fluorescence parameters in *C. inopinatum.*

4. 5. Change in enzyme activity

4.5.1 Superoxide dismutase

As discussed in the Introduction, desiccation is well known to promote the formation of potentially harmful ROS. An increase in the activity of ROS scavenging enzymes is a classic "inducible" stress tolerance mechanism in pteridophytes (Farrant et al. 2009; Wang et al., 2010; Živković et al. 2010; López-Pozo et al. 2018). In the present study, in *C. inopinatum* SOD

activity increased rapidly during the first 4 h of drying, and then remained constant until rehydration (Figure 13A). SOD synthesis presumably ceased when metabolic activity stopped, as after 4 h plants lost most of their water (Figure 10A) and their photosynthetic parameters dropped to values close to zero (Figure 12A, C, E). By contrast, in *L. abyssinica* SOD activity slowly increased for the first 24 h of drying and then slightly decreased until rehydration, but activities were only about 15% those of *C. inopinatum* (Figure 13B).

4.5.2 Peroxidase activity

The peroxidases of the model species of *C. inopinatum* and *L. abyssinica* both display maximum rates of metabolism using *o*-dianisidine as a substrate. The advantage of *o*-dianisidine is that it shows high sensitivity for reaction and results in stable coloured oxidized products (Bania and Mahanta, 2012). Peroxidase in both species was able to catalyse the oxidation of ABTS at lower pH (Figure 6C) compared to the oxidation of DMP catalysed at slightly higher pH (Figure 6D). The effect of pH on ascorbate peroxidase activity was also tested and found to increase with pH. However, the assay for Class I POX proved difficult to carry out on these species, and it was hard to get reproducible results. This was probably because many compounds absorb at 280 nm, the wavelength used to check for H_2O_2 breakdown. While future experiments should study whether, for example, desiccation upregulates Class I POX activity, it may be easier to use alternative methods, e.g. transcriptomics. The optimum pH of Class III enzyme was around 5 for both species (Figure 6A), and this pH was used in all future experiments. The kinetic parameters of K_m and V_{max} in both model species of peroxidase enzyme was determined by using *o*-dianisidine as a substrate. *L. abyssinica* had a lower K_m and V_{max} value compared to *C. inopinatum,* suggesting that peroxidase from *L. abyssinica* has a higher affinity for *o*-dianisidine compared to that of *C. inopinatum*. However, in higher plants, variations in the affinity of POX for *o*-dianisidine affinity exists (e.g. K_m and V_{max} values of 0.370 mM and 11.111 µM min^{-1} for *B. oleraceae capitata* peroxidase, 0.250 mM and 9.095 µM min^{-1} for *N. tabaccum* peroxidase and 0.277 mM and 10.012 µM min^{-1} for *R. sativus* peroxidase) (Bania and Mahanta, 2012).

As for SOD, absolute Class III POX activities are much higher (about ten times higher in this case) in *C. inopinatum* (Figure 14). POX activities of both species are maintained at more or less on the same levels in the desiccated and undesiccated fronds upon slow drying and rehydration (Figure 14A, B). By contrast, POX activity are clearly induced in both species upon rehydration after 1 week of dehydration with silica gel (Figure 14C, D, E, F). Activity in *C. inopinatum* was contrary downregulated (Figure 14C) and maintained slightly higher (Figure 14E) after 1 week desiccation above silica gel. However, material used in Figure 14C

was fully deacclimated compared to the material used in Figure 14E, suggesting that, not only the photosynthetic fluorescence (Figure 11A), but also peroxidase in *C. inopinatum* are sensitive to desiccation following long period of deacclimation (Figure 14C). Maintenance of good activities of antioxidant enzymes upon desiccation is very important to prevent ROS formation or damage at any particular stage during the wetting and drying cycles. This has been regarded as a typical feature of DT higher plants (Farrant et al., 2009). Shivaraj et al. (2018) suggested that more desiccation sensitive species suffer a failure of their antioxidant protection mechanisms following prolonged duration desiccation, consistent with the reductions in POX activity following storage over silica gel observed here. In a similar study with *Selaginalla tarmaricina,* SOD, ascorbate peroxidase and gluthione reductase were upregulated upon rehydration, presumably to prevent ROS damage (Wang et al., 2010). Related studies of peroxidase in ferns such as *Dicranopteris linearis* (Kavitha and Murugan, 2016) and *Asplenium ceterach* (Živković, et al., 2010) have demonstrated upregulation of peroxidase activity upon desiccation. It has been suggested that in these examples the upregulation of POX is more concerned with lignification for repair rather than ROS scavenging (Cosio and Dunand, 2009; Kavitha and Murugan, 2016).

4.5.3. Gel electrophoresis analysis of peroxidase isoforms

In both species, peroxidase activity occurs largely as a single isoform (Figure 15B, D). Usually most plant species have multiple peroxidase isoforms involved in different functions, but to test this for the ferns studied here would require whole genome sequencing / transcriptome analysis, recombinant DNA combined with biochemical approaches (Cosio and Dunand, 2009). In higher plants, Class III peroxidases are often involved not only in the scavenging of ROS, but also in the production of ROS, which in various ways form an important component of stress tolerance mechanisms (Almagro et al. 2009). However, although superoxide producing bands could be visualized (Figure 15A, C), these did not correspond to those of the peroxidases. Surprisingly, there appear to have been no studies on the enzymes responsible for ROS production in ferns. In the pteridophytes studied here production presumably occur by enzymes such as NADPH oxidases, which are responsible for ROS production in some higher plants (Marino et al., 2012).

4.6. Change in soluble sugars

Soluble sugars have been widely reported to protect plants against several abiotic stresses, and are very important component of DT in the so-called “modified” DT plant (Oliver et al., 1998). In *C. inopinatum* 48 h of slow drying increased the concentration of soluble sugars during the

first 4 h (Figure 16A), corresponding to the time when the fern was still metabolically active (Figure 12A, C, E), and thereafter concentrations remained constant until rehydration (Figure 16A). In *L. abyssinica*, concentrations rose more slowly (Figure 16B), again over the drying time when the fronds remained metabolically active (Figure 12A, C, E). Clearly, even if a fern dries quickly, it may still possess an inducible DT mechanism. In both fern species, the concentration of soluble sugars increased even if drying was rapid (Figure 16C, D), although the total amount of sugars induced were lower than those synthesised during slow desiccation. When both species were rehydrated, concentrations rapidly fell, and remained low following moist storage. The actual sugars synthesised were not determined here. However, in *Mohria caffrorum*, the most intensively studied pteridophyte for carbohydrate metabolism, desiccation increased the concentrations of sucrose, raffinose family oligosaccharides, and cyclitols (Farrant et al. 2009). Preliminary data from other species suggests that the major sugars involved in protective mechanisms are species-specific (López-Pozo et al. 2018).

As discussed earlier, it seems likely that the "sugars could act to stabilise membranes and proteins in the dry state by maintaining hydrogen bonding within and between macromolecules" (Denev et al., 2014). Second, the sugars could vitrify the cell contents and stabilise internal cell structure (Voytena et al., 2014), by forming a glass and protecting cellular integrity and drying out (Liu et al., 2008; Yobi et al., 2012). Soluble sugars can also act as a source of energy upon rehydration (Lopez-Pozo et al., 2018). Even so, on their own, soluble sugars are generally regarded as insufficient to protect cellular structures, and organisms probably use additional mechanisms e.g. specialized drought related proteins (Hellwege et al., 1994).

4.7. Effect of ABA treatment on the tolerance of photosynthesis to desiccation

The use of exogenous ABA application is well known to study DT in plants. In the filmy fern *Hymenophyllum caudiculatum* ABA treatment can increase DT by apparently stabilizing PSII (Garces et al., 2018). Similarly, exogenous application of ABA in *Polypodium virginianum* enables them to survive rapid desiccation through the synthesis of certain proteins (Oliver et al., 1998). In the present study, ABA treatment slightly improved the performance of *L. abyssinica* during desiccation and rehydration (Figure 18) but had no or little effect on the DT of *C. inopinatum* (Figure 17). A possibly explanation for the results obtained here is that a harsh, rapid treatment was used (1 week dehydration in silica gel). Desiccation may have occurred so suddenly that DT could not be induced by the ABA. The slower rate of desiccation

in *L. abyssinica* may have enabled ABA to have induced DT mechanisms. It is possible that in *C. inopinatum* the small reduction in fluorescence parameters could have been a protective mechanism against damage effect of rehydration. However, even after 1 week of rehydration, none of the parameters recovered to initial values, it seems more likely that the *C. inopinatum* had suffered severe damage because of desiccation. It should be noted that the experiments with ABA were carried out with material that had been stored for more than one week, and therefore had deacclimated, probably explaining the failure of *C. inopinatum* to recover to initial values. There are many examples where stress tolerance is induced in plants without the involvement of ABA, and it seems likely that these "ABA independent" pathways operate in the two species of ferns studied here (Yoshida et al., 2014).

Chapter 5: General Conclusions and Recommendations

5.1. General conclusions

DT in plants is undoubtedly based on more than one underlying mechanism, conferred by a variety of genes although the precise nature of the ‘‘desiccome’’ has not yet been unraveled (Kranner et al., 2008). On the cellular level, various biochemical mechanisms have been suggested to contribute to an organism’s ability to survive desiccation. Two of the most important of these are effective systems that reduce stress-induced ROS formation, and the synthesis or accumulation of high concentrations of sugars (Kranner and Birtic, 2005). The work presented here compared these mechanisms in species with and without cuticles. Taken together, results suggest that both *Crepidomanes inopinatum* and *Loxogramme abyssinica* exhibit dominant modified IDT mechanisms, especially with respect to SOD and soluble sugars. Even when *C. inopinatum* was subjected to very rapid drying above silica gel, some protective mechanisms were induced. Our original hypothesis was that constitutive mechanisms would predominate in the filmy fern, while inducible mechanisms would be found in the species with a cuticle. Results of this study show that inducible DT mechanisms occur even in filmy ferns that desiccate rapidly. The sensitivity of deacclimated *C. inopinatum* to rapid desiccation suggests that it needs at least some time to induce tolerance mechanisms. This is supported by the lack of an effect of ABA on DT in this species, presumably because desiccation was too rapid to allow development of tolerance mechanisms. Overall, both species are considered DT since they are able to recover from extreme water loss with minimal damage, depending on the rate of dehydration. Nevertheless, while both species totally survived slow desiccation, deacclimated *C. inopinatum* showed signs of injury when desiccation was rapid (Figure 11). Similar results were observed in the moss *P. patens*, where extended dehardening reduced DT (Greenwood, 2017). Interestingly, a slow drying pretreatment followed by rehydration increased the tolerance of *P. patens* to subsequent rapid drying, clearly showing that hardening can take place. It has been argued by Stark (2017) that it is important to distinguish “recovery from field stress” from “de-hardening” and suggested that it is essential to fully deharden mosses once collected before studying DT. Clearly, the “stress history” of field collected plants will usually be unknown but may influence physiological experiments. The effect of deacclimation is very crucial, as some mosses like *Crossidium* can take up to 7 d to deacclimate, while *Sphagnum* may take up to 3 weeks (Stark, 2017). This illustrates that deacclimation times are species specific. In the conditions used in the present study, slow deacclimation occurred in *C. inopinatum* but not *L. abyssinica*. Any future work on these two

species needs to take this deacclimation response into consideration when devising experiments on DT.

5.2. Future recommendations

DT in plants, and in pteridophytes in particular, is still very poorly understood. Future work should include characterizing the exact forms of sugars synthesised during desiccation, and studying precisely how these increase DT. The role of other DT mechanisms e.g. other ROS scavenging enzymes, LEA and HSP proteins could probably best be studied using next-generation "omics" approaches. To date, there are very few examples of where they have been used to study DT in ferns, but they have considerable potential to elucidate DT mechanisms. A rigorous comparison of *C. inopinatum* with *L. abyssinica* may yield valuable information on potentially contrasting strategies of DT mechanisms. Such methods should be coupled with the use of a variety of desiccating salts to give varying rates of drying. It has also been suggested by Stark (2017) that the duration of desiccation is important when conducting DT experiments, and this could certainly be varied in future experiments, especially as in the field both species appear to remain dry for many months over the dry winter period in KwaZulu-Natal (KZN).

In the future, it should be possible to improve the drought tolerance of crops by transferring genes from DT plants such as pteridophytes e.g using *Agrobacterium*-mediated genetic transformation. For this approach to be successful, it will be very important to know exactly the genes involved in DT. However, it should be noted that only limited success has been achieved to date in engineering stress tolerance in plants e.g. tomato has been genetically engineered for anti-frost using anti-freeze genes from an Arctic fish (Hightower et al., 1991) and several species like tobacco, arabidopsis and *Brassica* spp. have been genetically engineered for salt tolerance and other abiotic stresses (Roy et al., 2011). One of the very few studies that have involved pteridophytes attempted to produce "genetically engineered plants for increased tolerance to heat stress". In this study, conducted at the University of Florida, a gene involved in heat tolerance called PvGrX5 (a glutaredoxin) was transferred from *Pteris vittata* fern into a rice plant (Sundaram and Rathinasabapathi, 2010). Although the study was successful, but it hasn't been introduced commercially. Given future scenarios of climate change causing more frequent and more severe droughts in Southern Africa, understanding how plants tolerate water shortage is key to ensuring security of food production in the region. Given that poikilohydric ferns are phylogenetically closer to crop plants than, for example,

mosses and lichens, they could prove to be among the most valuable repositories of valuable genes for increasing abiotic stress.

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