University of Naples “Federico II”
Department of Agriculture
Ph.D. in Agriculture and Food Sciences
XXXII Cycle

Woodwardia radicans in the Ferriere Valley: ecophysiology of photosynthetic processes to study light and water stress

Tutor
Prof. Stefano Mazzoleni

PhD candidate
Gabriella N. M. Giudici

Contents

CHAPTER I

1. INTRODUCTION

Chapter II

1. CHARACTERISTICS OF LIFE CYCLE, MORPHOLOGY, REPRODUCTION AND ECOLOGICAL PHYSIOLOGY OF EVERGREEN TROPICAL FERN WOODWARDIA RADICANS: A REVIEW

Chapter III

3. HOW THE OLDEST ORGANISMS COPE WITH EXCESSIVE LIGHT IN EXTREME ENVIRONMENT?
Photoinhibition of primary photosynthetic processes in hydrated Polytrichum commune: analysis of non-photochemical quenching affecting species resistance

Chapter IV

4. DEHIDRATION: MEASURING THROUGH PHOTOSYNTHESIS A STRESS OCCURRING IN DIFFERENT AND DISTAT ENVIRONMENTS
Comparative research of photosynthetic processes in selected poikilohydric organisms from Mediterranean and Central-European alpine habitat

Chapter V

5. DAILY LIGHT COURSE OF PHOTOSYNTHESIS: ETR MEASUREMENTS IN GREENHOUSE CULTIVATION
Acclimation of primary photosynthetic processes to light in three fern species from a Mediterranean moist habitat, Ferriere Valley, Italy

.

Chapter VI

6. DAILY LIGHT COURSE OF PHOTOSYNTHESIS: WHAT REALLY HAPPENS IN THE FIELD?
Short-term photosynthetic adjustment of *Woodwardia radicans* and coexixting species to light regime. A field study by OJIP.

Chapter VII

7. COPING WITH STRESS: A COMPARATIVE STUDY ON DRIVING SPECIES OF *WOODWARDIA RADICANS* COMMUNITY IN THE FERRIERE VALLEY

Chapter VIII

8. CONCLUSIONS

PREFACE

First chapter is introducing reasons and methods of the research. In the second chapter there is an updated review of the central species of the research. The third chapter deals witht the application of the method, in the most extreme conditions, to the oldest terrestrial autotrophs who have developed resistence to the studied stress factors. In the fourth chapter a comparison is carried out on drought stress in extreme environment and the area of interest. The fifth is dealing with dayly light acclimation in field-like cultivation conditions. The sixth chapter investigates dayly light acclimation in the field. In the seventh chapter are presented the experiments in the lab-simulated stress conditions that could occurr in the field. Finally in chapter eighth are summarized results and personal obsarvation from the lab and the field

CHAPTER I

1. INTRODUCTION

1.1. GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

Woodwardia radicans is a relic of European paleotropical climate; it is currently present in small areas with specific microenvironmental conditions allowing the species to survive; these areas are generally deemed to be glacial refugia, have specific microenvironment conditions which make them hotspot of biodiversity. In Campania region, apart from a site in Ischia, not monitored from a long time, the main location of *W. radicans* is in the Ferriere Valley, in the south of the Sorrento Peninsula. This site is a protected natural area and is home to many protected species and to a habitat, enlisted "priority habitat" in the Habitat Directive. *W. radicans* itself is included in the IUCN Red List of Threatened species with the EN (endangered) status(Gargano D. et al. 2016), so it needs protection and monitoring. In Italy *Woodwardia radicans* areas are to be found: in Campania, Ferriere valley (South-Eastern Sorrento peninsula) and Ischia island, in many sites in Calabria and in Sicily. In the last overall monitoring, along 36 still existing sites, at least 24 areas were found disappeared over the last 50 years at a calculated rate of 8-10% of presence (Spampinato et al. 2008).

In IUCN Red List of Threatened Species in Italy, *Woodwardia radicans* is classified EN (endangered) (Rossi G. et al. 2013), while EU conservation status is "near threatened" IUCN.

1.1.1. *Woodwardia radicans* environment

Woodwardia radicans lives in microclimates providing environmental needed conditions: high soil and air humidity, mild temperatures throughout the year with low and gradual variations, and shelter from high light radiance. In current climate of the Mediterranean regions these conditions are met with in moist ravines, often located in coastal forest at limited altitude, in proximity of streams, falls or waterbodies able to maintain air moisture elevated for most part of the year. In many coastal regions *W. radicans* could be found on rocky or earthy walls rich in humidity or dripping water, in gorges, ravines often facing north or anyway shaded and well protected from low temperature and sudden variations. One of these ravines, in the Ferriere Valley was the area of our study.

1.1.2. The research

Although *Woodwardia radicans* vegetative forms are well established and thrive well in different areas in Italy, they do not increase their populations; in the Ferriere valley gamic reproduction is estimated low or absent or even considered non-existent in nature (Guadagno 1922). Although this view has been disputed (Caputo & De Luca 1968), it is true that in most cases it is rare to find new sporophytes originated by gamic reproduction (personal observations in the Ferriere Valley). Many studies have been carried out over reproductive stages, with special attention to spore germination and gametophyte production (Carafa 1984, Quintanilla *et al.*2000), but, little has been done in the field (Quintanilla et al 2002). So, there is a gap to be filled in the knowledge of the physiology of the species in the wild, when affected by environmental factors.

Even though most of the official reports about *Woodwardia radicans* conservation list among other threads, deforestation and water bodies conditions (Spampinato et al. 2008, D. Gargano et al. 2016,), experimental evidences are still to be provided to these statements. Because the immediate effects of the above threats are dramatic increase of light radiation and potential loss of water potential, we focused our research on the effects on *Woodwardia radicans* of high light and desiccation.

The main aims of our study have been:

- to provide a better knowledge of *W. radicans* functional responses to environmental factors
- to provide experimental evidence for a *W. radicans* risk assessment to high light and water deficiency stress

- to try to provide a new state of conservation monitoring tool based on *W. radicans* performances to assess the state of conservation of its habitat and to better implement the conservations measures required under article 17 of the Habitat Directive.

To this purpose photosynthetic performance was deemed to be the most appropriate indicator of the health status of the species and of its fitness to cope with environmental changes.

Chlorophyll fluorescence (ChlF) measuring techniques were used to evaluate photosynthetic performances.

1.2. MATERIALS AND METHODS

1.2.1. Chlorophyll fluorescence

Chlorophyll fluorescence (ChlF) is the radiation emitted by excited chlorophyll (Chl) electrons returning to their ground electrically neutral level. The higher energy level acquired by Chl electrons is channelled through different pathways that are complementary:

- electron and proton transport chain of photosynthetic processes
- non-photochemical quenching
- chlorophyll fluorescence

The amount and time course of ChlF parameters provide qualitative and quantitative information on photosynthetic processes. In physiological conditions average 80% of absorbed light energy is channelled to the photosynthetic pathway, while 2-5% is conveyed to ChlF and the remnant to heat dissipation.

ChlF analysis is a powerful, non-invasive tool with a wide range of applications in autotroph research. It has been particularly used in studies on adaptive mechanisms developed by plants to cope with environmental stressors such as high light, heath, cold and freezing, salinity, drought, pollution.

1.2.2. Chlorophyll fluorescence measurements techniques

Fast and slow Kautsky kinetics are ChlF techniques based on Kautsky effect, by which, when a pre-darkened photosynthetic material (leaf, chloroplast suspension etc.) is illuminated by high light, it shows a quick rise in ChlF, followed by a slow decline. Parameters related to this kinetic are used to investigate efficiency of light harvesting complexes (LHC), efficiency of PSII, electron transport rate (ETR), non-photochemical quenching.

1.2.3. Fast Chl induction kinetics (FIK or OJIP curve)

It is the transient measured for few seconds in a dark-adapted sample after being exposed for 2 seconds to actinic light (fig. 1.1). The OJIP name is after four characteristic point of a polyphasic curve related to specific level of ChlF. O and P points indicates respectively background and maximum emission levels; the interval 0 J is related to the equilibrium of the electron exchange Quinone A - B, it is an indicator of the functional modifications of LHC and efficiency of PSII; while the following J I interval is related to PSII – PSI exchanges and is functionally sensitive to temperature. After the P point, with the end of excitations the curve decreases in an exponential, sometimes polyphasic way, functionally corresponding to QA re-oxidation and quenchers activity.

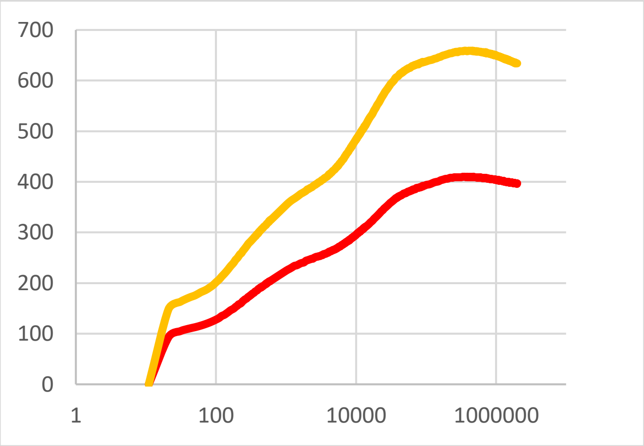


Fig. 1.1 Example of Fast Chl induction kinetics (or OJIP curve). The yellow line is control, the red one is the same sample after stress induction (here high light). The stress effect results in a lowering of the JIP values and a flattening of the curve shape.

1.2.4. Slow Chl induction kinetics

On a pre-darkened sample background (F_0) Chl is measured applying a weak, non-actinic light. Immediately after, a saturation light pulse is applied to induce maximum fluorescence (Fm). This peak is caused by all electron acceptors being reduced and so unable to accept energy, which is then reemitted as ChlF. The difference Fm-F0 is a measure of the maximum potential for photosynthesis. Then, under exposure to actinic light the transient shows a slow decline, during which electron transport and coupled photochemical reactions move toward an equilibrium, found at steady state (Fs). Then a new peak appears when a saturation pulse is applied. This second peak (Fm’) is a measure of the effective capacity of PSII and is always lower than Fm because of non-photochemical quenching processes involved. Finally, the actinic light is switched off, a far red light pulse applied and the minimum Chl value (F0’) recorded (fig. 1.2).

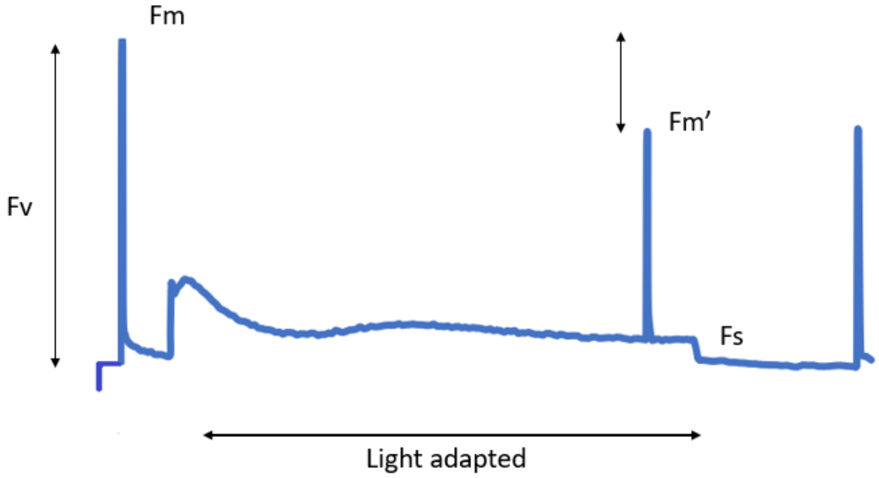


Fig. 1.2 Example of slow Kautsky kinetics: the first segment with non-actinic light measures the F_0 value, then Fm peak is the response to the saturating pulse, with the following dark relaxation. A new saturating pulse during actinic light, determines the Fm’ peak. The difference Fm-Fm’ is the value of non-photochemical quenching.

1.2.5. Electron transport rate (ETR)

This method measures ΦPSII under increasing intensities light pulses; rapid light curves (RLC) are obtained in a 20 seconds sequence of 10 increasing PAR pulses, each followed by a saturation pulse to evaluate ΦPSII (effective quantum yield of photosystem II).

1.3. HYPOTHESES AND EXPERIMENTS

1.3.1. Hypotheses

We hypothesized that

- Light and water could be driving factors affecting photosynthetic performance and their effect to be species-specific
- Light radiance could induce photosynthetic performance adjustment and that adjustment to be species-specific
- acclimation time would differ between species according to their shade tolerance

Based on these hypotheses we wanted to evaluate:

- Level of light tolerance
- Level of desiccation tolerance
- How protecting mechanisms where activated and when they were appearing during life stages

Pursuing these objectives, we designed the following experiments

1.3.2. Lab-based experiments

Photo-inhibition experiments were carried out exposing the samples to increasing dose of light (i.e. increasing in PAR intensity and/or time of exposure). Time course of ChlF parameters were recorded and analysed. (tested species: *W. radicans,* all phenological stages, *Adiantum capillus-veneris,* all phenological stages, *Polypodium vulgare* mature leaves, *Pellia endiviifolia*, *Palustriella commutata, Conocephalum conicum*, mature thalli/shoots)

Desiccation experiments were conducted continuously recording ChlF parameters during desiccation. Relative water content related ChlF parameters were recorded and analysed. (tested species: *W. radicans,* gametophyte, *Pellia endiviifolia*, *Palustriella commutata, Conocephalum conicum*, mature thalli/shoots)

1.3.3. Greenhouse experiments

Photosynthetic electron transport rate (ETR), rapid light response curves (RLC) of ETR and initial slope RLC (α) were evaluated in a 19-day long experiment under controlled conditions in greenhouse cultivation. We added a short-term high light treatment, studied by fast chlorophyll fluorescence transient (OJIP). (tested species: *W. radicans, Polypodium vulgare* and *Adiantum capillus-veneris*)

1.3.4. Field experiment in the Ferriere Valley

Daily light course ChlF parameters were recorded in two different days by OJIP measurements every 20 minutes. Climate data were recorded as well. Time courses of ChlF parameters were analysed in response to environmental factors. (tested species: *W. radicans, Conocephalum conicum).*

1.3.5. Plant material from *W. radicans* habitat

Apart from *Woodwardia radicans*, two ferns (*Polypodium vulgare* and *Adiantum capillus-veneris*) were analysed because they shared the same environment but in a gradient of light intensity.

Conocephalum conicum, a liverwort, was included because part of the *Conocephalo-Woodwardietum radicantis* Brullo, Lo Giudice & Privitera 1989, the typical *W. radicans* association.

Palustriella commutata (syn. Cratoneuron commutatum) was included because it is the driving species of the priority habitat (7220) *Cratoneurion*, “Petrifying springs with tufa formation habitat”, found under W. radicans leaves.

Pellia endiviifolia, a liverwort, was included because always found along *P. commutata*.

1.4. REFERENCES

Carafa A. M. (1984). Light dependent spore germination in *Woodwardia radicans* (L) Sm. Giornale botanico italiano, (118)5-6, 245-253.

COUNCIL DIRECTIVE 92 / 43 / EEC 1992

D. Gargano D., M. Vena, L. Bernardo, N.G. Passalacqua, A. Santangelo., 2016. *Woodwardia radicans* (L.) Sm. in: Ercole S., Giacanelli V., Bacchetta G., Fenu G.,

Genovesi P. (ed.). Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie vegetali. ISPRA, Serie Manuali e linee guida, 140/2016.

Rossi G., Montagnani C., Gargano D., Peruzzi L., Abeli T., Ravera S., Cogoni A., Fenu G., Magrini S., Gennai M., Foggi B., Wagensommer R.P., Venturella G., Blasi C., Raimondo F.M., Orsenigo S. (Eds.), 2013. Lista Rossa della Flora Italiana. 1. Policy Species e altre specie minacciate. Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare

Quintanilla, L. G., Amigo, J., Pangua, E., & Pajarón, S. (2002).Biogeographic analysis of the Pteridophyte flora of the Capelada Range La Coruña Spain Lazaroa 23: 17-24. 2002

Quintanilla, L. G., Pajarón, S., Pangua, E., & Amigo, J. (2000). Effect of Temperature on Germination in Northernmost Populations of Culcita macrocarpa and *Woodwardia radicans*. Plant Biology, 2(6), 612–617. https://doi.org/10.1055/s-2000-16638

Spampinato G, Cameriere P, Crisafulli A, Gangale C, Picone R. M, Santangelo A, Uzunov D. 2008 - *Woodwardia radicans* (L.) Sm,.Informatore botanico italiano 40(S1), 2008, pp. 132-134, ISSN: 0020-0697

2. CHARACTERISTICS OF LIFE CYCLE, MORPHOLOGY, REPRODUCTION AND ECOLOGICAL PHYSIOLOGY OF EVERGREEN TROPICAL FERN *WOODWARDIA RADICANS*: A REVIEW

Gabriella Nora Maria Giudici [a] *, Josef Hájek [b] **

Submitted to FLORA 31rst Januaru 2020

[a] University of Naples Federico II, Department of Agriculture, 80055 Portici, Italy
[b] Department of Experimental Biology, Division of Plant Physiology and Anatomy, Faculty of Science, Masaryk University, Laboratory of Photosynthetic Processes, Kamenice 5, 625 00 Brno, Czech Republic

* Corresponding author, * * author submitting to the Editorial Manager

2.1. ABSTRACT

Woodwardia radicans is a tropical fern species with limited distribution in Europe (Canaries, Madera, Azores, southwest Italy, Sicily). *W. radicans* is considered Macaronesian relict, because it is the remnant of the tropical vegetation which covered Europe in the Tertiary. In this review paper, taxonomy and evolution of *Woodwardia* sp., habitat characteristics and species distribution, and interactions with other community-forming species is given. Moreover, life cycle, morphology as well as spores, gametophyte and sporophyte description is reviewed. Ecophysiological background of species reproduction, i.e. spore maturation, release and germination, gametophyte formation and sex expression is described. Attention is paid to overview of recent knowledge on adaptive strategies and capacity of particular physiological processes in of *W. radicans.*

Key-words

Gametophyte, sporophyte, germination, sex expression, nitrogen, photosynthesis

Acknowledgements

The authors are grateful for the support provided by the Laboratoty of Photosynthetic Processes (LPP), Department of Experimental Biology, Masaryk University, Brno. The authors thank the LPP staff members for critical reading and valuable remarks during the manusript preparation.

Authors' contributions

GG suggested the outline of the manuscript, made substantial part of resourse browsing and data mining. Handling with gained data was done by GG with help of JH. GG wrote the paper with review and substantial editing of JH. Both authors read and approved the final manuscript.

Funding

The review was written thanks to the stay of the first author at the Masaryk University, Brno, Czech Republic. The stay was supported by the ERASMUS exchange framework between the Masaryk University and the University of Naples Federico II (Italy).

2.1.1. Background

Woodwardia radicans is an evergreen fern growing to the height of 1.8 m having individual leaves up to 0.6 m long. It is rhizomatous ferns with deciduous or evergreen, pinnate to bipinnate fronds, bearing spores in chain-like lines on the undersides. Throughout literature, there are many synonyms for the species (see below). Since *W. radicans* is quite rare to Europe because of being considered tropical relict, we present an overview of recent knowledge about the species. The review inteds to provide a comprehensive information about the species, ranging from taxonomy and geographical distribution to important aspects of general biology of the species including reproduction strategy and ecophysiological chracteristics. Finally, the review brings some information about recent and future trends in research in *W. radicans*, its photosynthetic processes in particular.

WOODWARDIA RADICANS (L.) Sm
Syn. Blechnum radicans L., Blechnum stans (Cav.) Poir., Woodwardia stans Cav., Blechnum houttuynii Poir., Blechnum radicans L., Blechnum stans (Cav.) Poir.
Order Polipodiales

Suborder Aspleniineae

Family Blechnaceae

Subfam. Woodwardioieae

2.1.2. Taxonomy and evolution

Blechnum is the biggest and most widely distributed (mainly in austral region) genus in the blechnoid group; other genera are smaller and located in small regions, except for world-wide *Woodwardia*. Bower (1928) considered all of them derived from an old genus, Ching (1940), Copeland (1947), Alston (1956) in turn grouped them as a separate family, with some small differences in the genera they included. Both *Blechnum* (Bower, 1928; Copeland, 1947) and *Woodwardia* (Holttum, 1954) have been regarded as the primitive genus from which the others derived. About the relation with other ferns, it is commonly accepted that the type of *sorus*, common to all genera of the group, has a different origin than that of other similar elongated *sori* parallel to the pinna midrib (Nayar, 1966). Cranfill and Kato (2003) indicated that the *Woodwardioid* ferns form a monophyletic clade representing the sister group to the remainder of the *Blechnaceae*. They recognized 14 species but separated *W. areolata* and *W. virginica* into two segregated genera, *Lorinseria* and *Anchistea*, respectively. Li et al. (2016) study based on rbcL tree provided, with new samples from Himalaya region, confirmation to Cranfill and Kato's hypothesis that first differentiation in *Woodwardia* occurred in North America during Paleocene (56.51 ± 2.89 Ma). Then, following a migration in central Asia trough Bering area, four newly diverged cladia (middle Eocene 45.04 ± 6.02Ma) radiated toward their current geographical areas; *Woodwardia radicans* arrived in Europe during Middle Miocene (ca.14.00 Ma) proceeding from Himalayan species. The same authors consider all current *Woodwardia* species in America part of the same lineage which migrated back from central Asia differentiation area (Oligocene 27.45± 8.29 Ma). Although two possible pathways (Beringia and north-American north-European land bridge) are suggested for Asian flora spread to North America, the authors suggest the first to be consistent with their findings (Li et al., 2016).

The most recent comprehensive classification of both ferns and lycophytes (PPG I 2016) is based on a community-based approach, using monophyly as primary key; the authors tried as well to include the most widely accepted classifications.

Woodwardia radicans with *Blechnum spicant* is the only European *Blechnacea*. Along with other species, *W. radicans* is called Macaronesian relict, because it is the remnant of the tropical vegetation which covered Europe in the Tertiary and which is now largely present in Canaries, Madera, Azores (Macaronesia). It is also present with small and disjointed populations in the Mediterranean area.

Fossils of the genus *Woodwardia* date back to early Tertiary (Paleocene) in America and in the Rhone Valley (France) to the Pliocene. Fossils of genus *Woodwardites*, related to *Woodwardia* have been discovered in Schliesen and Croatia (Miocene), Saxony and Franken (Oligocene). Currently, *Woodwardia* genus occupies three disconnected geographic areas with different number of species: one which has been thought to have been the first center of the genus differentiation, extending from Japan to the Philippines and Java, including the Himalaya and with five species; a second one including north Atlantic Spain and Portugal, Macaronesia, and small locations in southwest Italy, Sicily, north Africa, with only one species; the third area is localized in the North America on the Pacific coast and in the central America, and presents two species, (Pichi Sermolli, 1979).

Fossils of *Woodwardia radicans* from a fossil bed in São Jorge (Madeira), dated from 7 to 1.8 Ma., were described by Heer (1857), Bunbury (1859) and Hartung and Mayer (1864). Cóis-Marques et al. (2018) in their review of specimen descriptions of these previous authors, identified and confirmed the presence of *W. radicans* in a floristic association typical of warm humid climate, showing that the current laurel forest community was already established in the Macaronesia island between 2.58 and 1.80 Ma., when most of paleotropical flora was already extinct in Europe.

2.1.3. Geographical distribution and habitat

W. radicans occurs in ravines, 200 to 700 a.s.l., in humid, warm-temperate microclimate, with constant temperature and low light intensity. It grows in acid soil although its distribution demonstrates it is indifferent to soil pH, being found in basic soils as well (Quintanilla et al., 2002a). It is often found near waterfalls, on riverbanks, or in woods; it is present in laurel forest belt, at middle semi-humid elevations, in western Canary Islands (Hollermann, 1981). The Capelada Range (La Coruña, Spain) is reported to be the northernmost site in the European range of *W. radicans* (Quintanilla et al., 2002a). It is currently reported in Spain, Italy, Portugal, Crete, Algeria, Azores, Madeira, Canary Isl. The species is also reported from California, and Florida (Hassler, 2017) where it is introduced. In Italy, it has been observed in the south and in the largest islands as well as in coastal and sub-coastal areas in the central-northern part of the country (Biondi et al., 2014).

2.1.4. History of vegetation

Current laurel forest of the Canary Islands is a relic of Neogene Tethys coastal vegetation. During the geological period, climate was typically temperate with rainy summers. Due to the transition to a drier and cooler climate, the forest community retracted to coastal areas and mountain humid areas. The ocean surrounding the Canary Islands mitigated climatic oscillations of Pleistocene and provided adequate shelter from freezing and desiccation which were occurring in central Europe and southern Mediterranean. Mountains of individual island provided altitudinally differentiated biotopes for species to migrate and adjust to climatic oscillations of Pleistocene (Hollermann, 1981). In the same way in continental Europe, ravines in woodland, narrow, often north-oriented valleys, close to coast or to rivers, provided shelter from aridity and cool temperatures. The whole map of European *W. radicans* range is that of *glacial refugia*. Skov and Svenning (2004) in their study on climatic changes effect on European flora distribution, stated that the shift in distribution of *W. radicans* would be towards northwest (withdrawing from Mediterranean basin because of the expected decrease in precipitations). They also postulated that *W.*

radicans could increase its total occupation area under the new climate conditions, provided that sufficient potential dispersal routes would be present.

2.1.5. Biological interaction with other species

The community is *Conocephalo-Woodwardietum radicantis* (Brullo et al., 1989): rocky plant community, dominated by big ferns, rich in mosses and lichens, which grows on rocky or earthy walls, in habitats with high soil humidity, in narrow and shadowy gorges. It is mainly distributed in the Mediterranean coasts. For Italy, it is reviewed by Biondi et al. (2014). *W. radicans* is often found along with *Culcita macrocarpa* in wood habitats. *W. radicans* spores are known to be fed on by the Azores bullfinch during the winter (Arosa et al., 2009). Arbuscular mycorrhizal fungi were reported for *Woodwardia orientalis* (Walker, 2013). Arbuscular mycorrhiza is reported also in the Living Collections at the Royal Botanic Garden Edinburgh.

2.1.6. Morphology

The rhizome is thick and far creeping, erect in old branches, so that Quintanilla et al. (2002a) considered *W. radicans* acting as Chamaephyte even if it is classified as Hemicryptophyte. Leaves are monotype (no difference between fertile and vegetative leaves), bipinnatifid fronds, up to 2m long, with stipe (the frond stalk) long as the blade. They show circinate vernation, meaning that the newly formed frond is tightly curled so that the growing tender tip is protected within a coil which unfold with the leaf growth. This typical shape is called *crozier* or even *fiddlehead*. At the tip of mature fronds, there are bulbils capable of producing new individuals. Sporangia are clustered in elongated *sori* set in lines close and parallel to the pinna midrib, like in a chain, which provided the common name: chainfern.

2.1.7. Life cycle

A haploid spore develops a gametophyte, a small, leafy heart-shaped structure that produces antheridia or/and archegonia. The sperms produced by the former swim to the latter to fertilize the eggs inside. The zygote divides mitotically, developing a diploid sporophyte, the perennial and most conspicuous phase of the life cycle. On the lower face of the sporophyte leaves, inside the sporangia, spores are produced by meiosis. A spore starts a new cycle.

Spores

Spores are monolete (a single line, *laesura*, in the spore indicates the axis along which the mother spore split in four by meiosis), and relatively large (40 x 60 x40 µm). They contain one single nucleus, many pale green plastids and large yellow oil globules. Untreated fresh spores are yellow brown, exine is light brown, 2 µ thick, smooth. The perine is hyaline, very thin, granulose, loose and highly wrinkled into folds (4-6 µ tall); it is a distinctive character of the spore which in other ferns could be: very closely adherent to the exine, wrinkled with differently patterned folds, or absent (Carafa, 1984; Nayar et al., 1966). Spores are brought inside elongated *sori* parallel and near the midrib of the pinna, closed by an *indusium* which opens on the lateral side. This character is common to all *Blechnoid* ferns.

Gametophyte development

Gametophyte development was studied under controlled conditions (Carafa, 1985, 1990; Nayar et al., 1966). Before germination, the spore expands due to imbibition and become roughly round; during germination *laesura* opens producing a fast-growing cell rich in chloroplasts, from which, by asymmetrical divisions, a rhizoid and a prothallium are formed. The last one divides transversally producing 5-6 elongated cells; at this stage (4-6 days after germination) the subterminal cell (or the one before it) starts dividing longitudinally. After many longitudinal and transversal divisions, a prothallium plate is formed, showing dish-like cell and a hair formation coming from the apical cell (Carafa, 1990; Nayar, 1965). The prothallium grows asymmetrically and a meristematic cell is formed after 8-9 days from germination. Hair is formed on a marginal cell and the meristematic cell evolves in a meristematic tissue, located in the notch of a heart shaped prothallium. After 4 weeks at 24 ±1 °C (photoperiod L/D 16/8 experimental conditions), the new gametophyte is a cordate prothallium showing an apical notch bearing the meristematic tissue in its center. At the same time, unicellular hair is present along the edges. Then a midrib is formed with rhizoids sprouting from its base and the form becomes heart shaped. This is marking the stage of sexual maturity (Carafa, 1990). Under controlled conditions (24 ±2 °C, photoperiod LD 12/12, sterile agar medium), maturity is reached after 8 weeks from germination, but the new gametophyte continues to grow long after maturation (Nayar et al., 1966). The growth activity of the apical notch is high and powerful. It has been reported for other ferns that a removed notch, by sharp cutting, continues to grow and a longitudinally cut apical notch can reproduce the whole gametophyte from each half (Albaum, 1938).

Antheridia and archegonia

Antheridia are composed of three parts: a basal cell, an anular cell and an opercular cell; the spermatozoids inside are generated by a single cell (Nayar et al., 1966). The cell walls bend during development and the mature antheridium has a barrel-like shape with a stretched opercular cell at the top, deiscent at maturity. Archegonium is bottle-shaped with an elongated neck bended away from the apex of the prothallus and towards the rhizoids. It is originated by a layer of three cells, the middle one pushing the upper one which protrudes and divides in four cells with 90° walls. these cells form the archegonium neck by dividing 4 or 5 times (Carafa, 1990). The middle cell divides in turn producing upwards the neck canal cell and downwards the egg cell. At maturity, the canal cell of the neck and the basal canal cell are highly vacuolated.

Juvenile sporophyte

Nayar et al. (1966) described the juvenile sporophyte: after fertilization from the zygote develops a new sporophyte from inside the archegonium. The juvenile leaf is short and stalked; the lamina is semicircular, the margin undulated, pale green, and bluish. The vascular structure fans out in the lamina by forking of the bundle in the stipe. Usually, in the first leaf, the midrib is absent and becomes visible from the second or third leaf. As the lamina increases its size in successive leaves, it becomes trilobed, usually from the seventh, tenth leaf. The successive leaves become more and more elongated in shape after the midrib is well developed and forming closely forked lateral veins on both sides. At this stage, the leaf margin is undulated and starts to indent producing the pinnate shape. The rhizome of the juvenile sporophyte is short, juvenile leaves bear unicellular papillate hairs on the stipe and lamina (Nayar et al., 1966).

Vegetative propagation

W. radicans can reproduce vegetatively via adventitious buds growing at the tip of the leaf; when they touch the moist soil they can develop a new plant, identical to the parental one (Spampinato et al., 2008).

2.1.8. Reproduction physiology

Spore maturation and release

Timing of spore maturation and release have been studied in the field by Arosa et al. (2009) in one of Azores islands at three different altitudes. The authors observed no difference in the time of maturation depending on altitude or canopy cover; they supposed spore production to be controlled by stored photosynthates and observing that canopy cover did not affect the date of spore maturation, they assumed that *W. radicans* has a low light level for photosynthetic saturation such as filmy-ferns of the same environment. The authors also observed that maturation occurred in December and that there was a long delay until spore released. The release occurs in the late winter. The delay was found constant, meaning that spores matured earlier were released earlier regardless of weather conditions. The authors would so exclude interference of environmental factors, even though they did not suggest any biological reasons. The existence of spore predating bullfinch is not so extended to provide evolutionary adaptations. Finally spore release is dependent on drier weather, being mechanically controlled. Aborted spores number in *W. radicans*, when reported, was found low (≤8%) (Arosa et al., 2009) and this is consistent with the general rule that sexual ferns taxa have lower spore abortion rate (SAI) than apomictic taxa (Hornych and Ekrt, 2017).

Spore germination

Germination starts with the coat splitting along *laesura* and the internal cell dividing. For experimental comparisons, the most used definition is the emergence of the first cell of protonema or rhizoid; this is because mechanical or environmental factors could cause spore coat rupture without further development (Miller 1968). Carafa (1984) studied spore germination in *W. radicans* finding that spores can germinate only in the light and that gibberellic acid cannot remove the dark inhibition (as it happens in other light dependent fern spores). Blue or green light do not induce germination, and far red light can reverse the promoting effect of red light. The author suggested that germination may be under phytochrome control. Photosensitivity occurs only in imbibed spores and increase with imbibition time, showing the maximum after six days. Dried spores do not respond to light promoting effect. Germination is unaffected by mineral nutrients and can occur even in water-soaked filter paper (Carafa, 1984). Thus, water is the most important factor for germination since apparently spores are released with sufficient nutrients supply to complete germination in favorable conditions, although further development is affected by nutrients (Miller, 1968). Growth medium effect has not been tested for *W. radicans* but for other ferns is controversial. Douglas (1994) reported lower germination on agar (solid) than in liquid media whereas Sheffield et al. (2001) found the reverse for *Pteridium aquilinum* spores. Germination temperature ranges from 15 °C to 30 °C, the optimum being 25 °C (Quintanilla et al., 2000) or 24 °C (Carafa, 1990). Quintanilla et al. (2000) found that germination percentage and time is dependent on both temperature and population. They also suggested that differences among populations could be induced by the differently stressful environmental conditions (in their experiment the two populations with the lower rate and the higher time of germination come from the most extreme conditions of *W. radicans* habitat). Viability of spores have been studied with regard to storage techniques, storage temperature and their interaction by Quintanilla et al. (2002a, b). They found that all factors affected germination rate and that 6 or 12 months of dry storage killed most of the spores as well as wet storage at -20°C. Although in short term (1 month), storage technique made no difference, one-year viability could be reached only with wet storage at 5°C or 20°C. They reported that *W. radicans* germinated in the dark under wet storage at 20°C even though gametophytes were filamentous instead of the typical two-dimensional development. This is in contrast with Carafa's (1984) results and could be attributed to light induction on imbibed spores before storage. Although Hill (1971) showed that the spores frozen and stored in liquid medium for 1 month produced high germination percentage in *W. virginica*, this technique has never been tested on *W. radicans*.

Gametophyte development

Light has a strong impact on gametophyte development (Carafa, 1985); in fact red light does not allow the two dimensional growth and the prothallus continues to grow a filament, never reaching the thick, heart-shaped form of the mature gametophytes; white light radiations can restabilish the normal growth pattern. Gametophyte development pattern is not affected by temperature which only determines the development time; on the contrary it is strongly affected by nutrients, growth medium, sowing density and light. Gametophytes provide great variety of forms: they can be archegoniate, antheridiate, hermaphrodite (bearing both antheridia and archegonia) and even sterile (Carafa, 1990; Nayar et al., 1965). When they produce both gametangia, complex patterns are followed depending on environmental conditions. Nayar et al. (1966) observed that sometimes early antheridia are found before the completion of the prothallus, but usually they appear after the full development of the prothallial plate, positioned on the lower surface near the midrib. Carafa (1990) indicated fertilization and sporophyte formation is dependent on a film of water and every gametophyte produces only one sporophyte, whatever the number of archegonia on it; Miller (1968) suggested this is because sperms cannot reach more than one archegonium while Carafa (1990) hinted that some physiological barrier are pro after the first fertilization.

Sex expression

Sex expression is dependent on many factors and their co-actions. The sex expression of gametophytes might be affected by sowing density, by the presence of antheridiogen in the culture medium and by the nutritional conditions of gametophytes (Carafa, 1990). Moreover, De Soto et al. (2008) showed that some stress factors, such as limited nutrient supply, and crowding affects sex expression in *W. radicans* gametophytes. Medium type (solid or liquid) play a role: Carafa (1990) reported that in liquid medium, archegonia are never formed, on the other hand on solid substrate the first appearance is usually of antheridia unless sowing density is very low; in which case gametophytes continue to grow and develop archegonia when sufficiently large (2 mm). The author also observed that single sowed spores grow in a nodular cellular mass with no differentiation nor development. Poor nutrients conditions induce mostly antheridia that could also be produced by little irregular gametophytes sprouted by old archegoniate gametophytes not fertilized and declining. On the other hand, isolated cultured gametophytes never produce antheridia, regardless of nutrients availability (Carafa, 1990; Quintanilla et al., 2005). Näf (1958) proposed that in small male gametophytes, nutrients flow goes to and is utilized for reproduction instead of vegetative growth, but it has also been observed that male gametophytes could produce archegonia after increasing their size (Carafa, 1990, 1985). It has already been proved that gametophytes are subject to spore density (Miller 1968), which was afterwards recognized as antheridiogen effect (Näf et al., 1975; Korpelainen, 1995). Carafa (1990) also showed that *Pteridium aquilinus* growth medium extracts were more effective than *W. radicans* ones in promoting antheridia differentiation in *W. radicans* gametophytes. Old archegoniate prothallia can produce many small antheridiate gametophytes, confirming antheridiogens to be produced by mature, full grown, female gametophytes Carafa (1985). Finaly time of development was determined only by temperature Carafa (1990), with no differences among populations Quintanilla et al. (2005).

In general, all the above mentioned factors affecting sex expression are, in a way, compatible with Environmental Sex Determination (ESD) concept. Under good growth conditions, such as e.g. low density, high nutrient availability, gametophytes mature sexually at a relatively large size and turn into females and subsequently into bisexuals. Under harsh growth conditions, however, gametophytes mature sexually at a smaller size and turnes into males (De Soto et al., 2008).

Sex differentiation sequence

Gametangia sequence is complex and has been for a long time controversial: Carafa (1985, 1990) found antheridia always preceded archegonia, in contrast with Klekowski (1969); Quintanilla et al. (2005) confirmed Klekowski's model of gametophytes transforming from archegoniate to hermaphrodite, in both isolated and coupled gametophytes cultures, the rate of transition depending on the specific population. On the other hand, one of the same authors (De Soto et al., 2008), in subsequent studies, confirmed Carafa's data of sexual transition from male to bisexual. Both models are not in contrast because they depend on different experimental conditions, showing that ontogenetic sequence does not follow a rigid plan, but develops conforming to environmental conditions (De Soto et al., 2008).

Biochemistry of sex expression

It has already been shown that sex expression is regulated by two, alternatively functioning, genes: FEM1 promotes male differentiation, TRA induces archegonia differentiation (Tanurdzic and Banks, 2004). The supposed pattern for antheridiogens is that they act, trough regulator genes, activating FEM1 and inhibiting TRA. (Strain et al., 2001). Many authors showed relationship between small size and maleness for different ferns; the same condition is proposed for *W. radicans* by Carafa (1990) and confirmed by Quintanilla et al. (2007a,b) who indicated size as the path through which antheridiogens act. A threshold size is required for sex differentiation which is inversely related to stress conditions.

Breeding system

W. radicans is a homosporous fern, meaning a species producing spores that can develop gametophytes producing both sperms and eggs, on the same or different individuals. Adding this potential to high environment-induced differences in spore germination, the breeding system of this fern is very complicated and differentiated. Klekowski (1969) first defined three basic breeding systems: Intragametophytic selfing (the origin of both gametes from a single gametophytes), inter-gametophytic mating (each gamete originating from a different gametophyte but from the same sporophyte), intergametophytic crossing (the fusion of gametes from different gametophytes originating from different parental sporophytes). The first lineage should result in 100% homozygosity in one generation. The other two are difficult to detect without genetic analysis of the population. Klekowski (1969) found that *W. radicans* populations carry recessive deleterious genes, which is consistent with outcross breeding. In fact, gametophyte self-fertilization induces homozygosis, which in turn results in natural selection of lethal or deleterious genes, thus enhancing the fitness of population. Permanence of deleterious genes in the population is favored by heterozygosis which is the result of intergametophyte fertilization and outbreeding. Carafa (1990) observed that differences in time of spore germination and gametophytes development, the antheridiogen effect promoting sex differentiation inside populations and sex sequence in gametophytes, were all factors promoting outbreeding, because of the antheridiogen effect in promoting sex differentiation inside populations and sex sequence in gametophytes. Quintanilla et al. (2006) studies on isozymes variations in north Iberian populations of *W. radicans* discovered that the few loci being polymorph showed Hardy-Weinberg equilibrium, meaning an intergametophyte outbreeding system. They also showed no statistically significant differences among populations, meaning that the gene flow was active. They attributed the low genes polymorphism to genetic drift or founder effect, they both consistent with the glacial restriction of population and the postglacial colonizations (Quintanilla et al., 2006).

Genetics

W. radicans has 2*n* = 68 (Löve et al., 1977), (Brullo et al., 1982); it is a functionally diploid despite the high chromosome numbers (for a detailed overview of fern polyploids see Haufler, 2002).

2.1.9. Adaptive physiological traits to nutrients

Stewart et al. (1986) studied nitrate and ammonium enzymes in many ferns, to determine the distribution of nitrate assimilation in root and shoots and its relationship with different habitats and conditions. The authors indicated three models based on nitrate reductase presence and assimilation: root predominance, shoot predominance and shoot-root distribution. Their experiments showed that root nitrate reduction is important when nitrate supply is low and in general higher nitrate supply increased the shoot-root nitrate reductase, although this did not occur in *W. radicans*. They also demonstrated a direct relationship between nitrate reductase activity and glutamine synthetase, of which two forms, of equal amounts, were found in roots of *W. radicans*. Differently from some angiosperms, nitrate assimilation pattern was not differentiated in tropical and temperate ferns, as well as nitrate assumption capacity showed no differences between acidophilic or basophils ferns, which, on the contrary, is similar to what happens in many herbaceous angiosperms. *In vitro* tests showed that root nitrate assimilation was predominant in shade ferns which is consistent with the theory that root nitrate assimilation is more advantageous than leaf nitrate assimilation in low light conditions (Smirnoff and Stewart, 1985). The root nitrate assimilation, that was found predominant in the tested ferns, along with the generally low nitrate level of many ferns, made Stewart et al. (1986) consider it an eco-physiological trait adaptive to low nutrients and low light conditions.

2.1.10. **Ecophysiological characteristics**

In spite of several studies focused on fern responses to particular physiological processes to environmental factors (see e.g. Tosens et al., 2015), knowledge on ecophysiology of *W. radicans* is still rather fragmentary. In spite of the fact *W. radicans* is tropical species, it can withstand frosts to -5 °C. In gardens, occasionally, healthy plants have survived sub zero temperatures briefly down to -10 °C (Pyner, 2015). As it is generally less hardy than *W. unigemmata*, the latter is now more commonly planted in gardens. Photosynthetic performance of *W. radicans* has been investigated to only limited extend. Recent chlorophyll fluorescence-based study (Giudici et al., 2020, subm.) indicated that *W. radicans* belongs among shade-tolerant species capable to adjust photosynthesis to low light. The same study reports that the species is sensitive to photoinhibition induced by high light doses (1500 ▯mol m^{-2} s^{-1} for 60 min). *W. radicans*, however, showed partial recovery. The time, required for full recovery of photosynthetic processes, however, remained an open question because it depends on ecotype (sun and shade). Evaluation of heterogeneity of primary photosynthetic processes on a single leaf level as related to leaf age, orientation and prevailing light environment is another challenge (Giudici et al., MS in prep.). For future photosynthesis studies in *W. radiacans*, ecophysiological measurements exploiting the approach of simultaneous gas exchange and chlorophyll fluorescence evaluation might be beneficial. Such approach will allow to identify driving factors of photosynthesis related to particular sites in the field and help to understand species growth peculiarities from juvenile stages to mature plants and limited natural reproduction of the species under global climate change. For future studies focused on photosynthetic CO_2 fixation in *W. radicans*, stomatal and mesophyll conductance shoud be investigated in response to environmental factors as well as non-stomatal limitation of photosynthesis. This approach has been applied successfully in other species (e.g. Tosens et al., 2016).

2.2. REFERENCES

Albaum, H.G. 1938. Normal growth, regeneration, and adventitious outgrowth formation in fern prothallia. Am. J. Bot. 25(1), 37-44.

Alston, A.H.G. 1956. The subdivision of the Polypodiaceae. Tuxon 5, 23-25.

Arosa, M.L., Quintanilla, L.G., Ramos, J.A., Ceia, R., Sampaio, H. 2009. Spore maturation and release of two evergreen Macaronesian Ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an altitudinal gradient. Am. Fern J. 99(4), 260-272. https://doi.org/10.1640/0002-8444-99.4.260.

Biondi, E., Allegrezza, M., Casavecchia, S., Galdenzi, D., Gasparri, R., Pesaresi, S., Vagge, I., Blasi, C. 2014. New and validated syntaxa for the checklist of Italian vegetation. Pl. Biosystems 148(2), 318-332,
DOI: 10.1080/11263504.2014.892907.

Bower, F.O. 1928. The ferns (Filicales). Vol 3. Cambridge University Press, Cambridge. 306 p.

Brullo, S., Lo Giudice, R., Privitera, M. 1989. La classe Adiantetea in Sicilia. Arch. Bot. Ital. 65(1–2), 81-99.

Brullo, S., De Leonardis, W., Pavone, P. 1982. Chromosome numbers of some Sicilian ferns. Webbia 35(2), 275-281.

Bunbury, C.J.F. 1859. On some Vegetable Remains from Madeira. Quarterly Journal of the Geological Society 15, 50-59.

Carafa, A.M. 1984. Light dependent spore germination in *Woodwardia radicans* (L) Sm. Giorn Bot Ital (118)5-6, 245-253.

Carafa, A.M. 1985. Osservazioni sullo sviluppo del gametofito in *Woodwardia radicans* (L) Sm. Giorn. Bot. Ital. (119)2, 153.

Carafa, A.M. 1990. Gametophyte development of *Woodwardia radicans* (L) Sm. Effect of population density and antheridiogen on sex expression. Giorn. Bot. Ital. (124)5, 571-580, DOI: 10.1080/11263509009430257

Ching, R.C. 1940. On natural classification of the family ‘‘Polypodiaceae.” Sunyatsenia 5, 201–268.

Copeland, E.B. 1947. Genera Filicum. Chronica Bot. Co., Waltham, Mw., U.S.A.

Cranfill, R., Kato, M. 2003. Phylogenetics, Biogeography and Classification of the *Woodwardioid* Ferns (Blechnaceae). In: Chandra, S and Srivastava, M (eds.) Pteridology in the New Millenium. Kluwer Academic Press, Netherlands

De Soto, L., Quintanilla, L.G., Mendez, M. 2008. Environmental sex determination in ferns: Effects of nutrient availability and individual density in *Woodwardia radicans*. J. Ecol. 96(6), 1319-1327.

Douglas, G.E. 1994. An investigation into the growth, development and ultrastructure of fern gametophytes in existing and novel culture systems. PhD dissertation, University of Manchester, UK.

Giudici, G.N.M., Barták, M., Hájek, J. (2020 submitted) Acclimation of primary photosynthetic processes to light in three fern species from a Mediterranean moist habitat, Ferriere Valley, Italy. Submitted to Acta Physiologiae Plantarum.

Góis-Marques, C.A., Madeira, J., de Sequeira, M.M. 2018. Inventory and review of the Mio–Pleistocene São Jorge flora (Madeira Island, Portugal): Palaeoecological and biogeographical implications. J. Syst. Palaeontol. 16(2), 159-177, DOI: 10.1080/14772019.2017.1282991.

Hartung, G., Mayer, K. 1864. Geologische Beschreibung der Inseln Madeira und Porto Santo. Mit dem systematischen Verzeichnisse der fossilen Reste dieser Inseln und der Azoren von Karl Mayer. Engelmann, Leipzig, 364 p.

Hassler, M. 2017. *Woodwardia*. World Plants: Synonymic Checklists of the Vascular Plants of the World (2019). In: Roskov Y., Abucay L., Orrell T., Nicolson D., Bailly N., Kirk P., Bourgoin T., DeWalt R.E., Decock W., De Wever A., Nieukerken E. van, Zarucchi, J., Penev L., eds. 2017. Species 2000 & ITIS Catalogue of Life.

Haufler, C.H. 2002. Homospory 2002: An odyssey of progress in pteridophyte genetics and evolutionary biology. BioScience 52(12), 1081.

Heer, O. 1857. Ueber die fossilen Pflanzen von St. Jorge in Madeira. Neue Denkschriften der allgemeinen Schweizerischen Gesellschaft fur die gesamten Naturwissenschaften, 15, 1-40.

Hill, R.H. 1971. Comparative habitat requirements for spore germination and prothallial growth of three ferns in south eastern Michigan. Am. Fern J. 61, 171-182.

Hollermann, P. 1981. Microenvironmental studies in the laurel forest of the Canary Islands. Mt. Res. Dev. (1)3-4, 193-207.

Holttum, R.E. 1954. A revised flora of Malaya. Singapore: Government Printing Office.

Hornych, O., Ekrt, L. 2017. Spore abortion index (SAI) as a promising tool of evaluation of spore fitness in ferns: an insight into sexual and apomictic species. Plant Syst. Evol. 303(4), 497-507.

Klekowski Jr., E.J. 1969. Reproductive biology of the Pteridophyta. III. A study of the Blechnaceae. Bot. J. Linn. Soc. 62, 361-377.

Korpelainen, H. 1995. Growth and reproductive characteristics in artificially formed clonal gametophytes of Dryopteris filix-mas (Dryopteridaceae). Plant Syst. Evol. 196, 195-206.

Li, C.X., Lu, S., Junye, M., Gai, Y., Yang, Q. 2014. Phylogeographic history of the woodwardioid ferns, including species from Himalaya. Palaeoworld. 25. 10.1016/j.palwor.2014.10.004.

Löve, A., Löve, D., Pichi Sermolli, R.E.G. 1977. Cytotaxonomical atlas of the Pteridophyta. Cramer, Vaduz.

Miller, J.H. 1968. Fern gametophytes as experimental material. Bot. Rev. 34, 361-440.

Näf, U. 1958. On the physiology of antheridium formation in the Bracken fern *Pteridium aquilinum* (L.) Kuhn. Physiol. Plant. 11, 728-746.

Näf, U., Nakamishi, K., Endo, M. 1975. On the physiology and chemistry of fern antheridiogens. Bot. Rev. 41, 315-359.

Nayar, B.K., Bajpai, N., Raza, F. 1966. Morphological studies on some species of *Blechnum*, *Doodia*, *Woodwardia* and *Stenochlaena*: I. The gametophytes and the juvenile sporophytes. Bot. J. Linn. Soc. 59(380), 405–423. https://doi.org/10.1111/j.1095-8339.1966.tb00071.x

Nayar, B.K. 1965. Gametophytes and juvenile leaves of drynarioid ferns. Bot. Gaz. 126, 46-52.

PPG I (2016) A community-derived classification for extant lycophytes and ferns JSE 2016

Pichi Sermolli, R.E.G.P. 1979. A survey of the pteridological flora of the Mediterranean Region. Webbia 34(1), 175-242.

Pyner, T. 2015. *Woodwardia* in cultivation. https://www.rhs.org.uk/about-the-rhs/publications/the-plant-review/2015-Issues/september/Woodwardia-in-cultivation.pdf

Quintanilla, L.G., Amigo, J., Pangua, E., Pajarón, S. 2002a. Análisis biogeográfico de la pteridoflora de la sierra de la Capelada (La Coruña, España). Lazaroa 23, 17-24.

Quintanilla, L.G., Amigo, J., Pangua, E., Pajarón, S. 2002b. Effect of storage method on spore viability in five globally threatened fern species. Ann. Bot. 90, 461-467.

Quintanilla, L.G., De Soto, L., Jimenez, A., Mendez, M. 2007a. Do antheridiogens act via gametophyte size? A study of *Woodwardia radicans* (Blechnaceae). Am. J. Bot. 94(6), 986-990.

Quintanilla LG, Escudero A (2006) Spore fitness components do not differ between diploid and allotetraploid species of Dryopteris (Dryopteridaceae). Ann. Bot. 98, 609-618. doi: 10.1093/aob/mcl137

Quintanilla, L.G., Pajarón, S., Pangua, E., Amigo, J. 2000. Effect of temperature on germination in northernmost populations of *Culcita macrocarpa* and *Woodwardia radicans*. Plant Biol. 2(6), 612-617

Chapter III

HOW THE OLDEST ORGANISMS COPE WITH EXCESSIVE LIGHT IN EXTREME ENVIRONMENT?

CZECH POLAR REPORTS 9 (2): 160-169, 2019

Photoinhibition of primary photosynthetic processes in hydrated *Polytrichum commune*: Analysis of non-photochemical quenching affecting species resistance

Gabriella Nora Maria Giudici

University of Naples Federico II, Department of Agriculture, 80055 Portici, Italy

Abstract

A moss from the alpine environment of the Jeseníky mountains, *Polytrichum commune*, was studied under lab-induced light stress to analyse photoinhibition (PI) stress re-sponse; three PI doses were used: PAR 1500 µmol m^{-2} s^{-1} for 60 min., 1200 µmol m^{-2} s^{-1} for 60 min. and 1200 µmol m^{-2} s^{-1} for 30 min.; in the last one the added component of slight desiccation stress was added. Chlorophyll fluorescence parameters were plotted as time series, immediately before and after the PI treatments, then every 20 minutes for three hours (recovery period). F_V/F_M, Φ_{PSII} and NPQ parameters and quenching com-ponents were analysed. Decreasing courses and final values of F_V/F_M and Φ_{PSII} pa-rameters along with increased values of NPQ clearly indicated PI stress response, al-though not very severe. Quenching parameters analysis showed a dominant role played by xanthophyll pigments along with changes in PS II in the non-photochemical energy quenching. Dehydration contributed additional value to NPQ. All these factors are con-sistent with the adaptation of the species to harsh conditions of alpine environments.

Key words: NPQ quenching, *Polytrichum*, chlorophyll fluorescence, photoinhibition, lamellae, alpine environment

DOI: 10.5817/CPR2019-2-14

Introduction

Photoinhibition (PI) is a decrease of photosynthetic performance due to high light exposure. High light can cause clo-sure of photosynthetic reaction centres, therefore inducing an excess of adsorbed energy which in turn cannot be utilized through photosynthetic pathways and could be dangerous to structures and complexes of the whole chloroplastic apparatus. Moss-es and lichens in alpine environments are usually high light tolerant species as a re-sult of evolutionary adaptation to bright days which can occur through the year. Since the pioneering study of Murray et al. (1993), PI as well as photoprotective mech-anisms activated during PI has been in focus in a great variety of mosses. Major-ity of them were found PI tolerant or re-sistant. However, some sensitive species have been identified as well (*e.g. Pleuro-*

Received October 25, 2019, *accepted* December 18, 2019.

*Corresponding author: G. N. M. Giudici <gabriellanoramaria.giudici@unina.it>

Acknowledgements: The author wishes to thank the ECOPOLARIS project (CZ.02.1.01/0.0/0.0/16_013/0001708) for the infrastructure, Czech Ministry of Education for the facilities and the staff of the EEL Laboratory (CzechPolar2 infrastructure LM2015078).

zium schreberi from shade habitats - Hájek et al. 2009). Only few studies have dealt with mechanistic aspects of photoinhibition and photoprotection of desiccation-tolerant photoautotrophs (Heber et al. 2007, Veerman et al. 2007, Heber 2008, Heber et al. 2009). Apart from the studies focused on PI in mosses in wet state, there have been several others dealing with photopro-tective mechanisms in desiccating mosses (*e.g.* Csintalan et al. 1999, Nabe et al. 2007). High light resistance is the result of biochemical, physical and structural traits and protective mechanisms. Maximum and effective quantum yield (F_V/F_M, Φ_{PSII}) of photosystem II along with slow Kautsky kinetics are widely used to measure plant response to stress inducing factors, together with non-photochemical quenching (NPQ) values. Stress factors cause general lowering of F_V/F_M and Φ_{PSII} transient and flattening of shapes (*i.e.* general lowering of their peaks), see *e.g.* Jägerbrand and Kudo (2014). At the same time they in-duce increase in NPQ time course, whose components are indicative of the different activated mechanisms. In mosses, non-photochemical quenching has been studied as dependent on desiccation and photoinhibition Heber et al. (2006). It is generally accepted that there are three different components of non-photochemical quenching (reviewed by *e.g.* Yamakawa et al. 2012). The first mechanism, is active in hydrated mosses and controlled by the protonation of a thylakoid protein. Other two mechanisms are activated in desiccating thalli. One of them permits exciton migration towards the light-harvesting complexes, antenna pigments in particular, where fast thermal dissipation takes place. The third mechanism is based on the reversible photo-accumulation of a radical that acts as a quencher of excitation energy in reaction centres of photosystem II. The drought-induced quenching brings the acceleration of the chlorophyll fluorescence decay rate and rapid dissipation of excitation energy into heat (Yamakawa and Itoh 2013). Although many studies dealt with PI and desiccation, little has been written about photoinhibition in fully wet state. In the study we analysed chlorophyll fluorescence induction curve supplemented with analysis of quenching mechanisms in fully wet *Polytrichum commune*.

Material and Methods

General characteristics of Polytrichum commune

The species within the genus *Poly-trichum* have several adaptations helping them to keep water in the thallus and maintain photosynthesis in leaves. They comprise of 1) the ability of water trans-port from the base of the plant thanks to water-conducting cells (hydromes, in cen-tral strand of the stem, and hydroids, in the costa of leaves) and 2) special structures on the upper leaf surface (lamellae). The lamellae are ridges-like structures that run parallel to each other over the length of the leaf. They are several cell layers long and tall and a single cell wide (8 μm in our sample) (Fig. 1). The lamellae cells are rich in chloroplasts and increase the ef-fective area for photosynthesis (Thomas et al. 1996). They are effective in keeping water in photosynthesizing cells even dur-ing initial phase of desiccation. Moreover, moist air remains between the lamellae protecting the leaves from fast dehydration. Lamellar cells may undergo structural and functional changes in some periods of growing season, more pronounced in win-ter time (Ljubešic et al. 2005). *Polytri-chum commune* is easily identified because of the lamella apical cell which is indented or cup-like shaped (Fig. 1).

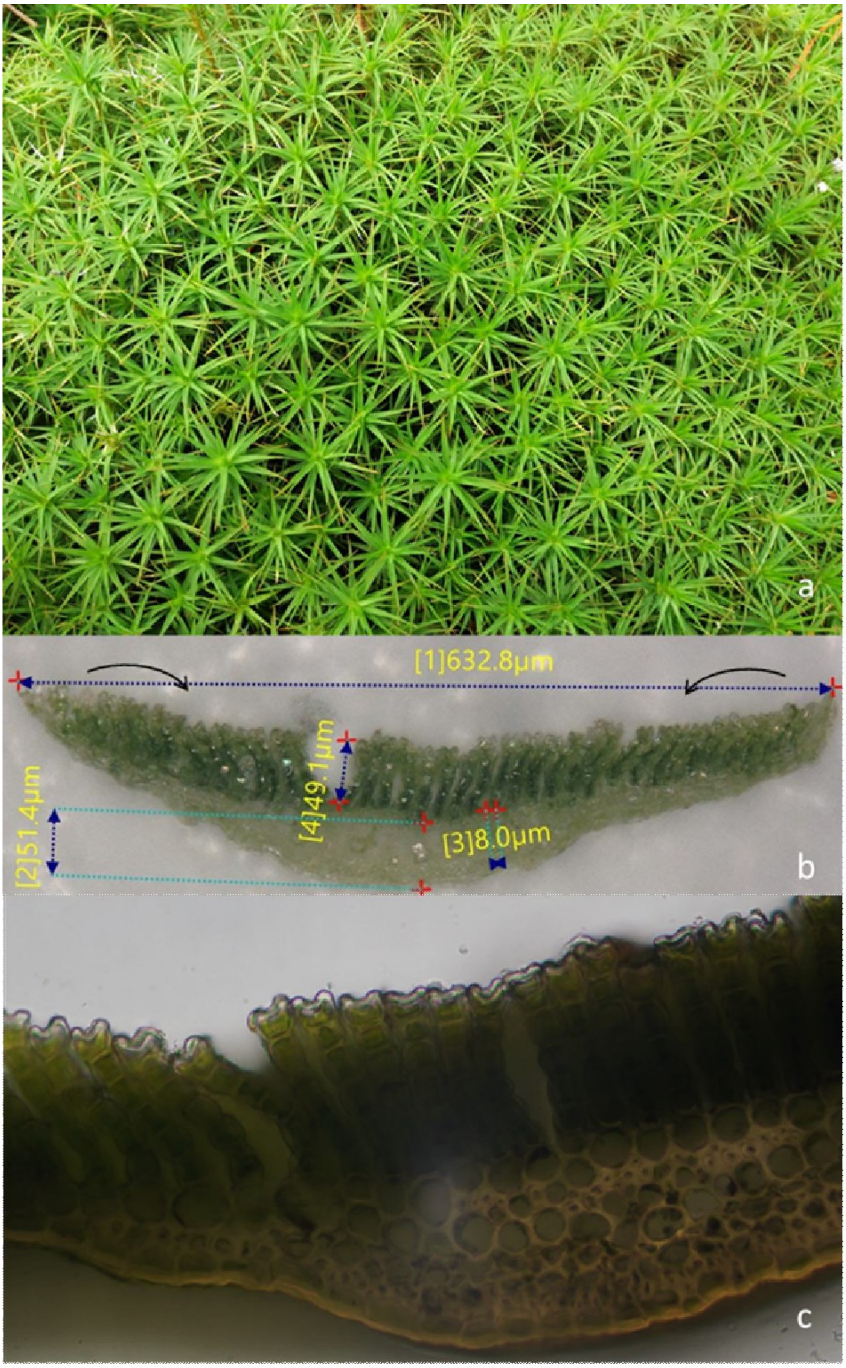


Fig. 1. *Polytrichum commune* leaves (a) and microscopy section of a single leaf showing lamellae (b). The arrows show the direction of the movement when the leaf folds inside to protect from dehydration. Apical cells (c) with the typical indented shape; chloroplasts are visible through the cells.

Experimental plants collection and handling

Shoots of *Polytrichum commune* were collected in Tabulové skály rocks, Jesení-ky Mts. (1415 m a.s.l.), and stored in wet condition (regularly sprayed) under dim light (20 μmol m^{-2} s^{-1}) at 5°C. Experiments were run soon after the collecting. Shoots were put in small containers with distilled water and kept fully wet by constantly spraying them with distilled water during the treatments and the measuring periods; ice was put around, but not in the con-tainers, to maintain low temperature (be-low 10°C). Three light treatments were in-duced (LED source PSI SL3500-498, Pho-ton Systems Instruments, Czech Republic) at PAR 1500 μmol m^{-2} s^{-1} for 60 min. (n°1), 1200 μmol m^{-2} s^{-1} for 60 min. (n°2) and 1200 μmol m^{-2} s^{-1} for 30 min. (n°3), in which we added the desiccation compo-nents, *i.e.* we used a set of shoots which were fully hydrated (100% RWC) along with another set which had 85-90% RWC at the beginning of the PI; during the photoinhibition, distilled water was sprayed around the samples and above them, enough to prevent rapid desiccation, but not increasing the actual RWC. During the three hours measurements, water was spray-ed around the samples but not directly on them. Chlorophyll fluorescence parameters were plotted as time series, immediately before and after the PI treatments, then every twenty minutes for three hours (re-covery period) after the end of the PI treatments. F_V/F_M, Φ_{PSII} and NPQ, were recorded after 5 min. pre-darkening with a PSI Handy Fluorcam HFC-010 (Photon Systems Instruments, Czech Republic). Oth-er parameters were calculated with the fol-lowing equations:

$\mathrm{NPQ} = (F_M{}^* - F_M')/F_M'$ *Eqn.* 1

$\mathrm{qN} = (F_M - F_0)^* – (F_M' - F_0')/(F_M - F_0)^*$ *Eqn.* 2

$\mathrm{qE} = (F_M{}^* - F_M'')/(F_M - F_0)^*$ *Eqn.* 3

$\mathrm{qI} = (F_M - F_0)^* – (F_M'' - F_0'')/(F_M - F_0)^*$ *Eqn.* 4

$F_V/F_M = (F_M - F_0)/F_M$ *Eqn.* 5

$F_M{}^*$ was always referred to control (before PI) values

$(F_M - F_0)^*$ F_M and F_0 were always referred to control (before PI) values

Results

Time courses analysis of F_V/F_M and Φ_{PSII}

In our experiment, both parameters F_V/F_M and Φ_{PSII} were indirectly correlated with the severity of light treatments, their values decreasing with increasing doses (*i.e.* time and intensity). The time courses in both parameters showed: first a clearly defined drop immediately after the end of PI, then a quick but smaller rise within the following 15 min. (fast phase recovery), finally a slower increasing recovery phase toward control levels (before PI), (slow phase recovery) (Fig. 2).

In Φ_{PSII}, only the second treatment (1200 μmol m^{-2} s^{-1} for 60 min.) showed a marked difference between the fast and slow phase of recovery, the other two treatments having a slight and average constant rate of the rising parameter. In all cases, the above-mentioned check points of the curves occurred always at the same time, in both parameters in all treatments. Ma-jority of recovery (*i.e.* above 75% of initial values) was reached within 15 min. after the end of PI in both parameters.

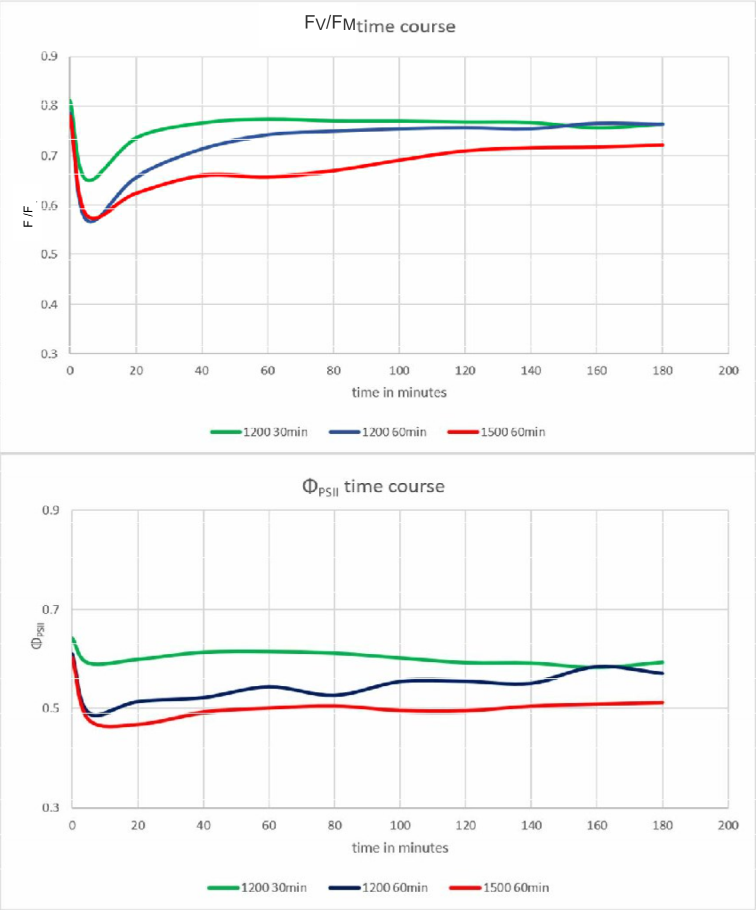


Fig. 2. F_V/F_M and Φ_{PSII} average time courses, before (control time = 0) and during three hours after the treatment (max value of standard deviation 0.11) .

Control values, before PI, were never reached after three hours of recovery period in any of the treatments for both parameters.

NPQ showed a rise peak in the first measurements after the end of PI, followed by a two-phase decline, first quick then slow. However, the least severe treatment (1200 µmol m^{-2} s^{-1} for 30 min.) showed constant rate of decrease during the recovery period (Fig. 3).

In contrast with F_V/F_M and Φ_{PSII} courses, there was no other synchronism check points on the curves related to different PI doses than the rising peaks.

Values of NPQ were found directly dose-dependent. Steepness of NPQ curve before reaching the peak was in a direct relationship with the PI dose, in contrast with time course of recovery phase (Fig. 4). Once again final NPQ values did not reach control values in any treatment.

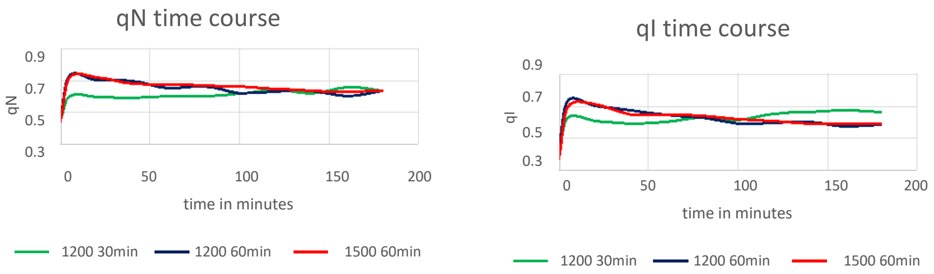


Fig. 3. NPQ, qN, qE, qI average time courses (max of average standard deviation 0.36).

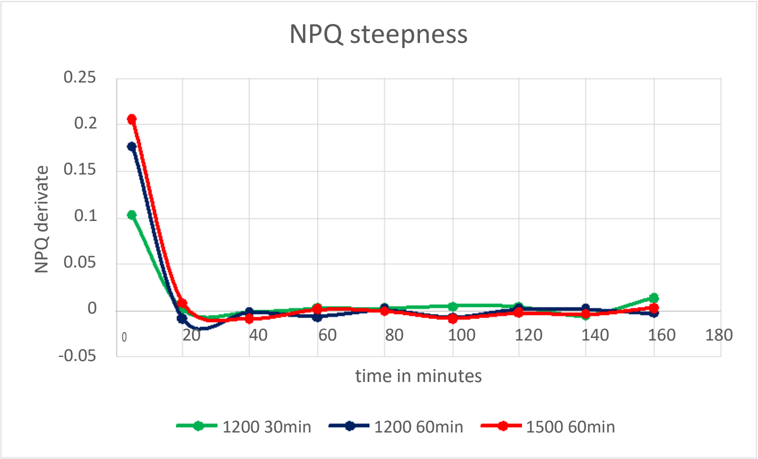


Fig. 4. NPQ steepness time course.

Quenching components

qN, qI and qE were considered. All showed comparable time courses, the general trend being comparable to that of NPQ, with more pronounced peaks and higher similarities among the curves. In all treatments, the fast phase of recovery was more prolonged in the highest dose.

Discussion

All treatments led to photoinhibition in the moss; it is clearly indicated by the lowering of both F_V/F_M and Φ_{PSII} time courses proportional to the PI dose, by the drop of F_V/F_M and Φ_{PSII} values immediately after the PI and by F_V/F_M and Φ_{PSII} final values (after three hours of recovery) lower than control (before PI). Moreover, photo-protecting mechanisms were activated as shown by the quick increase of NPQ immediately after the PI, once again proportional to PI dose. Light dose-dependent increase in NPQ was well documented in *P. formosum* (Marschall and Proctor 2004).

Non-photochemical quenching composing parameters showed a slightly higher proportion of the quick reversing component (qE), suggesting that a dominant role was played by the xanthophyll pigments cycle, as well as conformation and functional changes in PS II (related to the qI component). It must be noticed that in our data the addition of qE + qI is not below 1 because in the equation we used to calculate the parameters we kept constant F_M and F_0 values referred to control (*see* Equations 3 and 4).

It could be said that the doses we used induced a mild PI because of the following reasons:

1. After three hours of recovery, even though control values were not reached, the decrease of final values was less than 8% of initial values in all treatments (15% only in Φ_{PSII} final values of the most severe treatment, 1500 µmol m^{-2} s^{-1} for 60 min.)
2. The lowering of F_V/F_M and Φ_{PSII} curves was not substantial (less than 20%)
3. Majority of recovery during the recovery period was achieved quickly (70% in the first 10 min.).

Lowest treatment

The NPQ values in the lowest treatment (1200 µmol m^{-2} s^{-1} for 30 min.) showed a time course different from the others because of the additional effect of desiccation during the measuring time: the values that should decline quickly and continuously after the peak of the first measurement, decreased instead slowly, to rise again 40 min. after the end of the PI (dehydration effect). By the end of the recovery period, we observed NPQ values higher than the peak (Fig. 5); which could be explained by the additional effect of desiccation as was reported for *Polytrichum formosum* (Proctor et al. 2007) in which decreasing RWC caused increasing values of NPQ between 80 and 50% of RWC.

Confirmation is added by the decreasing course of Fs (Fig. 5), which is reported to be correlated with desiccating conditions. In desiccating mosses, Fs decreases thanks to desiccation-induced quenching as reported by *e.g.* Heber et al. (2007) for *Rhytidium rugosum*.

Capacity of protective mechanisms forming NPQ is species-specific in mosses. Some mosses have orange carotenoid pigments which may help prevent photosystem damage. For *Ceratodon purpureus,* photoprotective carotenoids including violaxanthin are reported (Post 1990). Proportion of underlying biophysical, biochemical and molecular responses activated in photoinhibited mosses and forming NPQ remains unclear because of the complex manner of PI response of chloroplastic apparatus. Mosses share many of photoprotective mechanisms with the vascular plants, however, there are some key differences in the photoprotection available (Robinson and Waterman 2014).

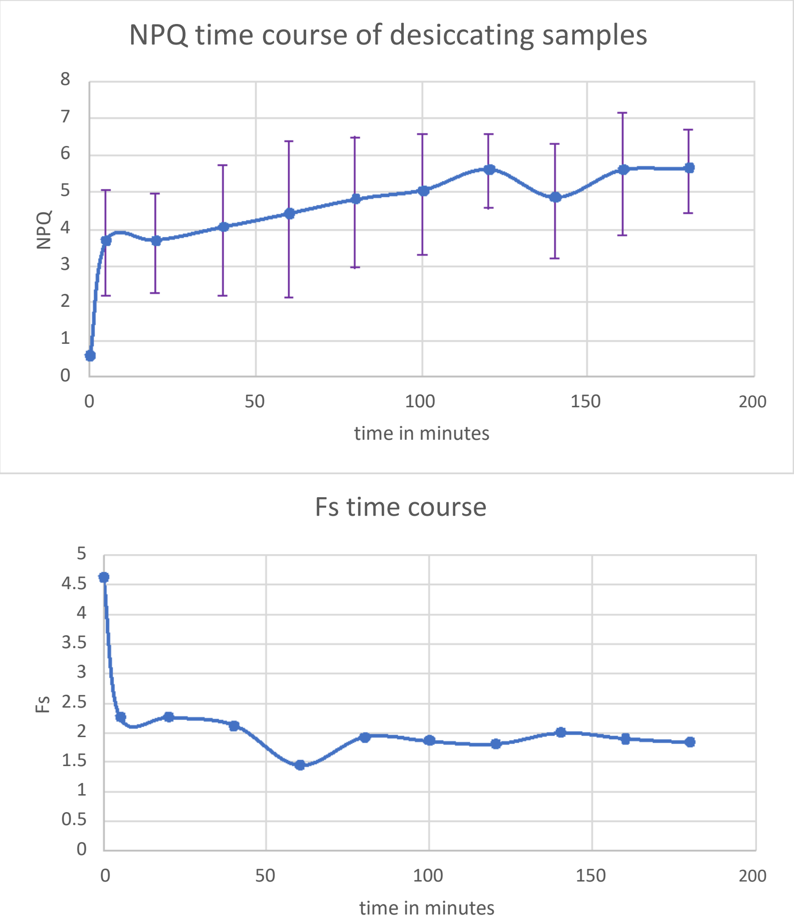


Fig. 5. NPQ and Fs average time course in the samples exposed to desiccation (*i.e.* samples which were not kept in fully wet state by constant spraying) in treatment n°3 (1200 µmol m^{-2} s^{-1} for 30 min.).

In mosses, fastest component of non-photochemical quenching occurs within PS II antenna system by the action of two essential light-harvesting complex (LHC)-like proteins, photosystem II subunit S (PSBS) in plants and light-harvesting complex stress-related (LHCSR) (Pinnola et al. 2015). Recent studies (*e.g.* Stella 2016, Dikaios et al. 2019) confirmed the role of LHCSR proteins in NPQ, particularly in thermal dissipation in photoinhibited mosses. Some studies, however, suggest that LHCSR proteins may be active in energy quenching (qE) as well (Alboresi et al. 2010, Gerotto et al. 2012).

Apparently, lichens possess a dominant mechanism of photoprotection which does not require zeaxanthin or a protonation reaction (Heber 2011).

Conclusions

According to our data, *Polytrichum commune* could be considered medium resistant to photoinhibition relating to the PI doses we used; moderate water loss could increase protective mechanisms activation (non-photochemical quenching in particular) but does not influence the overall photosynthetic performance. Protective mechanisms are activated in case of intense light and moderate water deficiency, which are both occurring in the mountains, after the snow cover is melted down and water availability is limited. Further studies should point to the extreme conditions of both light and desiccation levels which could be tolerated by this moss in the field. Since mosses are poikilohydric autotrophs active during winter period, low temperature photoinhibition of thalli in wet state should be taken into account when evaluating species-specific differences in resistance to photoinhibition (Lovelock et al. 1995a, b).

References

Alboresi, A., Gerotto, C., Giacometti, G. M., Bassi, R. and Morosinotto, T. (2010): *Physcomitrella patens* mutants afected on heat dissipation clarify the evolution of photoprotection mechanisms upon land colonization. *Proceedings of National Academy of Science* USA, 107: 11128-11133.

Csintalan, Z., Proctor, M. C. F. and Tuba, Z. (1999): Chlorophyll fluorescence during drying and rehydration in the mosses *Rhytidiadelphus loreus* (Hedw.) Warnst., *Anomodon viticulosus* (Hedw.) Hook. & Tayl. and *Grimmia pulvinata* (Hedw.) Sm. *Annals of Botany*, 84: 235-244.

Dikaios, I., Schiphorst, Ch., Dall'Osto, L., Alboresi, A., Bassi, R. and Pinnola, A. (2019): Functional analysis of LHCSR1, a protein catalyzing NPQ in mosses, by heterologous expression in *Arabidopsis thaliana*. *Photosynthesis Research*, 142: 249-264.

Gerotto, C., Alboresi, A., Giacometti, G. M., Bassi, R. and Morosinotto, T. (2012): Coexistence of plant and algal energy dissipation mechanisms in the moss *Physcomitrella patens*. *New Phytologist*, 196: 763-773.

Hájek, T., Tuittila, E.-S., Ilomets, M. and Laiho, R. (2009): Light responses of mire mosses: a key to survival after water-level drawdown? *Oikos*, 118: 240-250.

Heber, U. (2008): Photoprotection of green plants: A mechanism of ultra-fast thermal energy dissipation in desiccated lichens. *Planta*, 228: 641-650.

Heber, U. (2011): Lichens and Bryophytes: Light Stress and Photoinhibition in Desiccation/Rehydration Cycles – Mechanisms of Photoprotection. *In*: U. Lüttge, E. Beck, D. Bartels (eds.): Plant Desiccation Tolerance. Springer, 386 p.

Heber, U., Bilger, W. and Shuvalov, V. A. (2006): Thermal energy dissipation in reaction centres and in the antenna of photosystem II protects desiccated poikilohydric mosses against photo-oxidation. *Journal of Experimental Botany*, 57(12): 2993-3006. doi: 10.1093/jxb/erl058

Heber, U., Azarkovich, M. and Shuvalov, V. A. (2007): Activation of mechanisms of photoprotection by desiccation and by light: Poikilohydric photoautotrophs. *Journal of Experimental Botany*, 58: 2745-2759.

Heber, U., Bilger, W., Türk, R. and Lange, O. L. (2009) Photoprotection of reaction centres in photosynthetic organisms: mechanisms of thermal energy dissipation in desiccated thalli of the lichen *Lobaria pulmonaria*. *New Phytologist*, doi: 10.1111/j.1469-8137.2009.03064.x

Jägerbrand, A. K., Kudo, G. (2014): Short-term responses in maximum quantum yield of PSII (Fv/Fm) to ex situ temperature treatment of populations of bryophytes originating from different sites in Hokkaido, Northern Japan. *Plants* (Basel). 2016 Jun; 5(2): 22. doi: 10.3390/plants5020022.

LJUBEŠIC, N., WRISCHER, M., PREBEG, T. and DEVIDÉ, Z. (2005): Structural changes of lamellar cells in leaves of the moss *Polytrichum formosum* Hedw. during winter freezing and thawing processes. *Acta Botanica Croatica*, 64: 219-226.

LOVELOCK, C. E., OSMOND, C. and SEPPELT, R. D. (1995a): Photoinhibition in the Antarctic moss *Grimmia antarctici* card when exposed to cycles of freezing and thawing. *Plant Cell and Environment*, 18: 1395-1402.

LOVELOCK, C. E., JACKSON, A. E., MELICK, D. R. and SEPPELT, R. D. (1995b): Reversible photoinhibition in Antarctic moss during freezing and thawing. *Plant Physiology*, 109: 955-961.

MARSCHALL, M., PROCTOR, M. C. F. (2004): Are bryophytes shade plants? Photosynthetic light responses and proportions of chlorophyll *a*, chlorophyll *b* and total carotenoids. *Annals of Botany*, 94: 593-603.

MURRAY, K. J., TENHUNEN, J. D. and Nowak, R. S. (1993): Photoinhibition as a control on photosynthesis and production of *Sphagnum* mosses. *Oecologia*, 96: 200-207.

NABE, H., FUNABIKI, R., KASHINO, Y., KOIKE, H. and SATOH, K. (2007): Responses to desiccation stress in bryophytes and an important role of dithiothreitol-insensitive non-photochemical quenching against photoinhibition in dehydrated states. *Plant and Cell Physiology*, 48(11): 1548-1557.

POST, A. (1990): Photoprotective pigment as an adaptive strategy in the Antarctic moss *Ceratodon purpureus*. *Polar Biology*, 10: 241-245.

PINNOLA, A., GHIN, L., GECCHELE, E., MERLIN, M., ALBORESI, A., AVESANI, L., PEZZOTTI, M., CAPALDI, S., CAZZANIGA, S. and BASSI, R. (2015): Heterologous expression of moss Light-harvesting Complex Stress-related 1 (LHCSR1), the chlorophyll a-xanthophyll pigment-protein complex catalyzing non-photochemical quenching, in *Nicotiana* sp. *The Journal of Biological Chemistry*, 290: 24340-24354.

PROCTOR, M. C. F., LIGRONE, R. and DUCKETT, J. G. (2007): Desiccation tolerance in the moss *Polytrichum formosum*: Physiological and fine structural changes during desiccation and recovery. *Annals of Botany*, 99: 75-93.

ROBINSON, S., WATERMAN, M. (2014): Sunsafe bryophytes: Photoprotection from excess and damaging solar radiation. *In*: D. T. Hanson, S. K. Rice: Photosynthesis in bryophytes and early land plants. Springer, pp. 113-130.

STELLA, G. R. (2016): Light stress and photoprotection in green algae, mosses and diatoms. Ph.D. thesis, University of Verona and University Pierre et Marie Curie, 144 p.

THOMAS, R. J., RYDER, S. H., GARDNER, M. I., SHEETTZ, J. P. and NICHIPOR, S. D. (1996): Photosynthetic functions of leaf lamellae in *Polytrichum commune*. *The Bryologist*, 99: 11.

VEERMAN, J., VASILEV, S., PATON, G. D., RAMANAUSKAS, J. and Bruce, D. (2007): Photoprotection in the lichen *Parmelia sulcata*: The origins of desiccation induced fluorescence quenching. *Plant Physiology*, 145: 997-1005.

YAMAKAWA, H., FUKUSHIMA, Y., ITOH, S. and HEBER, U. (2012): Three different mechanisms of energy dissipation of a desiccation-tolerant moss serve one common purpose: To protect reaction centres against photo-oxidation. *Journal of Experimental Botany*, 63: 3765-3775.

YAMAKAWA, H., ITOH, S. (2013): Dissipation of excess excitation energy by drought-induced nonphotochemical quenching in two species of drought-tolerant moss: desiccation-induced acceleration of photosystem II fluorescence decay. *Biochemistry*, 52: 4451-4459.

Chapter IV

DEHIDRATION: MEASURING THROUGH PHOTOSYNTHESIS A STRESS OCCURRING IN DIFFERENT AND DISTANT ENVIRONMENTS

CZECH POLAR REPORTS 8 (2): 286-298, 2018

Comparative research of photosynthetic processes in selected poikilohydric organisms from Mediterranean and Central-European alpine habitats

Gabriella Nora Maria Giudici[1], Josef Hájek[2], Miloš Barták[2], Svatava Kubešová[3,4]

[1]*University of Naples Federico II, Department of Agriculture, 80055 Portici, Italy*
[2]*Masaryk University, Faculty of Science, Department of Experimental Biology, Laboratory of Photosynthetic Processes, Kamenice 5, Building A13, 625 00 Brno, Czech Republic*
[3]*Masaryk University, Faculty of Science, Department of Botany and Zoology, Kamenice 5, 625 00 Brno, Czech Republic*
[4]*Moravian Museum, Department of Botany, Hviezdoslavova 29a, 627 00, Brno, Czech Republic*

Abstract

Dehydration-induced decrease in photosynthetic activity was investigated in five poikilohydric autotrophs using chlorophyll fluorescence parameters recorded during controlled desiccation. For the study, two representatives of mosses from alpine zone (*Rhizomnium punctatum, Rhytidiadelphus squarrosus*) of the Jeseníky Mts. (Czech Republic) were used. Other two experimental species were mediterranean habitats liverwort (*Pellia endiviifolia*) and moss (*Palustriella commutata*), collected from under *Woodwardia radicans* canopy in the Nature Reserve Valle delle Ferriere (Italy). The last species was a liverwort (*Marchantia polymorpha*) collected from lowland site (Brno, Moravia, Czech Republic). We investigated the relationship between relative water content (RWC) and several chlorophyll fluorescence parameters evaluating primary photochemical processes of photosynthesis, such as effective quantum yield of photosynthetic processes in photosystem II (Φ_{PSII}), and non-photochemical quenching (qN). With desiccation from fully wet (RWC = 100%) to dry state (RWC = 0%), Φ_{PSII} exhibited a rapid (*R. punctatum*) and slow decline of Φ_{PSII} (*R. squarrosus*, *P. endiviifolia*, *M. polymorpha*, and *P. commutata*). Shapes of dehydration-response curves were spe-cies-specific. RWC$_{0.5}$, *i.e.* the RWC at which the sample showed half of maximum Φ_{PSII}, reflected the species-specificity. It reached 65% in desiccation sensitive (*R. punctatum*), 53% and 43% in semi-tolerant (*P. commutata* and *R. squarrosus*), 24% and 18% in desiccation-tolerant species (*P. endiviifolia* and *M. polymorpha*). In all experimental species, non-photochemical quenching (qN) of absorbed light energy showed high values at RWC = 100% and a slight increase with desiccation. Steady state chlorophyll fluorescence (F_S) remained high during desiccation and was not correlated with Φ_{PSII}.

DOI: 10.5817/CPR2018-2-24

Received November 6, 2018, *accepted* December 22, 2018.

[*] Corresponding author: G. N. M. Giudici <gabriellanoramaria.giudici@unina.it>

Acknowledgements: The authors thank the projects CzechPolar-II (LM2015078) for providing Brno-based facilities and the infrastructure for the research reported in this study. The authors thank also for the support from ECOPOLARIS project (CZ.02.1.01/0.0/0.0/16_013/0001708) aimed to fund the research reported in this paper and Carabinieri per la biodiversità, UTB Caserta, for field support in collecting samples from *Woodwardia radicans* restricted area. The help of the members of the Extreme Environments Life (EEL) laboratory (Masaryk University, Department of Experimental Biology, Brno) provided during data processing is also acknowledged.

Key words: chlorophyll fluorescence, moss, liverwort, dehydration, photosynthesis, *Woodwardia radicans*

Introduction

Mosses and liverworts, being desicca-tion tolerant poikilohydric autotrophs, cope well with dehydration/rehydration cycles and are capable to restore their photosyn-thetic activity soon after being rehydrated (Proctor et Smirnoff 2000). There are sev-eral ecophysiological adaptations, mainly protective mechanisms (see *e.g.* Green-wood 2017, for review), helping such or-ganisms to inhibit and recover their photo-synthetic activities during desiccation and rehydration, respectively. Most moss spe-cies are highly resistant to desiccation. The process of drying and rehydration can be repeated several times without causing major changes in the functioning of the organism (Stoklasa-Wojtasz et al. 2012). According to their capabilities to restore physiological characteristics after repeated cycles of hydration-dehydration, moss spe-cies are divided into two categories: desic-cation tolerant or desiccation sensitive. This concept, however, is considered not generally valid recently since there are many interacting factors, such as *e.g.* phe-notypic (Proctor et al. 2007) end ecotypic plasticity of the species, physiological ′his-tory′ of the sample, microclimate effects, the effects of the rate of desiccation/de-hydration that may co-act and change the desiccation tolerance/sensitivity. Although many of protective mechanisms of bryo-phytes are common with the higher plants, there are fundamental interspecific differ-ences in their response to desiccation. Study of Marschall et al. (2018) reported desiccation-tolerant (*Porella platyphylla*) and desiccation-sensitive (*Sphagnum angus-tifolium*) mosses. In slowly desiccating mosses, desiccation tolerance may increase thanks to the involvement of ABA (May-aba et al. 2001, Wise et Tunnacliffe 2004). Early stages of moss desiccation are associated with an increase in thermotolerance, *i.e.* increase of thermal stability of chloro-plastic photosynthetic apparatus, as shown for *Homalothecium lutescens* by Dulai et al. (2004). Recently, the phenomenon of inducible desiccation tolerance is studied in mosses. Generally, majority of mosses are considered desiccation-tolerant, while *e.g.* *Marchantia polymorpha* (liverwort) be-longs to desiccation sensitive species (Na-be et al. 2007). Also *Pellia endiviifolia* is considered desiccation-intolerant liverwort species (Deltoro et al. 1998b). Since the 90-ies (*e.g.* Deltoro et al. 1998a, Csintalan et al. 1999) of the last century, chlorophyll fluorescence technique has been extensive-ly used for the evaluation of dehydration effects on moss and liverwort photosyn-thesis. The approach was applied also in other poikilohydric organisms, such as *e.g.* *Nostoc commune* colonies (Barták et al. 2016). Typically, the photosynthetic stud-ies of such autotrophs at different degrees of dehydration combine gas exchange and chlorophyll fluorescence measurements (*e.g.* Hu et al. 2016). Several previous studies (*e.g.* Heber et al. 2001, Pressel et al. 2006) have shown that chlorophyll fluorescence is almost totally suppressed during de-hydration in bryophytes. Therefore, the change in chlorophyll fluorescence emis-sion and chlorophyll fluorescence parame-ters is a very useful tool in the evalua-tion of negative effects of dehydration on moss/liverwort photosynthesis. It has been shown by *e.g.* Hájek et Beckett (2008) that critical relative water content (RWC) and/ or water potential of a moss thallus might be evaluated by chlorophyll fluorescence technique, the potential (F_V/F_M) and effec-tive quantum yield (Φ_{PSII}).

In this comparative study, we focused on the changes in primary photosynthetic processes monitored by chlorophyll fluorescence parameters during desiccation in five poikilohydric autotrophs. We expected species-specific responses in gradually desiccating samples, among these the water content at which the first signs of inhibi-tion of photosynthesis appear and the RWC at which a half of the maximum Φ_{PSII} is found. The emphasis was also given to the critical RWC, *i.e.* relative water content at which the individual species show full limitation of primary photochemical processes of photosynthesis.

Material and Methods

Sampling sites

Samples of *Rhizomnium punctatum* and *Rhytidiadelphus squarrosus* were collected from the Jeseniky Mts. (NE of the Czech Republic, Moravian-Silesian region). The samples of *R. punctatum* were collected from the ground close to the margin of a stream with dominant grass cover close to the Barborka chalet (1 320 m a.s.l.). Sam-ples of *R. squarrosus* were collected from the locality called the Kapitánská stezka (940 m a.s.l.). The samples were collected from the moss-dominated vegetation cover-ing a ground within a mature Norway spruce (*Picea abies* L.) stand (the Jeseníky Mts., Czech Republic). The climate of the Jeseníky Mts. is characterized by annual mean temperature of 1.1°C (from meteoro-logical station Praděd, 1 492 m a.s.l., Led-nický 1985). The coldest month is January with the monthly mean of -7.5°C. The warmest one is July (9.7°C). Annual sum of precipitation reaches 1 231 mm with monthly maximum found in June (180 mm) and minimum in October (69 mm). The samples of *Marchantia polymorpha* were collected from moist soil surface in ornamental flower gardens (Brno, South Moravia, Czech Republic) located close to the Svratka river. Mean annual air temper-ature is 9.1°C, mean annual precipitation is 490 mm (meteorological station Brno-Tuřany, Dobrovolný et al. 2012). The sam-ples were delivered to the laboratories, during the autumn 2018, then dried and stored for 3 weeks until the beginning of experiments.

Samples of *Pellia endiviifolia* and *Palustriella commutata* were collected in the Valle delle Ferriere (Italy), 33 km SE of Naples and 17 km west of Salerno. The locality is a deep valley rich in water and waterfalls, with a permanent stream at its bottom (Canneto). The area is located in the southern side of the Sorrento penin-sula, made by Mesozoic limestone, trans-gressive Miocene calcarenite and flysch, with occurrences of volcanic deposits from Somma-Vesuvius. The main valley is crossed by secondary valleys and the un-derground faults along with the karst sys-tem regulate the water flow and drainage, generating many springs. It is surrounded by mountains and peaks, the altitude of which ranges from 300 to 1 203 m a.s.l. The climate is warm temperate (20°C to 5°C). The average annual rainfall of 1 500 (mountain) to 1 000 (sea level) mm, hits its maximum in the autumn - winter period. The proximity of the mountains to the coast is the cause of the high rainfall and mists because of condensation of humid air masses brought from the sea by wind. The particular orography creates very dif-ferent microclimates and altitude plant dis-tribution shows thermal inversion, *i.e.* me-sophyll woods are at the bottom of the valleys and Mediterranean vegetation is on higher slopes and mountain ridges. Because of these very particular microclimatic con-ditions, the valley is home to many dif-ferent vegetal and animal species listed in EU Red List, *e.g.* *Woodwardia radicans* and *Pteris cretica*, both tropical relicts. Three of its seven EU enlisted habitats are priority habitat types including "*Petrifying springs with tufa formation*", hard water springs with active formation of travertine or tufa, on the rocky walls and forest, dominated by bryophyte vegetation (*Cratoneurion commutati*).

Species characteristics

The above-specified experimental species were determined and the nomenclature of the species follows Hill et al. (2006).

Pellia endiviifolia

P. endiviifolia is a thallose liverwort with green or blackish-green thalli up to about 1 cm wide, without reddish tinges. Narrow (6 mm wide) and numerous new branches are developed at their tips in autumn or early winter. *Pellia* is dioicous with the female organ protected by a vertical tooth-mouthed tube. *P. endiviifolia* occurs in base-rich sites, is found by watercourses, springs, shaded moist underwood, fens and dune slacks, dripping rock outcrops (Atherton et al. 2010).

Marchantia polymorpha

M. polymorpha is a thallose liverwort which typically forms flattened thalli. Thal-li of the liverwort are green, pale green on the ventral side. They are usually sized 4-6 cm even or sometimes up to 15 cm long and about 1-2 cm wide. The thalli are dichotomously branched. Thallus margins could be slightly uplifted, gently sliced, sometimes with a blurred, black stripe in the middle. The middle rib located at the bottom is not very distinct from the basic tissue. Clear breathing holes sized 60-100 × 40-80 μm can be distinguished on upper surface. Thalli bear scattered, cup-shaped gemma receptacles. *M. polymorpha* grows on rocks, stones in/beside streams, in springs, beside reservoirs and in man-made habitats in gardens, on footpaths, on walls, and on old bonfire sites (Atherton et al. 2010).

Rhizomnium punctatum

R. punctatum is a moss species common to wet places in forests. It is medium-sized moss forming rather loose tufts of dark green color, about 1-10 cm high. The cauloid is typically red or brown, with noticeable rhizoids at the bottom. The leaves (phyloids) are thin, round to broadly oblique, with a rounded toe. The species has oval- or egg-shaped leaves that are usually broader above the middle. When dry, the leaves are contorted and twisted. The edge of the entire petal is lined with a number of prosenchymatic cells. The rib ends at the tip, or is rarely a little truncated. In the Czech Republic, *R. punctatum* is a very common species growing in damp, shaded places. It grows on the ground, rocks, rotting wood, especially along the streams, from the lowlands to the mountains.

Rhytidiadelphus squarrosus

R. squarrosus is a strong, green to yellowish-green moss species forming usually dense carpets. The shoots of this extremely common moss are about 10-15 cm long, sometimes branched, and distinctive in the way the tapered part of the 2-2.5 mm long leaves bend back at a right angle to the base so that they spread out and away from the stem in all directions, giving shoots a star-like appearance. The stem leaves are straight, ovate to broadly ovate, and tapered in the long, narrow to truncated tip, thinly edged. The broad leaf base completely sheathes the red stem. The leaf has lightly toothed margins and a short double nerve. *R. squarrosus* is a ubiquitous species growing in open and shady habitats. It is common in unimproved or semi-improved grasslands. It tolerates a wide variety of soil conditions, from calcareous grassland to acid heaths.

Palustriella commutata

Shoots of *P. commutata* are typically green or yellowish-brown, 4-6 cm long or more, with a densely and regularly pinnate pattern of branching. This type of branching looks rather feather-like, but calcareous deposits on the shoots make them feel stiff and rough. The stem is covered by tiny leaf-like structures and red-brown rhizoids. The species has triangularly heart-shaped leaves that are 2-2.5 mm long, pleated, and have a very stout nerve that extends to the long, fine tip. *P. commutata* occurs in a variety of wet, base-rich habitats. The species is particularly characteristic of wet cliffs, springs and flushes. It often forms extensive patches, and may drape dripping, calcareous cliffs (Atherton et al. 2010).

Chlorophyll fluorescence in response to dehydration

Measurements of chlorophyll fluorescence parameters were done in samples desiccating from fully hydrated to fully dry state in laboratory. During the desicca-tion, room temperature was kept constant (23°C, 40% RH) and bryophyte thalli were left in open Petri dishes to desiccate natu-rally. Before the experiments, and repeat-edly during the sample desiccation, rela-tive water content (RWC) was evaluated using a gravimetric method. Samples were weighted on an analytical Mettler scale (Mettler AS100, Germany), and RWC cal-culated according to the equation 1:

$$RWC\ (\%) = [(F_M - D_M)/(F_W - D_M)] * 100$$

where, F_M is the actual fresh mass (weight) of a sample during the measurements, D_M is the mass of fully dry sample, and F_W is the mass of fully wet sample. From fully wet (RWC = 100%) to dry (RWC = 0-10%) state of the moss/liverwort thalli, chlorophyll fluorescence parameters were measured repeatedly. They comprised (1) effective quantum yield (Φ_{PSII}) of photo-system II, (2) non-photochemical quench-ing (qN), and (3) steady state chlorophyll fluorescence F_S (for parameters definition and equations *see* Roháček et Barták 1999).

For chlorophyll fluorescence measure-ments, a PAM-2000 fluorometer (H. Walz, Germany) with a custom-programmed measuring routine was used. Saturation pulses were applied on the samples at light-acclimated state (exposed to 20 μmol m^{-2} s^{-1} of PAR) at 10 min. intervals. Since the chlorophyll fluorescence measurements were taken simultaneously with RWC eval-uation, the dehydration-response curves of

Φ_{PSII}, qN, and F_S could be plotted (*see* Figs. 1 and 2) and analyzed. Species-specific responses in the dehydration-induced decline in photosynthetic parameters were pointed out and RWCs at which the functional changes happened were evaluated.

Results and Discussion

The chlorophyll fluorescence data recorded during dehydration from fully wet (RWC = 100%) to dry state (RWC = 0%) revealed species-specific response of chlorophyll fluorescence level measured at steady state (F_S), effective quantum yield of photosystem II (Φ_{PSII}), and non-photochemical quenching (qN).

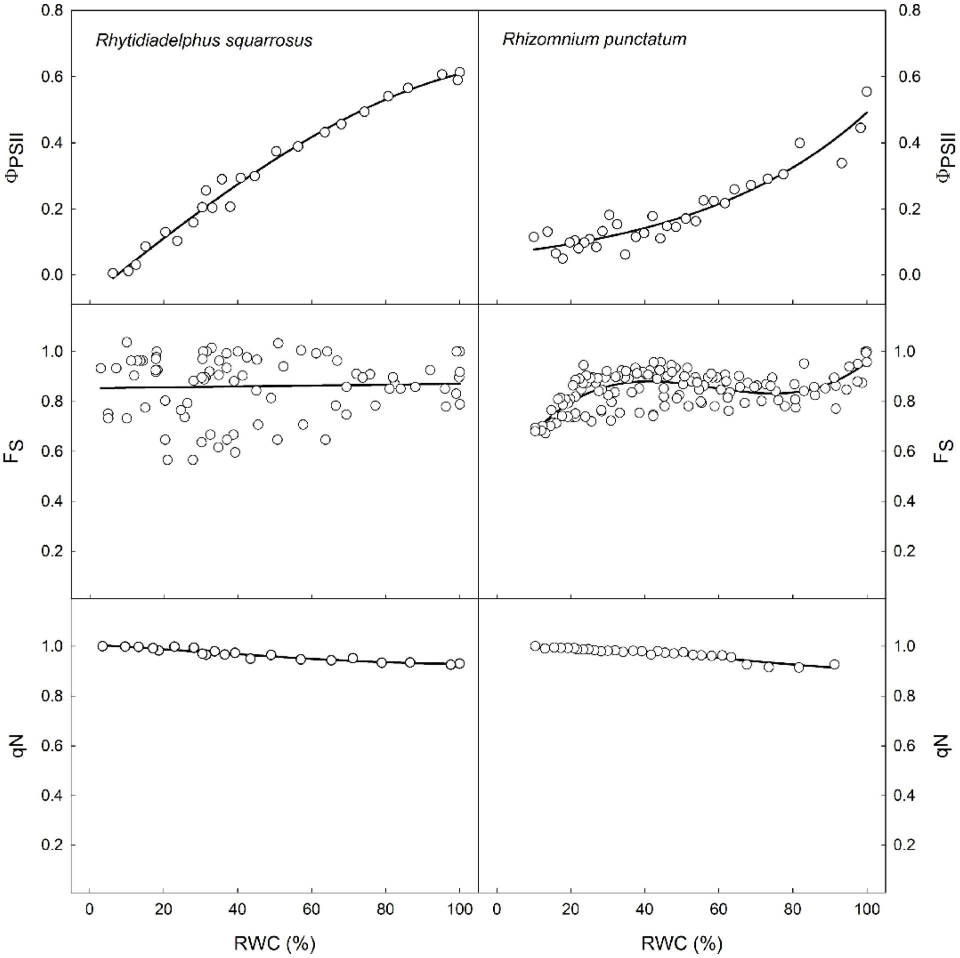


Fig. 1. Dehydration response curves of chlorophyll fluorescence parameters: effective quantum yield of photosynthetic processes in PS II (Φ_{PSII}), non-photochemical quenching (qN - normalized to maximum value), and steady-state chlorophyll fluorescence (F_S) in *Rhytidiadelphus squarrosus* (left) and *Rhizomnium punctatum* (right). *Key to the abbreviations*: RWC - relative water content.

Dehydration-response curves of Φ_{PSII} had, apart from *R. punctatum*, typical bi-phasic character with 2 main phases that differed between the studied species (Figs. 1, 2). At the RWC ranges decreasing from 100 to 80% (*R. squarrosus*, *P. commutata*), 100 to 70% (*P. endiviifolia*), and 100 to 60% (*M. polymorpha*), Φ_{PSII} decreased in a slow rate. With gradual dehydration from RWC of 60/70/80 to 0%, a rapid decline appeared in the four species following an S-curve. At RWC of 5% (*P. endiviifolia*, *R. squarrosus, M. polymorpha*) and 15% (*P. commutata*), critical point for photosyn-thetic processes (Φ_{PSII} = 0) was found. In *P. endiviifolia*, the second phases of the response curves were similar in shape to those reported by Nabe et al. (2007) with a typical S-curve at low RWCs.

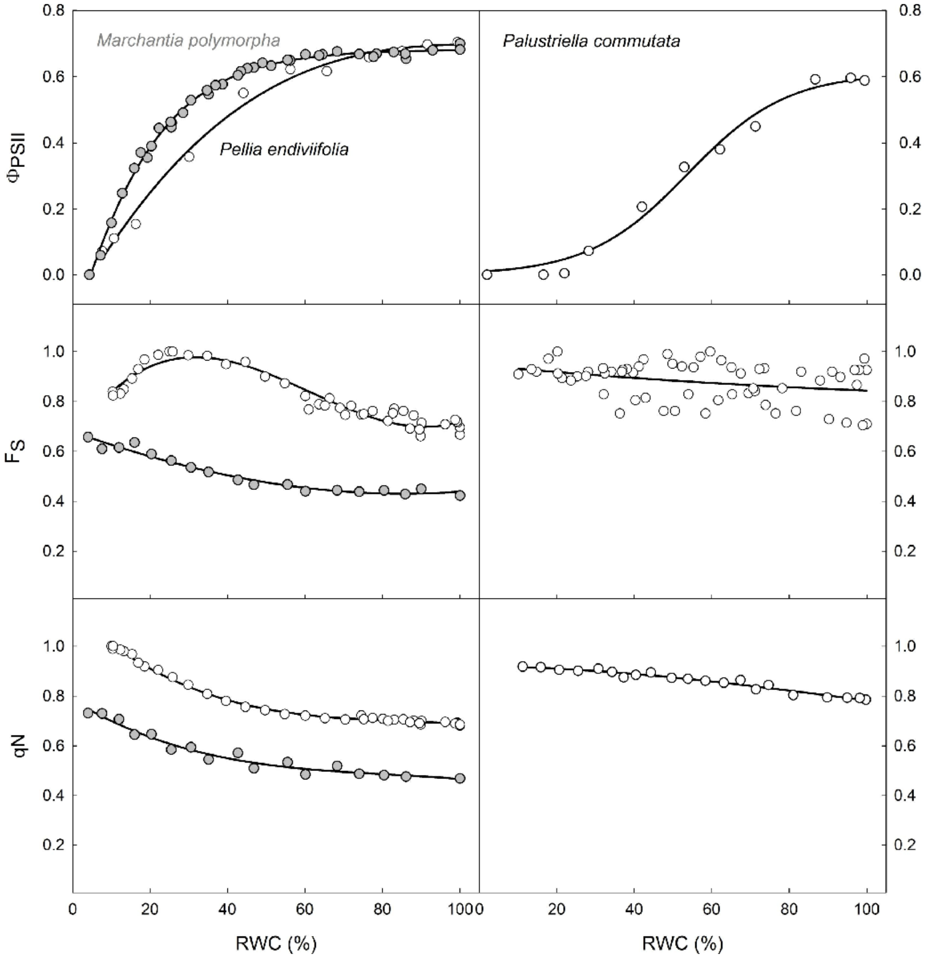


Fig. 2. Dehydration response curves of chlorophyll fluorescence parameters: effective quantum yield of photosynthetic processes in PS II (Φ_{PSII}), non-photochemical quenching (qN - normalized to maximum value), and steady-state chlorophyll fluorescence (F_S - normalized to maximum value) in *Pellia endiviifolia* and *Marchantia polymorpha* (left) and *Palustriella commutata* (right). *Key to the abbreviations*: RWC - relative water content.

Among the studied species, *R. punctatum* exhibited different desiccation-response curve: Φ_{PSII} declined in an exponential manner showing the fastest decline within 70-100% RWC and 0 point at 8.2 RWC.

Half of Φ_{PSII} was reached at high level of RWC by *R. punctatum* (65%), medium RWC by *P. communtata* and *R. squarro-sus* (53 and 43%), low RWC by *M. poly-morpha* (18%). These data confirm that tolerance to desiccation is high or medium for the five species except *R. punctatum*. The data show that primary photosynthetic processes in photosystem II are still active even at severe desiccation, which may have consequences for the species functioning under adverse environment conditions (*e.g.* high water demand). Being the four species able to cope with severe dehydration at the level of primary photosynthetic processes, further investigations should clarify the particular co-action of high light and tem-perature during desiccation.

Our experimental species proved, except *R. punctatum*, to be desiccation tolerant or semitolerant, since the majority of down regulation of primary photochemical processes, *i.e.* rapid decline of Φ_{PSII} and in-crease in qN were found at the RWC be-low 70%. This is comparable to a large number of moss species, however, some mosses are reported to be desiccation in-tolerant (*e.g. Sphagnum* - Marchall et Bor-bely 2011). In our study, *P. endiviifolia* and *M. polymorpha* were found tolerant, because the rapid decline in Φ_{PSII} appeared at about 50-60% RWC. These findings do not agree with Nabe et al. (2007) who ranks

M. polymorpha among desiccation sensitive bryophytes. On the other hand Hatanaka et Sugawara (2010) report high desiccation tolerance of *M. polymorpha* suspension-cell culture which is consistent with our data (*see* Fig. 2). In tolerant lichens, the RWC in which the decline is apparent, is even lower, *i.e.* 30-40% (Barták et al. 2018). Since all the studied species showed slow but permanent increase in qN with the RWCs decline from 100 to 0%, it might be suggested that the protective mechanisms involved in non-photochem-ical quenching were activated throughout desiccation. For mosses and liverworts, the functional background of desiccation-induced quenching is still not well under-stood. The desiccation-tolerant bryophytes are characterized by the fact that they do not suffer from photooxidative damage due to the coexistence of zeaxantin-de-pendent and dehydration-induced thermal energy dissipation (Marschall et al. 2018). Quenching due to the interconversion of xanthophyll cycle pigments was reported in bryophytes during desiccation under light conditions (Deltoro et al. 1998b, Nabe et al. 2007). Additionally, Heber et Shuva-lov (2005) suggested that reaction centre of PS II (P680) might be converted into Chl_{720} which is effective in quenching. More recently, two mechanism were pro-posed by Heber (2012). One of them faci-litates energy dissipation in the antenna of photosystem II which is faster than ener-gy capture by functional reaction centres. When this mechanism is insufficient for full photoprotection, the other one permits energy dissipation in the reaction centres themselves. The latter mechanism is based on the involvement of an efficient spill-over, *i.e.* energy transfer from PS II to PS I as suggested by (Slavov et al. 2013). The phenomenon is named desiccation-induced quenching and was described as a property of photosystem II reaction centres in *R. squarrosus* (Heber et al. 2006). The au-thors found that during desiccation, quench-ers accumulate and are stable in the ab-sence of water but revert to non-quenching molecular species on hydration. Together with zeaxanthin-dependent energy dissipa-tion, desiccation-induced thermal energy dissipation protects desiccated poikilohy-dric mosses against photo-oxidation, ensur-ing survival during drought periods. This mechanism requires light but not forma-tion of transthylakoidal delta pH (proton) gradient. The follow-up study done on the same species (Yamakawa et al. 2018) re-

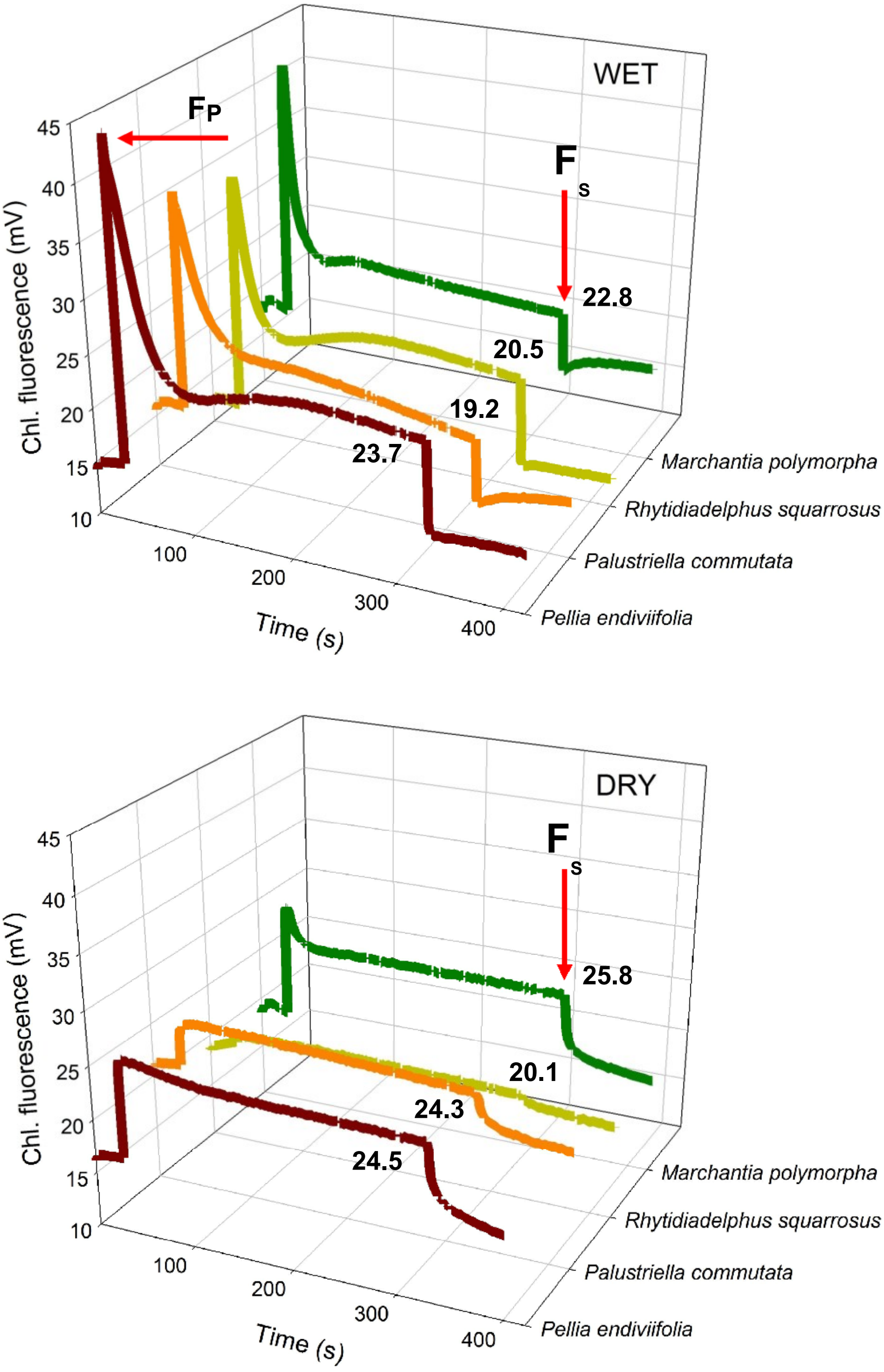


Fig. 3. Record of slow Kautsky kinetics for selected experimental species in wet (90% RWC) and dry state (10% RWC). Note that, irrespective of species, F_S are almost the same value in wet and dry state. Desiccation leads to diminishing of P peak (indicated by an arrow).

vealed that the quenching did not fully accelerated the PS II decay. The authors tentatively denoted this as type-B quenching. The desiccation-induced quenching in mosses is in strong contrast to the light-induced non-photochemical quenching as seen in higher plants affected by photoinhibitory light doses (Bilger 2014).

Complex character of protection of photosynthetic apparatus in desiccating mosses is documented by Yamakawa et al. (2012) in desiccation-tolerant moss *Rhytidium rugosum*. The authors report three different types of non-photochemical de-excitation of absorbed light energy and conclude that the slow drying moss (under light) has a better photo-protection than the same one slowly drying in darkness. Antioxidative substrates and enzymes might be involved into quenching as well since they represent effective agents acting against the ROS formed during desiccation. Paciolla et Tommasi (2003) studied ascorbate contents in wet and dry *Brachytheciastrum veluti-num* (syn. *Brachythecium velutinum*) and *Marchantia polymorpha*. They report more efficient ascorbate recycling in the moss than in the liverwort, and suggest higher desiccation tolerance in the moss than the liverwort. Recently, an abcisic acid–independent cold signalling pathway was found in *Marchantia polymorpha* that leads to specific gene expression that is related to freezing and desiccation tolerance (Minami et al. 2005). However, the extent of involvement of such mechanism during desiccation and the co-action with photosynthetic performance remain unclear.

Steady-state chlorophyll fluorescence (Fs) did not show rapid decline with progressive desiccation. The response of Fs was species-specific: It slightly decreased (*R. punctatum*), stayed more or less constant (*R. squarrosus*), slightly increased (*P. commutata*), and showed an increase followed by a decrease (*P. endiviifolia*). Similarly, Bartošková et al. (1999) reported almost constant Fp chlorophyll fluorescence signal in desiccating in *R. punctatum.* Generally, the responses were different from lichens that show a rapid Fs decline with RWC decrease from 100 to 0%. The response of Fp, however, seems to be species-specific. Tuba et al. (1996) reported big differences in Fp during desiccation in *Tortula ruralis*. Therefore, in follow-up studies, attention will be payed to the changes in Fp and Fs in moss species during desiccation. Mosses and liverwort exhibited more or less constant Fs within a wide range of RWC as documented from slow Kautsky kinetics (*see* values in wet and dry state, Fig. 3).

Concluding remarks

The research reported in this study was carried out at the Jeseníky mountains (Czech Republic) and in the Valle delle Ferriere (Italy). The Jeseníky Mts. are under strict natural protection and comprise preserved nature of the highest peaks having the character of the northern tundra similar to that found in the Alps and Polar regions. The natural treeless landscape above the upper border of the forest at an altitude of 1 350 m has a bare and rocky Alpine character. The other location, the Valle delle Ferriere, is a unique landscape of high natural biodiversity and historical value; the valley is included in many protected areas lists: Nature reserve 1972 (DM 427 29/03/1972), part of the Regional Park Monti Lattari (LR 33 01/09/1993), SPA (Sorgenti del Vallone delle Ferriere di Amalfi) IT8050045, SCI (dorsale dei Monti Lattari) IT8030008, UNESCO site Costiera Amalfitana IT 830 inscription 1997.

References

ATHERTON, I., BOSANQUET, S. and LAWLEY, M. (Eds.) (2010): Mosses and liverworts of Britain and Ireland – a field guide. British Bryological Society, Plymouth, 848 p.

BARTÁK, M., HAZDROVÁ, J., SKÁCELOVÁ, K. and HÁJEK, J. (2016): Dehydration-induced responses of primary photosynthetic processes and spectral reflectance indices in Antarctic *Nostoc commune*. *Czech Polar Reports*, 6: 87-95.

BARTÁK, M., HÁJEK, J., MORKUSOVÁ, J., SKÁCELOVÁ, K. and KOŠUTHOVÁ, A. (2018): Dehydration-induced changes in spectral reflectance indices and chlorophyll fluorescence of Antarctic lichens with different thallus color, and intrathalline photobiont. *Acta Physiologiae Plantarum*, 40: 177-187.

BARTOŠKOVÁ, H., KOMENDA, J. and NAUŠ, J. (1999): Functional changes of photosystem II in the moss *Rhizomnium punctatum* (Hedw.) induced by different rates of dark desiccation. *Journal of Plant Physiology*, 154: 597-604.

BILGER, W. (2014): Desiccation-Induced Quenching of Chlorophyll Fluorescence in Cryptogams. *In*: B. Demmig-Adams, G. Garab, W.W.Adams III, and Govindjee (Eds.): Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria. Springer Dordrecht, New York, Tokyo, Heidelberg, New York, pp. 409-420.

CSINTALAN, Z., PROCTOR, M. C. F. and TUBA, Z. (1999): Chlorophyll fluorescence during drying and rehydration in the mosses *Rhytidiadelphus loreus* (Hedw.) Warnst., *Anomodon viticulosus* (Hedw.) Hook. and Tayl. and *Grimmia pulvinata* (Hedw.) Sm. *Annals of Botany*, 84: 235-244.

DELTORO, V. I., CALATAYUD, A., GIMENO, C. and BARRENO, E. (1998a): Water relations, chlorophyll fluorescence, and membrane permeability during desiccation in bryophytes from xeric, mesic, and hydric environments. *Canadian Journal of Botany*, 76: 1923-1929.

DELTORO, V. I., CALATAYUD, A., GIMENO, C., ABADÍA, A. and Barreno, E. (1998b): Changes in chlorophyll a fluorescence, photosynthetic CO_2 assimilation and xanthophyll cycle interconversions during dehydration in desiccation-tolerant and intolerant liverworts. *Planta*, 207: 224-228.

DOBROVOLNÝ, P., ŘEZNÍČKOVÁ, L., BRÁZDIL, R., KRAHULA, L., ZAHRADNÍČEK, P., HRADIL, M., DOLEŽELOVÁ, M., ŠÁLEK, M., ŠTĚPÁNEK, P., ROŽNOVSKÝ, J., VALÁŠEK, H., KIRCHNER, K. and KOLEJKA, J. (2012): Klima Brna. Víceúrovňová analýza městského klimatu. 1. vyd. Brno: Masarykova univerzita, 200 p. ISBN 978-80-210-6029-6. (In Czech).

DULAI, S., CSIZI, K., SASS-GYARMATI, A., ORBÁN, S. and MOLNÁR, I. (2004): Combined effects of Thylakoid Energisation Level and Water Deficit on Thermal Stability of Photosystem II in a Dessication Tolerant Moss. *Acta Academiae Paedagogicae Agriensis*, Sectio Biologiae, XXV: 127-126.

GREENWOOD, G. L. (2017): Factors influencing induction of desiccation tolerance in bryophytes: redefining fundamental aspects of the organism's relationship with the environment in xeric habitats. Ph.D. thesis, University of Nevada, Las Vegas, 118 p.

HÁJEK, T., BECKETT, R. P. (2008): Effect of water content components on desiccation and recovery in *Sphagnum* mosses. *Annals of Botany*, 101: 165-173.

HATANAKA, R., SUGAWARA, Y. (2010): Development of desiccation tolerance and vitrification by preculture treatment in suspension-cultured cells of the liverwort *Marchantia polymorpha*. *Planta*, 231: 965-976.

HEBER, U., BUKHOV, N. G., SHUVALOV, V. A., KOBAYASHI, Y. and LANGE, O. L. (2001): Protection of the photosynthetic apparatus against damage by excessive illumination in homoiohydric leaves and poikilohydric mosses and lichens. *Journal of Experimental Botany*, 52: 1999-2006.

HEBER, U., SHUVALOV, V. A. (2005): Photochemical reactions of chlorophyll in dehydrated photosystem II: two chlorophyll forms (680 and 700 nm). *Photosynthesis Research*, 84: 85-91.

HEBER, U., BILGER, W. and SHUVALOV, V. A. (2006): Thermal energy dissipation in reaction centres and in the antenna of photosystem II protects desiccated poikilohydric mosses against photo-oxidation. *Journal of Experimental Botany*, 57: 2993-3006.

HEBER, U. (2012): Conservation and dissipation of light energy in desiccation-tolerant photoautotrophs, two sides of the same coin. *Photosynthesis Research*, 113: 5-13.

HILL, M. O., BELL, N., BRUGGEMAN-NANNENGA, M. A., BRUGUÉS, M., CANO, M. J., ENROTH, J., FLATBERG, K. I., FRAHM, J.-P., GALLEGO, M. T., GARILLETI, R., GUERRA, J., HEDENÄS, L., HOLYOAK, D. T., HYVÖNEN, J., IGNATOV, M. S., LARA, F., MAZIMPAKA, V., MUÑOZ, J. and GUERRA, J. (2006): An annotated checklist of the mosses of Europe and Macaronesia. *Journal of Bryology*, 28: 198-267.

HU, R., XIAO, L., BAO, F., LI, X. and HE, Y. (2016): Dehydration-responsive features of *Atrichum undulatum*. *Journal of Plant Research*, 129: 945-954. doi: 10.1007/s10265-016-0836-x.

LEDNICKÝ, V. (1985): Climate the Praděd Mt. Šumperk, Severní Morava, 49: 44-48. (In Czech).

MARSCHALL, M., BORBÉLY, P. (2011): Photosynthetic responses of the desiccation intolerant *Sphagnum angustifolium* in relation to increasing its desiccation tolerance by exogenous ABA. *Acta Biologica Szegediensis*, 55: 119-121. *Available*: https://www2.sci.u-szeged.hu/ABS/2011/Acta%20HP/55119.pdf.

MARSCHALL, M., BORBÉLY, P., PNÉ-KÓNYA, E. and SÜTO, S. (2018): Background processes and the components of photoprotection and regeneration under rehydration in desiccation-tolerant and desiccation-sensitive bryophytes. *In*: Abstract Book for the Plant Biology Europe Conference in Copenhagen, 80 p. (ISBN 978-87-996274-1-7).

MAYABA, N., BECKETT, R. P., CSINTALAN, Z. and TUBA, Z. (2001): ABA increases the desiccation tolerance of photosynthesis in the afromontane understorey moss *Atrichum androgynum*. *Annals of Botany*, 88: 1093-1100.

MINAMI, A., NAGAO, M., IKEGAMI, K., KOSHIBA, T., ARAKAWA, K., FUJIKAWA, S. and TAKEZAWA, D. (2005): Cold acclimation in bryophytes: Low-temperature-induced freezing tolerance in *Physcomitrella patens* is associated with increases in expression levels of stress-related genes but not with increase in level of endogenous abscisic acid. *Planta*, 220: 414-423

NABE, H., FUNABIKI, R., KASHINO, Y., KOIKE, H. and SATOH, K. (2007): Responses to desiccation stress in bryophytes and an important role of dithiothreitol-insensitive non-photochemical quenching against photoinhibition in dehydrated states. *Plant and Cell Physiology*, 48: 1548-1557.

PACIOLLA, M., TOMMASI, F. (2003): The ascorbate system in two bryophytes: *Brachythecium velutinum* and *Marchantia polymorpha*. *Biologia Plantarum*, 47: 387-393.

PRESSEL, S., LIGRONE, R. and DUCKETT, J. G. (2006): The effects of de- and rehydration on food-conducting cells in the moss *Polytrichum formosum* Hedw: A cytological study. *Annals of Botany*, 98: 67-76.

PROCTOR, M. C. F., SMIRNOFF, N. (2000): Rapid recovery of photosystems on rewetting desiccation - tolerant mosses: chlorophyll fluorescence and inhibitor experiments. *Journal of Experimental Botany*, 51: 1695-1704. *Available*: https://doi.org/10.1093/jexbot/51.351.1695.

PROCTOR, M. C. F., OLIVER, M. J., WOOD, A. J., ALPERT, P., STARK, L. R., CLEAVITT, N. L. and MISHLER, B. D. (2007): Desiccation-tolerance in bryophytes: a review. *The Bryologist*, 110: 595-622.

ROHÁČEK, K., BARTÁK, M. (1999): Technique of the modulated chlorophyll fluorescence: Basic concepts, useful parameters, and some applications. *Photosynthetica*, 37(3): 339-363.

SLAVOV, CH., REUS, M. and HOLZWARTH, A. R. (2013): Two different mechanisms cooperate in the desiccation-induced excited state quenching in *Parmelia* lichen. *The Journal of Physical Chemistry B*, 117 (38): 11326-11336.

STOKLASA-WOJTASZ, A., RZEPKA, A. and RIT, G. (2012): Responses of mosses species on environment stress factors. *In*: M. T. Grzesiak, A. Rzepka, T. Hura, S. Grzesiak (Eds.): Plant functioning under environmental stress. The F. Górski Institute of Plant Physiology, Polish Academy of Science, Cracow, Poland, pp. 69-83.

TUBA, Z., CSINTALAN, Z. and PROCTOR, M. C. F. (1996): Photosynthetic responses of a moss, *Tortula ruralis* ssp. ruralis, and the lichens *Cladonia convoluta* and *C. furcata* to water deficit and short periods of desiccation, and their ecophysiological significance: A baseline study at present-day concentration. *New Phytologist*, 133: 353-361.

WISE, M.J., TUNNACLIFFE. A. (2004): POPP the question: What do LEA proteins do? *Trends in Plant Science,* 9: 13-17.

Yamakawa, H., Fukushima, Y., Itoh, S. and Heber, U. (2012): Three different mechanisms of energy dissipation of a desiccation-tolerant moss serve one common purpose: to protect reaction centres against photo-oxidation. *Journal of Experimental Botany*, 63: 3765-3775.

Yamakawa, H., van Stokkum, I. H. M., Heber, U. and Itoh, S. (2018): Mechanisms of drought-induced dissipation of excitation energy in sun- and shade-adapted drought-tolerant mosses studied by fluorescence yield change and global and target analysis of fluorescence decay kinetics. *Photosynthesis Research*, 135: 285-298.

Chapter V

5. DAILY LIGHT COURSE OF PHOTOSYNTHESIS: ETR MEASUREMENTS IN GREENHOUSE CULTIVATION

Acclimation of primary photosynthetic processes to light in three fern species from a Mediterranean moist habitat, Ferriere Valley, Italy

Submitted to Acta Physiologiae Plantarum 30th January 2020

Gabriella Nora Maria Giudici [1*], Miloš Barták [2], Josef Hájek [2*]

[1] University of Naples Federico II, Department of Agriculture, 80055 Portici, Italy

[2] Department of Experimental Biology, Division of Plant Physiology and Anatomy, Faculty of Science, Masaryk University, Laboratory of Photosynthetic Processes, Kamenice 5, 625 00 Brno, Czech Republic

5.1. ABSTRACT

Adjustment of photosynthetic processes to driving environmental factors, light in particular, is of key importance for fern growth and successful reproduction. Ferns are generally shade tolerant/adapted plants with a great plasticity of photosynthesis in low light. In our study, we focused on acclimation of primary photosynthetic processes to low light during controlled greenhouse experiment. Photosynthetic performance was monitored by automatic chlorophyll fluorescence system in three ferns (*Adiantum capillus-veneris*, *Polypodium vulgare*, and *Woodwardia radicans*) during 19-day long cultivation. From gained data, daily courses of photosynthetic electron transport rate (ETR), rapid light response curves (RLC) of ETR and initial slope RLC (α) were evaluated. Additionally, sensitivity of the three species to a short-term high light treatment was studied by fast chlorophyll fluorescence transient (OJIP). Analysis of RLC revealed that, in spite of same photosynthetic performance before experiment, ETR decrease after 19-day cultivation was much more pronounced in *A. capillus-veneris* than in *P.vulgare*, and *W. radicans.* This suggest better acclimation to low light in *P. vulgare*, and *W. radicans*. Short-term photo-inhibitory treatment showed that *A. capillus-veneris* was more susceptible to photoinhibition than the other two species because of more pronounced high light-induced decrease in F_V/F_M and photosynthetic electron transport rate (ET_0/RC). Moreover, *A. capillus-veneris* showed uncompleted recovery after 60 min in dark. Analysis of OJIP-derived parameters indicated those responding to photo-inhibitory treatment sensitively: performance index PI_{ABS}/RC, absorption ABS/RC, effectivity (probability) for electron transport Phi_E_0, and thermal dissipation DI_0/RC). Those could be used in follow up photoinhibition studies focused on primary photosynthetic processes in ferns.

* - equal corresponding authors

Key words

Adiantum capillus-veneris, *Polypodium vulgare*, *Woodwardia radicans*, chlorophyll fluorescence, photoinhibition, rapid light curves

Acknowledgements

The authors thank the projects CzechPolar-II (LM2015078) and the from ECOPOLARIS project (CZ.02.1.01/0.0/0.0/16_013/0001708) for providing Brno-based facilities and the infrastructure for the research reported in this study. The authors thank also to Carabinieri per la biodiversità, UTB Caserta, for field support in collecting samples from *Woodwardia radicans* restricted area.

Author contribution statement

GG and MB designed research, GG and JH conducted experiments. GG and JH analysed data, GG and MB wrote the manuscript with review and substantial editing of JH. All authors read and approved the manuscript.

5.2. INTRODUCTION

In terrestrial ferns, photosynthetic performance is driven mainly by photosynthetically active radiation (PAR) available at the site, however, high temperature and low air humidity may limit photosynthesis because of stomatal closure. It is well established that in brightly lit sites with high heat loads and/or vapour pressure deficits, increased rates of photosynthetic carbon fixation due to increased light must be balanced with greater water loss and low leaf water potentials (Sessa and Givnish 2014), which can lead to leaf desiccation. Pioneering study of Hill (1972) made in three fern species from different genera (*Adiantum*, *Thelypteris* and *Woodwardia*) growing in different light regimes, reports that species from sunnier habitats had higher maximum photosynthetic rates, light compensation points and light saturation points than those from a shadier habitat. In general, maximum photosynthetic rate (Pn_{max}) in ferns is typically below 10 μmol m^{-2} s^{-1}, as shown e.g. for *Adiantum pedatum* (Nishida and Hanba 2017), *Dryopteris* sp. e.g. Bauer et al. (1991), Pittermann et al. (2011), Sessa and Givnish (2014). These studies, however, were based on gas exchange measurements exclusively, i.e. not taking any benefit from simultaneous CO_2 exchange and chlorophyll fluorescence measurements.

The studies exploiting chlorophyll fluorescence approach to evaluate photosynthetic processes in ferns are rather scarse (e.g. Gago et al. 2013, Tosens et al. 2016) and related rather to a narrow and/or specific part of photochemical processes of photosynthesis in majority of cases. Cyclic electron transport around PSII and PSI, as dependent on PAR, was evaluated for *Polypodium* sp. by (Ver Sagun et al. 2019). In this study, cyclic electron transport around PS I (expressed relatively to photosynthetic linear transport) was found high when compared to C3 and C4 grasses, *Zea mays*, and tree species. Sensitivity of ferns to photoinhibition of primary photochemical processes was assessed by Hietz and Briones (2001) as a light-induced increase in the background chlorophyll fluorescence (F_0). Information on light-response curves of effective quantum yield of photosystem II (Φ_{PSII}) and/or photosynthetic electron transport rate (ETR) are almost missing in ferns. The two chlorophyll fluorescence parameters, as dependent on light, were studied only scarcely. Minardi et al. (2014) measured ETR light response curves in order to quantify water deficit effect on photosynthetic processes. The authors report the photosynthetic active radiation (PAR) above 800 μmol m^{-2} s^{-1} to be inhibiting primary photosynthesis.

Acclimation studies on photosynthetic processes, in ferns adjusting to high/low light environment exploiting chlorophyll fluorescence methods are almost missing. Adjustment of chlorophyll fluorescence parameters to prevailing light environments of epiphytic and terrestrial ferns was studied by (Chen et al. 2019). The study focused on light-response curves based on gas-exchange method supplemented by the measurements of capacity of photochemical processes of photosynthesis in PSII (F_V/F_M). The authors, however, did not measure ⍰$_{PSII}$ and ETR. To fill the gap, we decided to study interspecific differences in photosynthetic processes in three Mediterranean species using the approach of long-term monitoring of ⍰$_{PSII}$ and ETR. Together with the measurements of environmental characteristics, such as photosynthetically active radiation (PAR) and leaf temperature (T) the approach leads to the evaluation of daily courses of ETR as dependent on PAR and/or T. Such method has been applied many times in vascular plants. The recent application of field ETR monitoring ranges from conifers (Porcar-Castel et al. 2011) to broad leaf woody species (Liu et al. 2019), tundra perennials (Barták et al. 2012), aquatic vascular plants (Ritchie et al. 2012), macroalgae Figueroa et al. (2014), diatoms (Torres et al. 2013).

Monitoring of *in situ* chlorophyll fluorescence during acclimation period and/or growing season, together with ETR data analysis represents one of the effective tools to study species-specific differences in photosynthetic processes driven by environmental factors. Supplemented by the measurements of environmental characteristics, such as PAR and leaf temperature, the approach leads to the evaluation of daily/seasonal courses of ETR as dependent on PAR and/or T. Such approach is used in the evaluation of seasonal acclimatory changes in greenhouse experiments (e.g. Ulqodry et al. 2014) as well. To evaluate inter-specific differences in ETR, we designed a greenhouse-based, long-term experimental monitoring of ⍰$_{PSII}$ and ETR in three fern-species (*Adiantum capillus-veneris*, *Polypodium vulgare*, and *Woodwardia radicans*). We hypothesized, that acclimatory changes in ETR, reflecting species adjustment to environmental factors, light availability in particular, will be species-specific. We also hypothesized that acclimation time will differ between the species according to their shade tolerance. Since the fern species, because of sunflecks, are exposed to short-term high light in the field, we expected species-specific differences in the sensitivity of particular species to high light as well. Therefore, sensitivity of the three fern-species to a short-term photo-inhibitory treatment was studied by the analysis of fast chlorophyll fluorescence transient (OJIP). The OJIP approach was used in ferns to monitor ozone-induced changes in primary processes of photosynthesis (Oliwa et al. 2019). Therefore, we hypothesized that simlarly to other stressors, such as high temperature, drought, salinity, and nutrient defficiency (for review see Kalaji et al. 2017), short-term high light stress would lead to species-specific differences in OJIPs and OJIP-derived chlorophyll fluorescence parameters.

5.3. MATERIAL AND METHODS

5.3.1. Plant material

Woodwardia radicans was taken from the Naples Botanical garden. It was agamically propagated from an apical leaf bud, typical of the species, more than 18 months before the start of the experiment. The potted plant has been sitting in the greenhouse for at least six weeks before the experiment, along with the other two. *Adiantum capillus-veneris* and *Polypodium vulgare* were both coming from the Ferriere Valley (Amalfi coast – Italy). *A. capillus-veneris* was taken just outside the highly restricted nature conservation area of *W. radicans*, under the tree canopy, while *P. vulgare* was collected outside the conservation zone along one of the paths, in a sunny location without any tree cover. The choice of the plant material was determined by the natural gradient of light which the plants were exposed to in the field.

During cultivation in greenhouse, the potted plants shared the same controlled environmental conditions and were watered in the same way. Mean air temperature was maintained 20 ±2.7 ^{o}C. Daily maxima and night minima ranged within the intervals of 22.1-26.4 ^{o}C and 15.2-15.8 ^{o}C, respectively. Air humidity was kept high (mean of 79 %) and the light was following the natural photoperiod of the season. The only differences were determined by the geometry of the plants themselves and by self-shadowing that was changing for *W. radicans* due to the high growing rate of new leaves. *Adiantus* as well was observed to change its canopy.

5.3.2. Ferriere valley

The Ferriere Valley (Amalfi coast – Italy) is an important hotspot of biodiversity. It is home to *Woodwardia radicans* (an enlisted and protected fern) and a glacial refugium for other paleotropical relicts (*Cyathodium Kunze* in Duckett & Ligrone

2006). It is a deep, V-shaped forested valley, rich in water and sheltered from pronounced and sudden climatic variations, with limestone rocky peaks and cliffs.

5.3.3. Short-term experiment

Light response curves of apparent electron transport rate (ETR) were measured on the three experimental fern species in untreated control and after photo-inhibitory treatment (1500 µmol (photons) m^{-2} s^{-1} for 120 min of recovery). For the purpose, a PAM-2500 fluorometer (H. Walz, Germany) was used. The method consisted in the measurements of rapid light curves (RLC - White and Critchley 1999), i.e. measurements of Φ_{PSII} in samples exposed to increasing intensities of actinic light (from 0 to 1500 µmol m^{-2} s^{-1} of PAR). The time interval of each PAR intensity level was 20 s. At the end of each PAR interval, the leaves were exposed to saturation pulse in order to evaluate the effective quantum yield of photosystem II (Φ_{PSII}). Then, photosynthetic electron transport rate was calculated according to the equation: ETR = Φ_{PSII} x PAR x a, where a is absorption divided by 2. This is because of the presumption that photosystem I, II equally receive light energy, i.e. the relative proportions of PSI and PSII light harvesting antennae are assumed to be 1 : 1 (Melis 1989). Numeric value of a was 0.42. Then, ETR data were plotted against PAR and α - Initial slope of RLC which is related to quantum efficiency of photosynthesis was calculated.

During the short-term experiment, OJIP curves were measured using a FluorPen fuorometer (Photon Systems Instruments, Czech Republic) before, immediately after photoinhibitory treatment, then after 60, 120 min recovery in dim light of 10 µmol m^{-2} s^{-1} PAR. Resulting curves were plotted against time and analyzed in terms of shape and the chlorophyll fluorescence values reached in O, J, I, P steps. From the OJIPs, chlorophyll fluorescence parameters. evaluating PSII performance, were calculated (see Table 1).

F_J ≡ F2ms	Fluorescence intensity at the J-step (2 ms) of OJIP

F_I $\equiv$ F30ms	Fluorescence intensity at the I-step (30 ms) of OJIP
F_0 $\cong$ F50µs or $\cong$ F20µs	Minimal fluorescence (all photosystem II reaction centers – PSII RCs are assumed to be open)
F_M	Maximal fluorescence, when all PSII RCs are closed
F_V $\equiv F_M - F_0$	Maximal variable fluorescence
F_V/F_M	Maximal quantum yield of PSII
F_V/F_0	Ratio of variable to minimal fluorescence
F_M/F_0	Ratio of maximal to minimal fluorescence
Mo	Normalized value of the initial slope of the curve
ABS/RC = $M_0(1 / V_J)(1 / \text{Phi\_P}_0)$	Absorption flux (of antenna chlorophylls) per RC
TR_0/RC = $M_0(1 / V_J)$	Trapped energy flux (leading to quinone A - Q_A reduction) per RC
ET_0/ RC = $M_0(1 / V_J)\text{Psi\_E}_0$	Electron transport flux (further than Q_A) per RC
V_J = $(F_J - F_0) / (F_M - F_0)$	Relative variable fluorescence at the J-step
V_I = $(F_I - F_0) / (F_M - F_0)$	Relative variable fluorescence at the I-step
Psi_0 = $1- V_J = ET_0 / TR_0$	Probability that a trapped exciton is used for electron transport beyond Q_A.
Phi_Pav = $\text{Phi\_P}_0 (S_M / t_{FM})$	Time to reach maximal chlorophyll fluorescence
DI_0 / RC = (ABS/RC) – (TR_0/RC)	The flux of dissipated excitation energy at time 0
Phi_D_0 = $1 - \text{Phi\_P}_0 = F_O/F_M$	Quantum yield (at t = 0) of energy dissipation
Phi_P_0 $\equiv TR_0 / ABS = [1-(F_0 / FM)]$	Maximum quantum yield for primary photochemistry
Psi_E_0 $\equiv ET_0 / TR_0 = (1-V_J)$	Efficiency (probability) for electron transport (ET), i.e. efficiency (probability) that an electron moves further than Q_A
Phi_E_0 $\equiv ET_0/ABS = [1-(F_0/F_M)]\psi E_0$	Quantum yield for electron transport (ET)
Pi_ABS = $(RC/ABS)[\text{Phi\_P}_0/(1-\text{Phi\_P}_0)]\ [\text{Psi\_0}/(1-\text{Psi\_0})]$	Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors

Table 1. Formulas and explanation of fast chlorophyll fluorescence (OJIP) derived parameters used in study (according to Strasser et al. 2004).

5.3.4. Long-term experiment

Long-term, continuous measurements of chlorophyll fluorescence of the three fern-species started on April 18th 2019 and ended on May 6th 2019. The ferns were placed into a greenhouse and exposed to natural variation of light and to controlled air humidity and temperature. A multichannel monitoring fluorometer Moni-PAM (Heinz Walz, Germany) was used to measure diurnal courses of effective quantum yield (Φ_{PSII}). The system consisted of control and data logging unit (MONI-DA), and three monitoring emitter-detector heads (probes). In our experiment, the probes were gently attached to the upper leaf surface using the metal frame of the holder (fig. 5.1). The probes with sample clips mounted at a distance of 25 mm from the probe´s end, formed an optical window arranged at an angle of 45° between the probe longitudinal axis and the sample. The optical window was a metal frame with a spot of white area (radiation sensor) measuring incident photosynthetically active irradiance. The optical window was placed directly on the leaf. The measuring spot over a leaf was selected carefully, so that the spot was representative of the leaf, naturally exposed to light.

Fig. 5.1 Photograph of the experimental set-up used in the greenhouse cultivation of three fern species.

Such set up allowed to use repetitive saturation pulse method. The pulses of photosynthetically active radiation (duration 1 s, intensity 3 500 µmol m^{-2} s^{-1} were applied on the spots in light-adapted state (natural irradiation inside the greenhouse following daily courses) each 10 min. In such a way, steady state chlorophyll fluorescence (F_S) and maximum chlorophyll fluorescence induced by a saturation pulse applied in light-adapted state (F_M') were measured and stored in the MONI-PAM memory. For each measurement, the monitoring fluorometer Moni-PAM calculated photosystem II-based photosynthetic electron transport rate (ETR) according to the equation: $ETR = 0.5 \times 0.84 \times PAR \times \Phi_{PSII}$ (Schreiber 2004, Baker 2008), where PAR is photosynthetically active radiation, and Φ_{PSII} is effective quantum yield of photosynthetic processes in PS II (Genty parameter, $F_M' - F / F_M'$ Genty et al. 1989). In automatic ETR calculation, absorptance (A) is assumed 0.84

(Baker 2008) which is valid for a vast majority of green plants. For vascular plants, however, the absorptance range of 0.551 - 0.902 is reported by Stemke and Santiago (2011). Recently, A is being studied across a wide variety of vascular plant species (e.g. Ritchie and Runcie 2014).

Vitality and overall photosynthetic performance of the three fern-species during the greenhouse experiment was assessed as a time course of potential quantum yield of photochemical processes in PSII (F_V/F_M). For F_V/F_M calculation, chlorophyll fluorescence data recorded after a saturation pulse during dark period (peak F_{Mdark}') of the day were used. For each day, 60 records of F_{Mdark}' (at PAR=0) were used and F_V/F_M calculated, using the equation of $(F_{Mdark}' - F_0) / F_{Mdark}'$. Mean F_V/F_M was an average value from the 60 measurements. Then, relation of daily mean F_V/F_M to the cultivation time was calculated.

To analyze the differences in primary photosynthetic processes between the 3 fern-species, Φ_{PSII} and ETR data were analysed by a modification of the diurnal regression method (Durako 2012) for each species. Light response curves (ETR to PAR) were plotted and the initial slope (⍰ parameter) of the relationship was calculated for the PAR interval 0-50 µmol m^{-2} s^{-1}. The alpha parameter (⍰) was considered a measure of the light harvesting effectivity of the chloroplast photosynthetic apparatus (Belshe et al. 2008). The change of the light-response curve of ETR with the time of cultivation in a greenhouse was evaluated by the comparison of the curves fitted for the data recorded on the 1st, 2nd and 19th day of cultivation. In the following text, day(s) are abbreviated as d1 to d19.

5.3.5. Statistical analysis

Univariate analysis of variance (ANOVA) and Duncan multiple comparisons test ($P < 0.05$) were employed (software STATISTICA) to determine significant differences between the species in the parameters measured.

5.4. RESULTS

5.4.1. Daily courses of ETR

The ETR of the three investigated ferns exhibited a strong diurnal pattern under experimental conditions, light variation in particular. ETR displayed differences between species as well (fig. 5.2. 5.3).

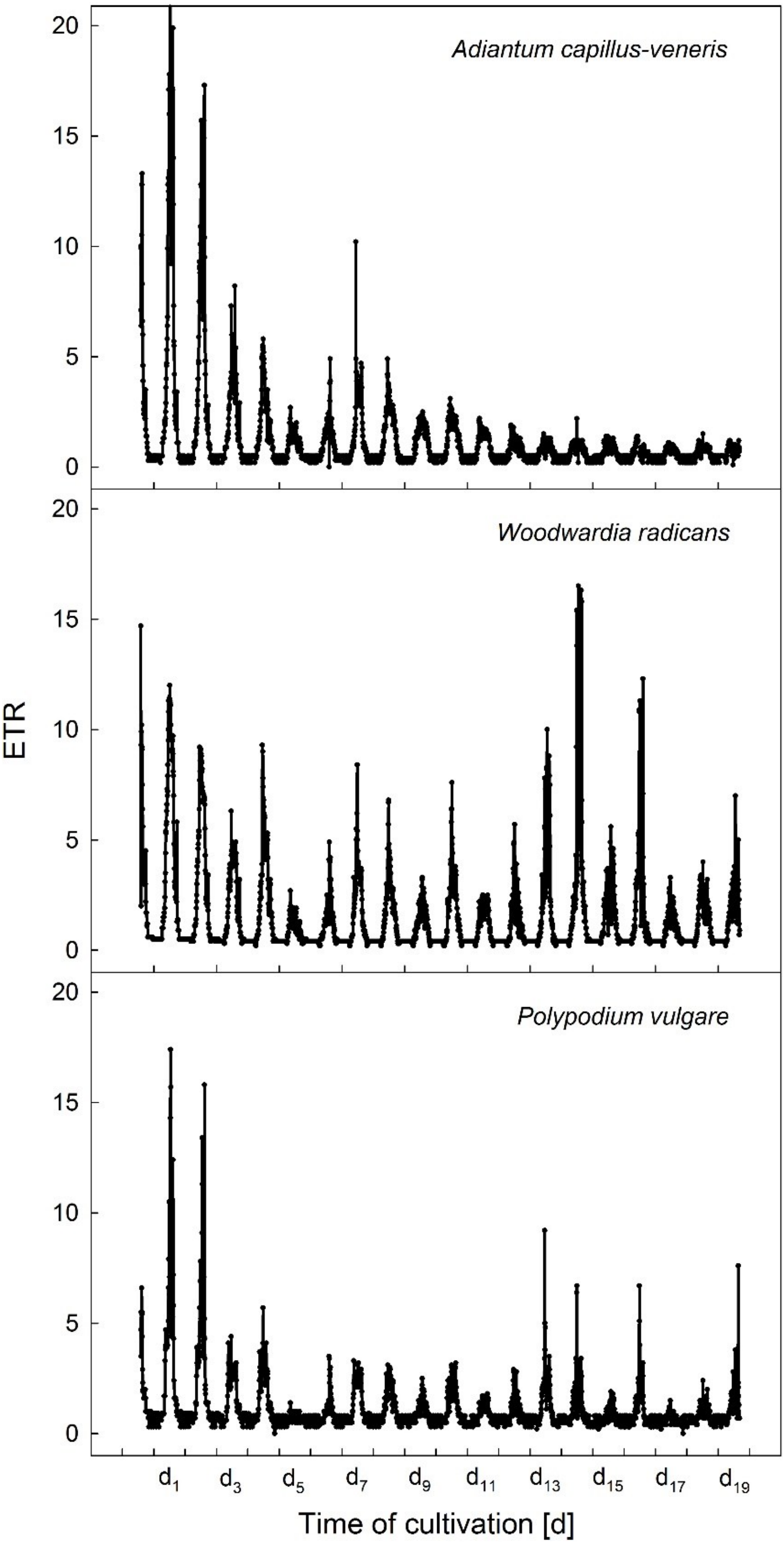


Fig. 5.2 Daily courses of photosynthetic electron transport rate (ETR) recorded for the three fern species: (1) *Adiantum capillus-veneris,* (2) *Woodwardia radicans*, and (3) *Polypodium vulgare*.

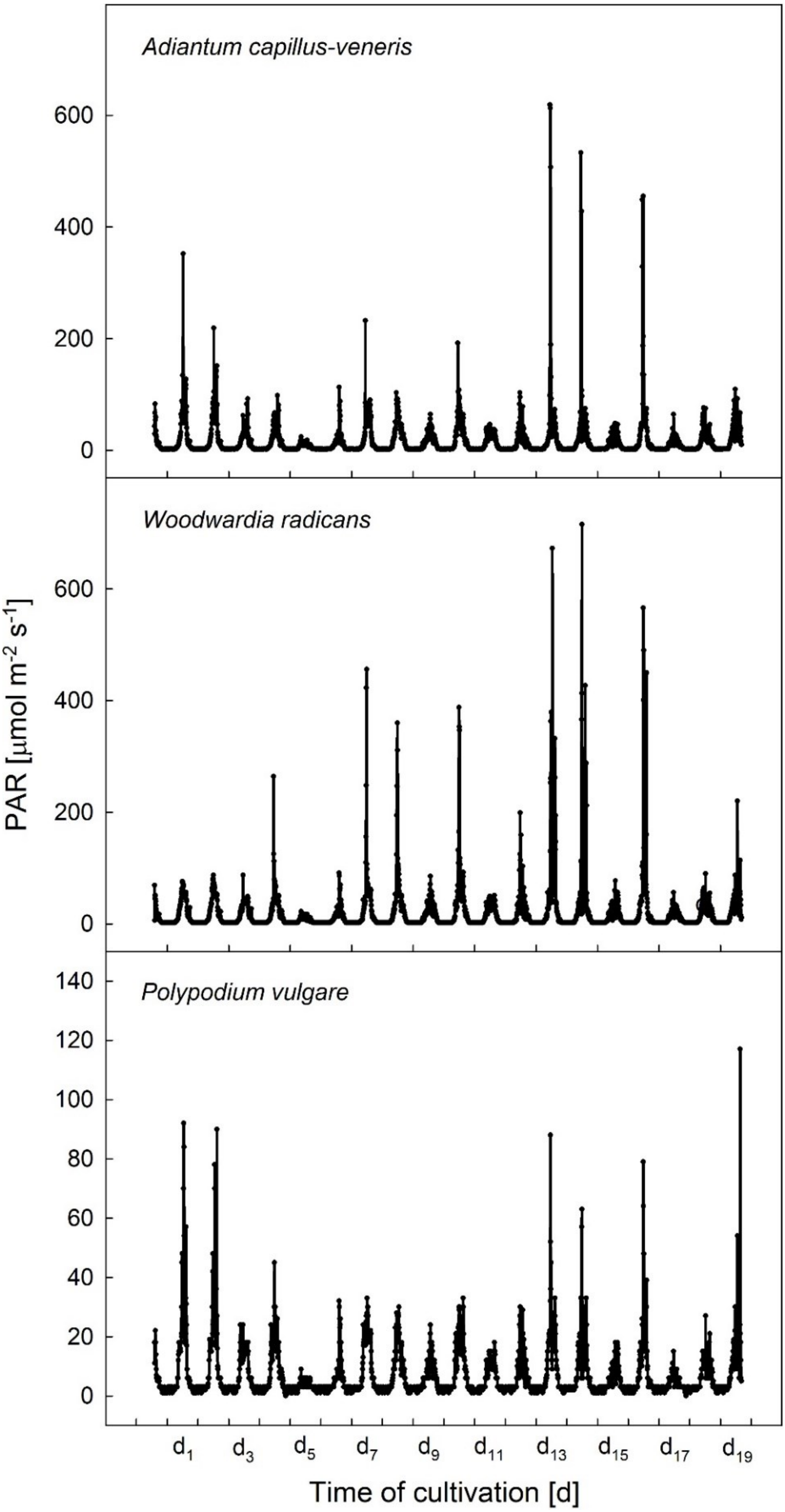


Fig. 5.3
Daily courses of photosynthetically active radiation (PAR) incident on the leaves of each fern species: (1) *Adiantum capillus-veneris*, (2) *Woodwardia radicans*, and (3) *Polypodium vulgare.* Please note apparently lower PAR values incident to the leaf of *P. vulgare* caused by self-shading effect of the newly-grown leaves during the cultivation period.

Except of *W. radicans*, the lowest ETR values for one day light period were observed in the second part of the cultivation period, i.e. around April 26th (d10) and after that day. Night minima of ETR=0 were obviously found for the dark period of the day, indicating full limitation of primary photosynthetic processes because of the lack of light. Maximal values of ETR (ETR_{max}) were reached around 14:00 to 15:00 h GMT each day. Absolute values of ETR_{max} reflected daily means of PAR. On sunny days, they were found higher than 10, except of the *P. vulgare*, which showed an ETR_{max} of only 6.3 because of the likely limitation imposed by low light (shading effect of neighbouring leaves) and of the leaf age.

According to the overall course of ETR, *A. capillus-veneris* seemed to be much more limited by cultivation conditions than the other two because ETR and ETR_{max} decreased dramatically at d5 and later exhibited values below 5 in the second part of cultivation period (d10-d19). *W. radicans*, on the other hand, exhibited ETR_{max} values constantly higher than 5 in about 50 % of days throughout the whole cultivation period, with no clear indication of ETR_{max} depression in the second part of the cultivation period. In the *A. capillus-veneris* and *P. vulgare*, contrastingly, apparent depression of ETR_{max} was found in the second part of the cultivation period indicating limitation of primary photosynthetic processes due to cultivation conditions, more apparently in *A. capillus-veneris.*

5.4.2. Vitality assessed as F_V/F_M

Under greenhouse cultivation conditions, F_V/F_M underwent a drop with cultivation time in all three species (fig. 5.4). The pattern of F_V/F_M decline, however, was species-specific. In *A. capillus-veneris,* it declined substantially from d1 to d2. Then, it showed irregular pattern of values ranging between 0.5 and 0.6. *Woodwardia radicans* showed biphasic decrease in F_V/F_M . Most remarkable decline was found between d1 and d2. Then it decreased much slowly but more or less regularly in the cultivation period delimited by d2 and d19. In comparison with *A.capillus-veneris,* however, *W.radicans* showed a decrease to the values below 0.5. *P. vulgare* had the highest F_V/F_M value at the beginning of cultivation experiment (0.748 on d1). Then, exponential-like F_V/F_M decrease was apparent from d2 to d11 followed by more or less constant F_V/F_M value of 0.6 found in the second period of cultivation time. The

F_V/F_M data suggest that the vitality of the ferns in terms of primary photosynthetic processes could be classified in the following (descendent) order: *P. vulgare*, *W. radicans*, *A. capillus-veneris.*

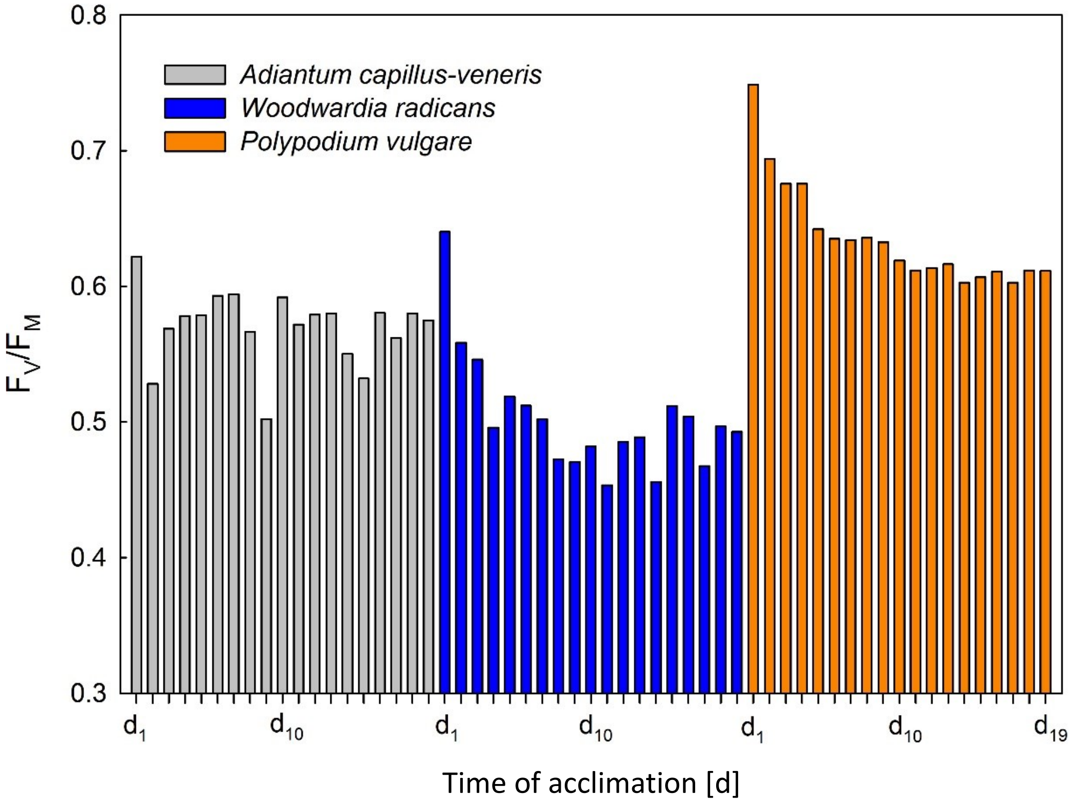


Fig. 5.4 Time courses of potential quantum yield of photosynthetic processes in PSII (Fv/Fm) recorded during the cultivation experiment in 3 fern species. Standard deviation is not indicated (it was less than 2 % of means). *A. capillus-veneris* (grey), *Woodwardia radicans* (blue), *P. vulgare* (orange).

5.4.3. Light-response curves of ETR

Measurements of ETR in the three fern- species revealed the fact that during the experimental period, gradual decrease in ETR for particular PAR appeared. The phenomenon was demonstrated as gradual flattening of the ETR light response curves with time of cultivation in a greenhouse (fig. 5.5 for d1, d2, and d19). The decline in ETR values was apparent even on d2, i.e. shortly after the start of experiment in the greenhouse. Then, in the course of cultivation time, ETR decreased in all species (see data for d19 in fig.5.5). In some cases (*A. capillus-*

veneris on d19), ETR remained below 2 in a wide range of PAR, indicating full inhibition of primary photosynthesis processes.

The ETR light response curves are presented here for d1 and d2 (left panels), d19 (right panels). *A. capillus-veneris* showed full inhibition of primary photosynthetic processes in PSII (upper right panel). The arrows indicate the alpha parameter (α), i.e. the slope of initial (linear) part of the curve. The linear slopes for the ETR (α parameter) recorded within the PAR range of 0-20 μmol m^{-2} s^{-1} were found

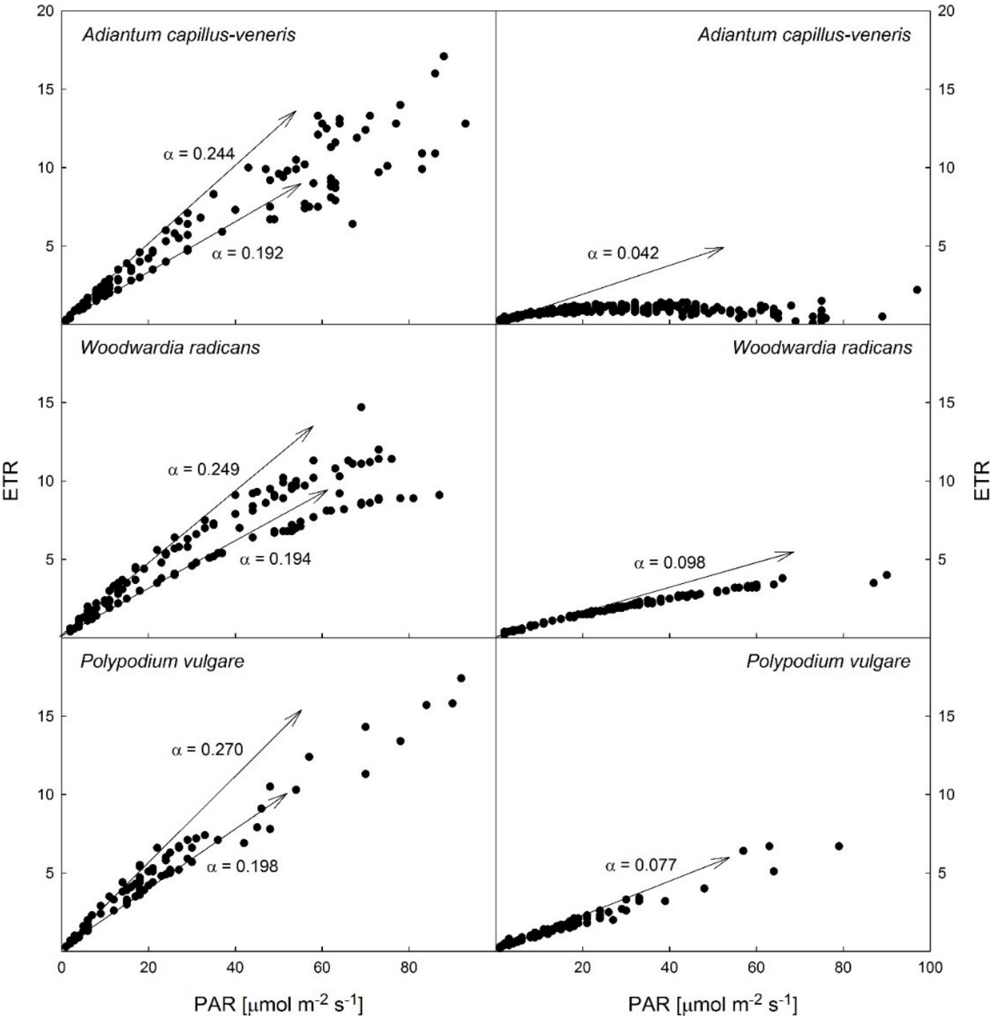


Fig. 5.5 Light response curves of photosynthetic electron transport (ETR) derived from chlorophyll fluorescence data for 1) *Adiantum capillus-veneris*, 2) *Woodwardia radicans*, and 3) *Polypodium vulgare*. In left column, light response curves of ETR for d1 and d2 are presented. In right column, ETR curves for d19 are presented. Parameter α referred to the initial slope of the ETR to PAR relationship was calculated for the PAR.

rather similar for all species in d1 (April 18th). Then, due to decline of the ETR in relationship to PAR, the slope was found species-specific for d2 and d19.

5.4.4. Short-term experiment - sensitivity to photoinhibition

OJIPs recorded immediately after the photo-inhibitory treatment and during dim light recovery showed species-specific response. Compared to pre-photoinhibition control, the three experimental species showed a strong decrease in Chl fluorescence values (flattening of the curve) throughout the OJIP curves recorded immediately after tthe photoinhibition treatment (fig. 5.6).

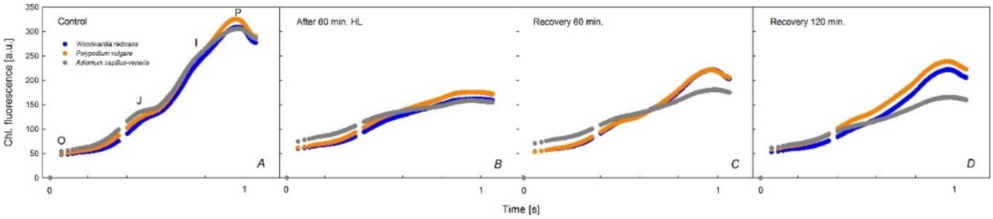


Fig. 5.6 Fast chlorophyll fluorescence transients (OJIPs) presented for untreated control (A), recorded immediately after photoinhibition (B), and after 60, and 120 min dim light recovery (C, and D). *Adiantum capillus-veneris* (grey), *Woodwardia radicans* (orange), and *Polypodium vulgare* (blue).

For chlorophyll fluorescence recorded at P point (F_P), the most pronounced decrease was found in *A. capillus-veneris* (42.7 % of initial pre-photoinhibition value), followed by *W. radicans* (52.4 %), and *P. vulgare* (54.1 %). A photo-inhibitory, treatment-dependent, increase of background Chl fluorescence (F_0 = O) was found in all experimental species. Chlorophyll fluorescence level recorded at I point (F_I) increased relatively to F_P (by 36.4, 30.7, and 28.8 % *W. radicans*, *P. vulgare*, and *A. capillus-veneris*) which is attributed to the decrease in effectivity of photosynthetic electron transport from Q_A to plastoquinone pool.

With progression of time recovery (60, 120 min), OJIP shapes and absolute values of Chl fluorescence showed a partial return to pre-photoinhibition values. However, this recovery did not reach the initial values after 60 min recovery (F_P of 74.3 % in *W. radicans*, 77.2 % in *P. vulgare*, and 42.7 % in *A. capillus-veneris* full recovery = 100 %). After 60 min of recovery, OJIP shape, i.e. Chl fluorescence levels within whole transient increased towards pre-photoinhibition values. The increase was more apparent in *W. radicans*, and *P. vulgare*, than in *A. capillus-veneris*.

Chlorophyll fluorescence parameters responded to photo-inhibitory treatment similarly in each species (fig. 5.7). However, some species-specific differences were found. Chlorophyll fluorescence

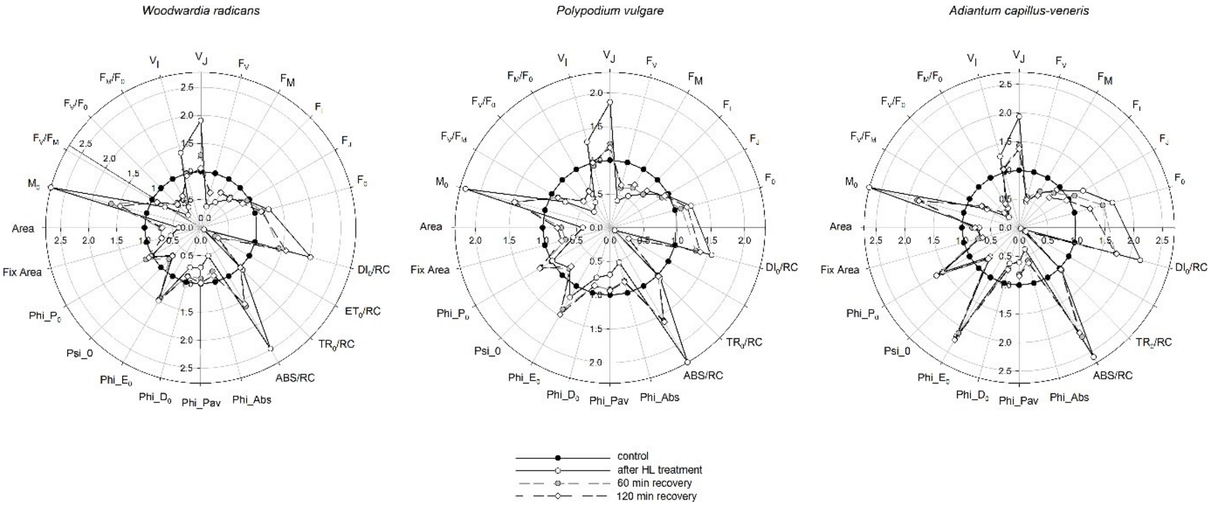


Fig. 5.7 Radar plot of chlorophyll fluorescence parameters derived from chlorophyll fluorescence transients (OJIPs) measured during a short-term photoinhibitory treatment (1500 µmol m^{-2} s^{-1} for 60 min): black symbol, full line - control, open symbol, full line - immediately after photoinhibitory treatment, grey symbol, dotted line - after 60 min recovery, open symbol, dotted line - after 120 min recovery.

signals (F_0, F_J, F_I, F_M) decreased along with V_J and V_I. Photo-inhibitory treatment induced a decrease in all parameters associated with PSII functioning (F_M/F_0, F_V/F_0, F_V/F_M) and electron transport (ET_0/RC). Photoinhibition-induced increase was found in thermal dissipation of absorbed light energy (Di_0), as well as in Mo and ABS/RC. Interspecific differences were found in ET_0/RC: *W. radicans* and *P. vulgare* showed partial recovery in ET_0/RC (an increase when compared to the value recorded immediately after photo-inhibitory treatment). However, ET_0/RC remained low in *A. capillus-veneris,* i.e. no recovery to pre-treatment values was apparent. Similar differences were found for ABS/RC: recovery was more apparent in *W. radicans*, and *P. vulgare* than in *A. capillus-veneris*. Photoinhibitory treatment-dependent thermal dissipation (DI_0/RC) increase was found smallest in *P. vulgare*, followed by *W. radicans*, and *A. capillus-veneris*. Inter-specific differences were found also in the extent of (DI_0/RC) recovery after 60 min: 1.4 of pre-photoinhibition value in *P. vulgare*, 1.5 in *W. radicans* and 1.7 in *A. capillus-veneris*. These differences suggest species-specific sensitivity of particular species to photoinhibition (see Discussion).

5.5. DISCUSSION

5.5.1. Daily courses of ETR

Daily courses of ETR reflected PAR daily courses (fig. 5.2). However, in the second part of the cultivation period, ETR peaks (see ETR_{max} below) were much less pronounced than PAR peaks. Such phenomenon relates to the fact that effective quantum yield (Φ_{PSII}) decreased with cultivation time for specific PAR level. This could be associated with the negative adjustment of primary photosynthetic processes in PSII in the three fern-species and associated with the decrease in ETR to PAR curves with the time of cultivation (fig. 5.4). Such conclusion is in agreement with acclimatory changes in photosynthetic parameters described for ferns growing in different (manipulated) light environments (Volkova et al. 2011). The authors report significant change in the light response curves of photosynthesis for summer and winter period in Australian wet forest. In our study, shading effect was an additional factor playing a role in the ETR decrease in the second part of the cultivation period (d10 to d19). This was mainly due to development of new foliage close and above the measuring spot in *P. vulgare* and *A. capillus-veneris* (see F ig. 1). Analysis of RLC revealed that, in spite of same photosynthetic performance before experiment, ETR decrease after 19-day cultivation was much more pronounced in A. capillus-veneris than in P.vulgare, and W. radicans. This suggest better acclimation to low light in P. vulgare, and W. radicans.

5.5.2. Vitality assessed by F_V/F_M

Since in all experimental fern species F_V/F_M declined with the cultivation time in the greenhouse, It might be concluded that vitality decreased with the cultivation time. The reason for such F_V/F_M decline could be insufficient light that did not exceed 200 µmol m^{-2} s^{-1} of PAR throughout the cultivation period. Therefore, the three fern-species suffered from low light which led to F_V/F_M decline. In all species, however, the cultivation conditions led to an acclimation (negative adjustment) of F_V/F_M since the F_V/F_M values were found more or less constant at the end of cultivation period. It might be concluded that F_V/F_M decreased thank to the combined effect of leaf age and environmental factors, especially increase of self-shading induced by newly developed leaves. For ferns, it is well established, that F_V/F_M declines with partial leaf dehydration, as shown by Larama et al. (2017) for *Hymenophyllum caudiculatum* and *H. dentatum*. In spite of environmental factors, some ferns are highly adaptive to changes in light availability (Weng et Wong 2015). These adaptational and acclimatory changes are sensitively followed by the changes in F_V/F_M happening in a time span ranging from several days to weeks. The adaptational/acclimatory changes may be caused by e.g. low light. Johnson et al. (2000) reported the changes for low-light thriving *Trichomanes speciosum* in which chlorophyll fluorescence parameters responded sensitively to even a small change on PAR.

5.5.3. Light-response curves of ETR

In our study, species-specific differences of light response curves of ETR (ETR to PAR relation) were found, more apparently during the second part of the experiment period thanks to co-action of environmental factors and leaf age. Species specifity of the ETR to PAR relation has been described several times for a wide variety of plants, such as e.g. macroalgae (Figueroa et al. 2014), lichens (Casanova-Katny et al. 2019), succulent plants, broadleaf trees (Pieruschka et al. 2014).

In fig. 5.5, the ETR to PAR curves are compared related to two specific days in the three species. This approach is well comparable to the diurnal rapid light curves (RLC) methods (see e.g. Belshe et al. 2008). RLCs of ETR, however, are based on the assumption that Φ_{PSII} measurements can be used routinely to calculate the rate of linear electron transport (ETR) from chlorophyll fluorescence data. For majority of plant species, calculated ETRs based have a linear relationship with photosynthetic CO_2 fixation. In some other species, however, ETR and CO_2 fixation only correlate at lower irradiances. With high light stress and the initial phase of photoinhibition, the ETR to CO_2 relation deviates from linearity. This is because of short illumination time at particular PAR levels used in the RLC method. Short time of PAR is a strong limitation, as the plant has insufficient time to equilibrate to the light level and resulting ETR values reflect are then negatively affected (Beer and Axelsson 2014).

Moreover, in plants exposed to high light, an increased portion of ATP and NADPH formed by photochemical processes (ETR) of photosynthesis is utilized in reparative and protective processes. Therefore, relative utilization of ATP and NADPH in biochemical processes of photosynthesis, decreases. In such circumstances, typically, ETR values are overestimated in comparison to the electron transport rates calculated from gas exchange data. In fact, ATP and NADPH (photochemically produced through ETR) are differently employed, depending on physiologically conditions. In stressed plants they are preferentially allocated to reparative and protective processes rather than to CO_2 fixation. In conclusion, acclimatory changes in ETR to PAR curves (d1 to d19) could be attributed to decreased photochemical processes of photosynthesis. Biochemical processes of photosynthesis acclimated as well since primary (photochemical) and secondary processes (Calvin-Benson cycle) are well coupled in plants (see e.g. Tosens et al. 2016). Acclimatory downregulation of photosynthesis is typically related to the changes in stomatal conductance (g_s) as affected by light (Carins Murphy et al. 2017), temperature, water saturation deficit and drought (McAdam and Brodribb 2012a). In ferns, mesophyll conductance (g_m) may play a role in acclimatory responses to light environment since it is reported generally low (Gago et al. 2013), much lower than reported for higher plants (reviewed by Flexas et al. 2012). However, g_s and g_m changes in a course of acclimation should be still investigated in ferns because operational stomatal conductance tends to disagree with maximum theoretical stomatal conductance (McAlwain et al. 2016). Such disparity may be the result of stomata not opening fully due to e.g. suboptimal water supply. Moreover, Volkova et al. (2009) reported that g_s did not respond to short-term high light treatment (simulated sunfleck). Similarly, McAdam and Brodribb (2012b) showed that ferns display a reduced stomatal response to transitions in light intensity without evidence of a feedback between assimilation and stomatal conductance, commonly observed in seed plants. On the other hand, non-stomatal limitations may be a substantial part of acclimatory responses of ferns to growth environment since it accounted for up to 70 % of photosynthesis limitation in *Osmunda regalis*, *Blechnum gibbum* and *Nephrolepis exaltata* (Gago et al. 2013).

Slope (α) of the initial part of the ETR to PAR curves showed day and species specificity (fig. 5.5). The α parameter is frequently used to denote the maximal quantum yield (or the "efficiency" of photosynthesis) of higher plants. It has been showed to respond sensitively to particular periods of growing season and/or day (White and Critchley 1999), leaf age and category (sun, shade - Paula et al. 2018), nutrient availability (Cai et al. 2012). Since in our experiment ⍰ differed in response to day of cultivation and species, it may be concluded that the method of Φ_{PSII} monitoring and subsequent ETR calculations proved to be sensitive enough to detect environmental-induced responses in ferns. Therefore, the method can be recommended for detailed analysis of *in situ* photosynthetic performace of ferns under a wide range of daily irradiance.

5.5.4. Sensitivity to photoinhibition

It is well established that Chl fluorescence values of OJIP transient decrease with photoinhibitory stress in PSII leading to a flattening of the OJIP curves. Such response is reported both in higher plants (Mlinaric et al. 2017) and lichens (e.g. Barták 2014). In our study, F_0 increased after the photoinhibitory treatment followed by a F_0 decrease during recovery (F_0, however, still higher than untreated control). Such behaviour is reported for many species (e.g. Manrique et al. 1993) and attributed to a direct effect of high light on the functioning of light harvesting complexes (LHCs). Severe photo-inhibitory treatment typically leads to changes in LHC arrangement which are manifested as F_0 increase. The consequence is a decrease of the F_V/F_M ratio which, due to the inactivation of reaction centers or the antenna detachment, could be attributed to an increase in F_0.

OJIP-based analysis of functional effectivity of LHCII and core of PSII under radiation stress has been studied recently (Stirbet 2013). The study suggested that connectivity between PSII, i.e. transfer of the excitation energy from a closed PSII RC to an open (active) PSII RC may be altered by photoinhibition as well. The OJIPs, flattened after photoinhibitory treatment, tended to reach pre-photoinhibitory shape in the course of recovery. Since *A. capillus-veneris* showed the lowest recovery to the initial shape among the three species, it might be concluded that it was the most susceptible to short-term photoinhibitory treatment. Such conclusion might be supported by the OJIP-derived parameters, ET_0/RC in particular. Recovery of ET_0/RC suggested that the photosynthetic performance after Q_A (to which this parameter is related) was much less affected by photoihibitory treatment in *W. radicans*, and *P. vulgare* than in *A. capillus-veneris*. Higher sensitivity to photoinhibition in *A. capillus-veneris* than in the two other species is supported by PI_{ABS}/RC value which recovered to only limited extent (about 50 %) in *A. capillus-veneris* after 120 min recovery. *W. radicans*, and *P. vulgare*, on the contrary, showed almost complete recovery of PI_{ABS} /RC (93 and 75 %). *A. capillus-veneris* was found most sensitive to photoinhibition, in spite of the fact it possesses a photoprotective mechanism related to light dose and spectral quality-induced chloroplast movements in leaf cells (Augustynowicz and Gabryz 1999, Suetsugu et al. 2017). Our data (fig. 5.8) supports the idea of generally high sensitivity of ferns to photoinhibition in shade-adapted and -tolerant species. Sensitivity to

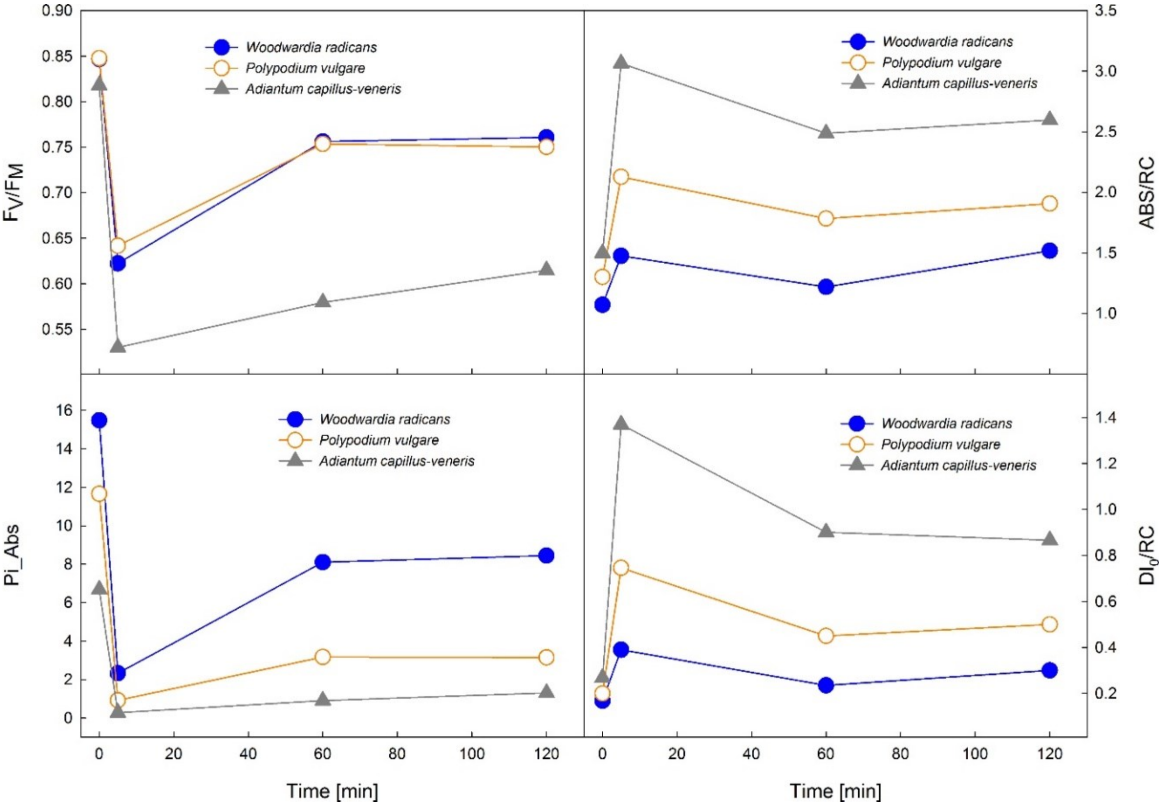


Fig. 5.8 Time courses of selected chlorophyll fluorescence parameters as affected by photoinhibitory treatment (1500 mol m^{-2} s^{-1} for 60 min) and recovery time. *Adiantum capillus-veneris* (grey), *Woodwardia radicans* (orange), and *Polypodium vulgare* (blue).

photoinhibition, however, might be low in shade-intolerant species such as e.g. tropical bracken (*Pteridium arachnoideum*) which occupies post-fire areas and high-altitudes natural pastures in tropical mountainous ecosystems (Bendix et al. 2009). Among the OJIP-derived parameters, several responded very sensitively to photoinhibitory treatment and, therefore, might be considered as useful indicators of species-specific differences in ferns exposed to short-term high light stress. For follow-up studies, V_J, relative variable fluorescence at the J-step, M_O - initial slope of relative variable ChlF curve (Küpper et al 2019), ABS/RC absorption, Phi_E_0 effectivity (probability) for electron transport, and DI_0/RC, thermal dissipation might be recommended.

In conclusion, all fern species treated by short-term high light showed downregulation (photoinhibition) of photosynthetic processes in PSII. Photoinhibition of PSII was previously considered as a solely negative mechanism to limit photodamage to PSII, effectively limiting the photosynthetic process. Recently, PSII photoinhibition is understood as protective mechanism to PSI (e.g. Tikkanen et al. 2014, Järvi et al. 2015), which is not equipped with its own repair mechanisms. Therefore, PSII photoinhibition represents a mechanism by which plants limit the photodamage to PSII but also preserves PSI. Unfortunately, only very limited data on photoinhibition of PSI in ferns (e.g. Huang et al. 2018) are available recently. This is a challenge to follow-up studies.

5.6. REFERENCES:

Augustynowicz J., Gabryš H (1999): Chloroplast movements in fern leaves: correlation of movement dynamics and environmental flexibility of the species. Plant, Cell and Environ, 22: 1239–1248.

Baker NR (2008) Chlorophyll fluorescence: A probe of photosynthesis *in vivo*. Annu Rev Plant Biol 59: 89-113.
https://doi.org/10.1146/annurev.arplant.59.032607.092759

Barták M, Váczi P, Hájek J (2012) Photosynthetic activity in three vascular species of Spitsbergen vegetation during summer season in response to microclimate. Polish Polar Research 33: 443–462. doi: 10.2478/v10183−012−0018−z

Bauer H, Gallmetzer C, Sato T (1991) Phenology and photosynthetic activity in sterile and fertile sporophytes of *Dryopteris filix-mas* (L.) Schott. Oecologia 86: 159–162.
https://doi.org/10.1007/BF00317526

Beer SL, Axelsson L (2004) Limitations in the use of PAM fluorometry for measuring photosynthetic rates of macroalgae at high irradiances. Europ J Phycol 39: 1–7. https://doi.org/10.1080/0967026032000157138

Belshe F, Durako M, Blum, JE (2008) Diurnal light curves and landscape-scale variation in photosynthetic characteristics of *Thalassia testudinum* in Florida Bay. Aquat Bot 89: 16-22. https://doi.org/10.1016/j.aquabot.2008.01.007

Bendix J, Silva B, Roos K, Göttlicher DO, Rollenbeck R., Nauss T, Beck E (2009) Model parameterization to simulate and compare the PAR absorption potential of two competing plant species. Int J Biometeorol DOI: 10.1007/s00484-009-0279-3

Cai, XL, Gao G, Tang XM, Dai JY, Chen D (2012) Photosynthetic response of *Vallisneria natans* (Lour.) Hara (*Hydrocharitaceae*) to increasing nutrient loadings. Photosynthetica 50: 437-446. https://doi.org/10.1007/s11099-012-0046-2

Chen Q, Sun J-Q, Song L, Liu W-Y, Yu F-H, Li S, Gong H-D, Lu H-Z (2019) Trait acclimation of the clonal fern Selliguea griffithiana to forest epiphytic and terrestrial habitats. Ecol Res 34: 406-414. DOI: 10.1111/1440-1703.12002

Casanova-Katny A, Barták M, Gutierrez C (2019) Open top chamber microclimate may limit photosynthetic processes in Antarctic lichen: Case study from King George Island, Antarctica. Czech Polar Reports 9: 61-77.
http://dx.doi.org/10.5817/CPR2019-1-6

Carins Murphy MR, Jordan GJ, Brodribb TJ (2017) Ferns are less dependent on passive dilution by cell expansion to coordinate leaf vein and stomatal spacing than angiosperms. PLoS ONE 12(9): e0185648. https://doi.org/10.1371/journal.pone.0185648

Durako MJ (2012) Using PAM fluorometry for landscape-level assessment of *Thalassia testudinum*: Can diurnal variation in photochemical efficiency be used as an ecoindicator of seagrass health? Ecol Indic 18: 243-251. https://doi.org/10.1016/j.ecolind.2011.11.025

Figueroa FL, Conde-Álvarez R, Bonomi Barufi J, Celis-Plá PSM, Flores P, Malta EJ, Stengel DB, Meyerhoff O, Pérez-Ruzafa A (2014) Continuous monitoring of in vivo chlorophyll a fluorescence in *Ulva rigida* (Chlorophyta) submitted to different CO_2, nutrient and temperature regimes. Aquat Biol 22: 195-2014. doi: 10.3354/ab00593

Flexas J, Barbour M, Brendel O, Cabrera HM, Carriqui M, Díaz-Espejo A, Douthe C, Dreyer E, Ferrio JP, Gago J, Galle A, Galmes J, Kodama N, Medrano H, Niinemets U, Peguero-Pina JJ, Pou A, Ribas-Carbo M, Tomás M, Warren RC (2012) Mesophyll diffusion conductance to CO_2: an unappreciated central player in photosynthesis. Plant Sci 193–194: 70–84

Genty B, Briantias JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta (BBA) - Gen Subj 990: 87-92. https://doi.org/10.1016/S0304-4165(89)80016-9

Gago J, Coopman RE, Cabrera HM, Hermida C, Molins A et al. (2013) Photosynthesis limitations in three fern species. Physiologia Plantarum 149: 599–611. doi:10.1111/ppl.12073

Hietz P, Briones O (2001) Photosynthesis, Chlorophyll Fluorescence and Within-Canopy Distribution of Epiphytic Ferns in a Mexican Cloud Forest. Plant Biol (Stuttg) 3(3): 279-287. https://doi.org/10.1055/s-2001-15198

Huang W, Tikkanen M, Zhang SB (2018) Photoinhibition of photosystem I in *Nephrolepis falciformis* depends on reactive oxygen species generated in the chloroplast stroma. Photosynthesis Research, 137: 129-140.

Järvi S, Suorsa M, Aro EM (2015) Photosystem II repair in plant chloroplasts. Regulation, assisting proteins and shared components with photosystem II biogenesis. Biochim. Biophys. Acta 1847, 900–909. doi: 10.1016/j.bbabio.2015.01.006

Johnson GN, Rumsey FJ, Headley AD, Sheffield E (2000) Adaptations to extreme low light in the fern *Trichomanes speciosum*. New Phytol 148: 423–431. https://doi.org/10.1046/j.1469-8137.2000.00772.x

Kalaji, HM, Jajoo A., Oukarroum A, Brestič M, Žifčák M, Samborska I, Cetner MD, Lukasik I, Goltsev V, Ladle RJ (2016) Chlorophyll a fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. Acta Physiol Plant, 38: 102. DOI: 10.1007/s11738-016-2113-y.

Kolari P, Chan T, Porcar-Castell A, Bäck J, Nikinmaa E, Juurola E (2014) Field and controlled environment measurements show strong seasonal acclimation in photosynthesis and respiration potential in boreal Scots pine. Front Plant Sci 5:717. doi.org/10.3389/fpls.2014.00717

Larama G, Ostria-Gallardo E, Berrios G, Gutierrez A, Ensminger I, Bravo LA (2017) The transcriptional dynamics of two filmy ferns from *Hymenophyllaceae* with different niche preferences unravel key aspects of their desiccation tolerance and vertical distribution along host trees. bioRxiv 228866. doi: https://doi.org/10.1101/228866

Liu Y-H, Liu F-L, Long B, Zhou X-L, Zhang X, Zhang Y, Wang W-L, Shen S-K (2019) Chlorophyll fluorescence characteristics and rapid light response curves of alpine rhododendron species across elevation gradients. Horticultural Science and Technology 37: 463-472. https://doi.org/10.7235/HORT.20190047

Manrique E, Balaguer L, Barnes J, Davison AW (1993) Photoinhibition studies in lichens using chlorophyll fluorescence analysis. Bryol 96: 443-449. doi: 10.2307/3243876

McAdam S, Brodribb T (2012a) Fern and Lycophyte Guard Cells Do Not Respond to Endogenous Abscisic Acid. The Plant Cell 24: 1510-21. DOI: 10.1105/tpc.112.096404

McAdam S, Brodribb T (2012b) Stomatal innovation and the rise of seed plants. Ecology letters, DOI:10.1111/j.1461-0248.2011.01700.x

McElwain JC, Yiotis C, Lawson T (2016) Using modern plant trait relationships between observed and theoretical maximum stomatal conductance and vein density to examine patterns of plant macroevolution. New Phytol 209: 94–103. https://doi.org/10.1111/nph.13579

Melis A (1989) Spectroscopic Methods in Photosynthesis: Photosystem stoichiometry and chlorophyll antenna size. Philosophical Transactions Royal Society B 323: 397–409. https://doi.org/10.1098/rstb.1989.0019

Minardi BD, Lorenzen Voytena AP, Santos M, Randi AM (2014) The epiphytic fern *Elaphoglossum luridum* (Fée) Christ. (Dryopteridaceae) from central and south America: morphological and physiological responses to water stress. Sci World J 2014, Article ID 817892. http://dx.doi.org/10.1155/2014/817892

Mlinaric, S., Atunovic Duncic, J., Skendrovic Babojelic, M., Ceasr, V., Lepeduš, H. (2017) Differential accumulation of photosynthetic proteins regulates diurnal photochemical adjustments of PSII in common fig (*Ficus carica* L.) leaves. Journal of Plant Physiology 209: 1–10.

Oliwa J, Stawoska I, Janeczko A, Oklešťková J, Skoczowski A (2019) Response of the photosynthetic apparatus in the tropical fern Platycerium bifurcatum to increased ozone concentration. Photosynthetica 57(4): 1119-1129. DOI: 10.32615/ps.2019.117

Paula SM, Boselli MA, Sanches MC (2018) Chlorophyll fluorescence and initial growth of two liana species, *Cuspidaria sceptrum* (Cham.) L.G. Lohmann and *Fridericia florida* (DC) L.G. Lohmann, under changes of natural light conditions. Photosynthetica 57: 170-180. doi: 10.32615/ps.2019.004

Pieruschka R, Albrecht H, Muller O, Berry JA, Klimov D, Kolber ZS, Malenovský Z, Rascher U (2014) Daily and seasonal dynamics of remotely sensed photosynthetic efficiency in tree canopies. *Tree Physiol* 34: 674-685. https://doi.org/10.1093/treephys/tpu035

Porcar-Castell A (2011) A high-resolution portrait of the annual dynamics of photochemical and non-photochemical quenching in needles of *Pinus sylvestris*. Physiol Plant 143: 139-153.
https://doi.org/10.1111/j.1399-3054.2011.01488.x

Ritchie RJ (2012) Photosynthesis in the blue water lily (*Nymphaea caerulea* Saligny) using pulse amplitude modulation fluorometry. Int J Plant Sci 17:124–136.
doi: 10.1086/663168

Ritchie RJ (2014) Photosynthesis in an encrusting lichen (Dirinaria picta (Sw.) Schaer. Ex Clem., Physiaceae) and its symbiont, *Trebouxia* sp., using pulse amplitude modulation fluorometry. Int J Plant Sci 175: 450-466. https://doi.org/10.1086/675575

Saroussi S, Beer S (2007) Alpha and quantum yield of aquatic plants derived from PAM fluorometry: uses and misuses. Aquat Bot 86: 89–92. https://doi.org/10.1016/j.aquabot.2006.09.003

Sessa EB, Givnish TJ (2014) Leaf form and photosynthetic physiology of Dryopteris species distributed along light gradients in eastern North America. Funct Ecol 28: 108-123. https://doi.org/10.1111/1365-2435.12150

Schreiber U (2004) Pulse-Amplitude (PAM) fluorometry and saturation pulse method. In: Papageorgiou G and Govindjee (eds.): Chlorophyll fluorescence: A signature of photosynthesis. Springer, Dordrecht, The Netherlands. pp. 279-319.

Stemke JA, Santiago LS (2011) Consequences of light absorptance in calculating electron transport rate of desert and succulent plants. Photosynthetica 49: 195-200. https://doi.org/10.1007/s11099-011-0026-y

Stirbet A (2013) Excitonic connectivity between photosystem II units: what is it, and how to measure it? Photosynth Res 116: 189–214. https://doi.org/10.1007/s11120-013-9863-9

Strasser RJ, Tsimilli-Michael M, Srivastava A (2004) Analysis of the Chlorophyll a Fluorescence Transient. In: Papageorgiou GCh etGovindjee (ed) Chlorophyll a fuorescence. Advances in photosynthesis and respiration. vol 19. Springer, Dordrecht, pp 321-362

Suetsugu N, Higa T, Wada M (2017) Ferns, mosses and liverworts as model systems for light-mediated chloroplast movements. Plant, Cell and Environment 40: 2447–2456.

Tikkanen, M., Mekala, N. R., Aro, E. M. (2014) Photosystem II photoinhibition-repair cycle protects photosystem I from irreversible damage. Biochim. Biophys. Acta 1837, 210–215. doi: 10.1016/j.bbabio.2013.10.001

Torres MA, Ritchie RJ, Lilly RMC, Grillet C, Larkum AWD (2013) Measurement of photosynthesis and photosynthetic efficiency in two diatoms. New Zealand J Bot 52: 6-27.
https://doi.org/10.1080/0028825X.2013.831917

Tosens T, Nishida K, Gago J, Coopman RE, Cabrera HM et al. (2016) The photosynthetic capacity in 35 ferns and fern allies: mesophyll CO_2 diffusion as a key trait. New Phytologist, 209: 1576–1590. doi: 10.1111/nph.13719

Ulqodry TZ, Matsumoto, F., Okimoto, Y, Nose A, Zheng SH (2014) Study on photosynthetic responses and chlorophyll fluorescence in *Rhizophora mucronata* seedlings under shade regimes. Acta Physiol Plant, 36: 1903-1917.

Ver Sagun J, Badger MR, Chow WS, Ghannoum O (2019) Cyclic electron flow and light partitioning between the two photosystems in leaves of plants with different functional types. Photosynth Res 142: 321-334. https://doi.org/10.1007/s11120-019-00666-1

Volkova L, Bennett LT, Tausz M (2009) Effects of sudden exposure to high light levels on two tree fern species *Dicksonia antarctica* (Dicksoniaceae) and *Cyathea australis* (Cyatheaceae) acclimated to different light intensities. Aust J Bot 57: 562–571

Volkova L, Bennett LT, Tausz M (2011) Diurnal and seasonal variations in photosynthetic and morphological traits of the tree ferns *Dicksonia antarctica* (Dicksoniaceae) and *Cyathea australis* (Cyatheaceae) in wet sclerophyll forests of Australia. Environ Exp Bot 70: 11–19

Weng J-H, Wong S-L (2015) Photosynthetic light responses of fern species adapted to different light regimes. In: Tuft J (ed): Ferns and Shrubs, Diversity, Cultivation, and Implications fo Environment. Nova Science Publishers, New York, USA. pp 57-76.

White AJ, Critchley C (1999) Rapid light curves: a new fluorescence method to assess the state of the photosynthetic apparatus. Photosynth Res 59: 63–72.
https://doi.org/10.1023/A:1006188004189

6. DAILY LIGHT COURSE OF PHOTOSYNTHESIS: WHAT REALLY HAPPENS IN THE FIELD?

Short-term photosynthetic adjustment of *Woodwardia radicans* and coexisting species to light regime. A field study by OJIP.

(Manuscript under revision for submission)

6.1. ABSTRACT

Two species from a natural protected environment were studied with OJIP Chlorophyll fluorescence (ChlF) techniques. *Woodwardia radicans* and *Conocephalum conicum* are a fern and a liverwort whose area is in the wet habitat of the Ferriere Valley – Italy. Daily light course responses were analysed to provide a scientific basis for *W. radicans* risk assessment and an easy tool to monitor the state of conservation of the species and of its habitat. OJIP has been proved sensitive to quick adjustment to short-term changes in the environment, then a useful tool for the purpose. Two-day measurements were carried out on the two species and climate parameters registered. Absolute and normalized by maximum OJIP curves were plotted and each segment analysed. Recorded data were plotted as time series of the ChlF parameters deemed more sensitive to the change. The choice of the parameters was aided by the radar plot, a graph showing variations of each parameters not in absolute value but as proportional to the first measurement (control). In this way parameters with values more distant from the initial level were the most affected by environmental factors. Since OJIP and OJIP-derived parameters represents an effective tool in the analysis of particular processes in PSII, a radar-plot provides an insight into their sensitivity to particular environmental factors; i.e. simultaneous analysis in ETo/RC and PI_{ABS} values together with DIo/RC allowed the conclusion of no photoinhibition during a short-term sunfleck periods, on the contrary, detailed analysis of a short-term increase of ETo/RC supported the idea of a short-term sunfleck-induced increase in photosynthesis in *C. conicum*

The Fv/Fm and PI_{ABS} data for *C. conicum* and *W. radicans* from the Ferriere Valley indicated no limitation of PSII and high photosynthetic for the environmental conditions of the days of measurements

Keywords: Chlorophyll fluorescence, OJIP, sunflecks, ferns, liverwort, Ferriere Valley

6.2. INTRODUCTION

Woodwardia radicans is a giant fern dating back to the tropical paleoclimate of Europe, which is now present as a glacial relict in limited and fragmented areas. One of these areas, the Valle delle Ferriere (Sorrento peninsula - Italy) is a protected natural reserve with multiple habitats, where *Woodwardia radicans* is one of the most important items of biodiversity. Many lab-based studies have been carried out on this species, but little has been done in the field. In addition, even though most of the official reports about *Woodwardia radicans* conservation are dealing, among others, with deforestation, (Spampinato et al. 2008, Rossi G. et al. 2013, Gargano D. et al. 2016), scientific basis has never been provided to these statements. Because the primary effect of deforestation is a dramatic change of light in the understory, we decided to carry out a study to get a preliminary view of the photosynthetic response to daily light course of the two driving species, (*Woodwardia radicans*, *Conocephalum conicum*) of the floristic association (*Conocephalo-Woodwardietum radicantis*) dominated by *W. radicans*. Our study had two main purposes: 1) to provide a scientific basis for *W. radicans* risk assessment, which is currently reported as EN (endangered) in Italian IUCN Red List of Threatened Species (Gargano D. et al. 2016). 2) to try to determine a quick and easy tool to monitor the state of conservation of the species and of its habitat. For this purpose, fast chlorophyll fluorescence transient (OJIP curve) technique was chosen because largely used on a variety of plants and found sensitive to detect short-term changes in photosynthetic performance caused by rapid changes of environmental factors (Strasser et al. 2004, Kalaji et al. 2011) and by stress factors such as low temperature (Kalaji et al. 2016, Marečková et al 2019), high temperature (Brestič et al 2013), photoinhibition (Živčák et al 2015), or dehydration (Živčák et al 2008).

Then daily light course response was measured, on the two species in the Ferriere Valley, in two days using OJIP.

OJIP kinetics are measured within the first 2 seconds after saturation pulse of strong actinic light on a predarkened sample. Measured ChlF shows a typical shape of kinetics. It starts from a minimal level F_0 (Ohen reaction centres of PSII are fully oxidized. Then, ChlF rises through two inflection points (J and I) to the maximal level of ChlF (F_P) at the peak P. The photochemical phase the OJIP kinetic (O-J rise) is more sensitive to changes in the number of absorbed photons. This part of OJIP kinetic is related to the balance of PSII primary electron acceptors reduction (QA) and its reoxidation by Q_B (Boisvert et al 2006). Therefore, it might be used for the evaluation of light-induced changes in the functioning of light harvesting complexes (LHC) and photosystem II (PS II). Thermal phase (J-I-P rise) is more sensitive to the temperature changes. The inflection point I reflects a balance of plastoquinone pool reduction by PSII and its reoxidation by PSI (Cyt b_6/F). The I-P phase is mostly connected to the PSI electron flow (Schansker et al 2005).

Along with the kinetics, several OJIP-derived parameters are calculated. The most commonly used is maximal quantum yield of PSII photochemistry (F_V/F_M), absorption flux of antenna chlorophylls per reaction centres (RC), (ABS/RC) or electron transport flux per RC (ET_0/RC).

6.3. MATERIAL AND METHODS

6.3.1. The area

The Valle delle Ferriere is a Natura 2000 protected site (IT8050045) of the Mediterranean biogeographical region, which includes 25 species protected under the Nature Directives and 7 habitat types of the Habitats Directive (NATURA 2000 - Standard Data Form2019). Our area of study is a humid ravine shaded by a canopy of mixed forest, rich in water with surface runoff for most of the year; *Woodwardia radicans* occupies the steep, earthy and dripping slopes while *Conocephalum conicum* is found on small rocky areas with flowing water. Their association, *Conocephalo-Woodwardietum radicantis* Brullo, Lo Giudice & Privitera 1989, is found across *Cratoneurion*, “Petrifying springs with tufa formation habitat” (fig.

6.1), a priority habitat (7220) listed in the Habitats Directive of the EEA (EC - DG Environment 2013).

Fig 6.1 Calcium deposits on *Palustriella commutata* (syn. *Cratoneuron commutatum*), typical of "Petrifying springs with tufa formation habitat", 7220 priority habitat. Ferriere Valley

6.3.2. Plant material

Woodwardia radicans (L.) Sm, is an evergreen, rhizomatous fern of the family Blechnaceae. The fronds, up to 2 mt. long, are bipinnated, with spores inside elongated sori, arranged in two underside lines parallel to the midrib of the leaflet. This chainlike arrangement gives the fern its common name "European chain fern". The species Latin name is given after the bulbils, on the underside of the frond tips, able to generate a new plant when touching the wet soil.

Conocephalum conicum (L) Dumort (Marchantiophyta) is a liverwort of the family of Conocephalaceae. The thallus, ribbonlike, deep green to dark green, is 5–20 cm long and 1–2.5 cm wide, forked, broadly lobed, with a middle groove, shiny with characteristic hexagonal patches bearing in the middle slightly raised hyaline airway openings, above the respiratory cavities (fig. 6.2). Unicellular rhizoids are numerous on the underside, anchoring the thallus to substrate. Vegetative propagules are produced on the underside of the thallus. The species is dioecious, with male gametes produced in antheridia submerged in flask-like cavities that open into conical papillae. The male gametes swim to the archegonia to fertilize the eggs inside. The spores are released by the rupture of sporangium produced at the end of the sporophyte.

Fig. 6.2 Leaf of *Conocephalum conicum* (L) Dumort showing the geometric patterns and the openings of the respiratory cavities

6.3.3. Measurements and climate parameters monitoring

During the field campaign (2 days of measurements), photosynthetic active radiation (PAR), temperature and relative humidity were continuously monitored by two dataloggers equipped with sensors: 1 Minikin THi and 1 Minikin RTHi/QTHi, (Environmental Measuring Systems - Czech Republic) placed in two points inside the experimental area. PAR spot measurements were carried out with a Quantum Sensor EMS 12 (Environmental Measuring Systems - Czech Republic).

OJIP measurements (FluorPen FP 100 - Photon System Instruments - Czech Republic) were recorded at 20 minutes intervals in six leaves of *W. radicans*, and five thalli of *C. conicum*, each of them in the same order and in the same spot to get a time series.

Measurements were carried out on April 26th and 28th starting at 10.30 and 09.30 a.m.

6.4. RESULTS

6.4.1. Environmental data

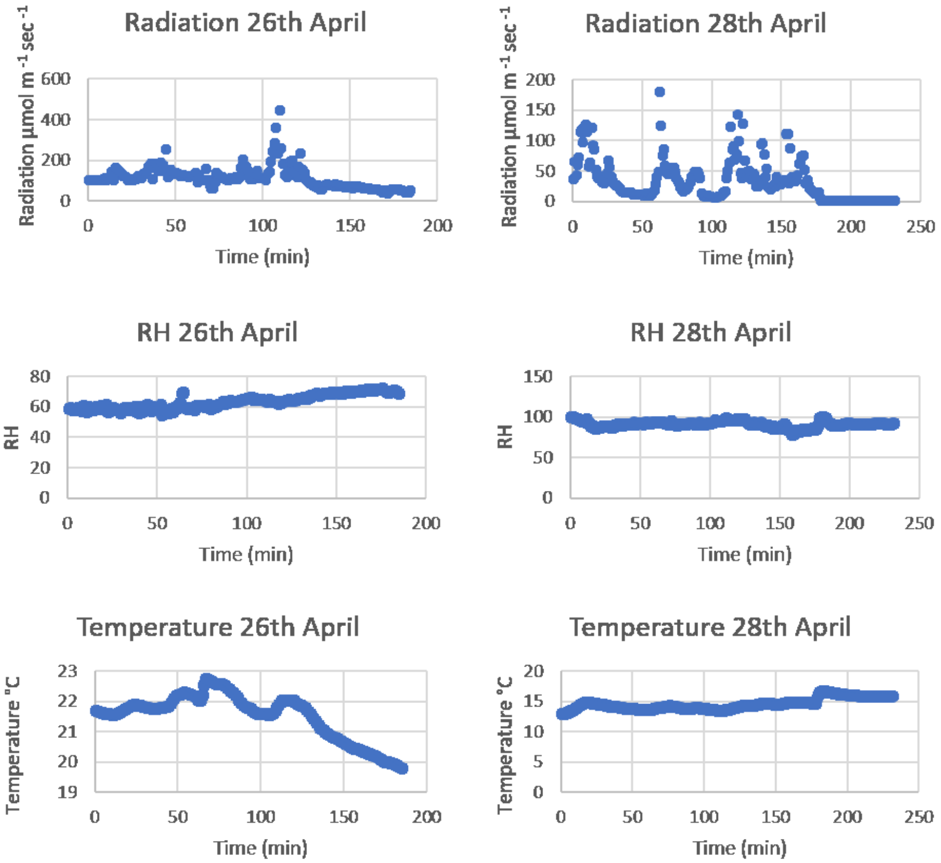


Fig. 6.3 Time course on 26th April (left) and 28th April (right) of light (top), relative humidity (middle), temperature (bottom)

6.4.2. Radar plot

Out of OJIPs (curves), several chlorophyll fluorescence parameters were calculated. To visualize the changes of specific processes happening in PSII and subsequent chloroplast photosynthetic processes (photochemical phase), the parameters were plotted in the form of Radar plot which is a frequently used approach in presentation of the plant studies using OJIPs. The first measurement was taken as reference while all subsequent values were expressed as relative value. This method allows to determine, in a single graph, which parameters are affected the most in the experiment in hand. The OJIP meter has a full set of parameters which are automatically recorded. In our case, among the variability shown by samples in both species and both days, clear indication was given that some presented more remarkable variations than others in the daily course of measurements (fig. 6.4). Among these some were deemed particularly interesting for our experiment. Below there is the list of the selected parameters, formulas for their calculation and description. The physiological values of each is briefly examined after.

F_0_norm ≅ F20µs = F_0 / $F_{0\ (100\%RWC)}$	Normalized minimal ChlF (all PSII RCs are assumed to be open)
Fv/Fm	Maximal quantum yield of PSII photochemistry
Fv/F_0	Ratio of variable to minimal ChlF
Fm/F_0	Ratio of maximal to minimal ChlF
ABS/RC = M_0(1 / V_J)(1 / Phi_P_0)	Absorption flux (of antenna chlorophylls) per RC
TR_0/RC = M_0(1 / V_J)	Trapped energy flux (leading to QA reduction) per RC
ET_0/ RC = M_0(1 / V_J)Psi_E_0	Electron transport flux (further than QA) per RC
DI_0 / RC = (ABS/RC) – (TR_0/RC)	The flux of dissipated excitation energy at time 0
P_{hi}_D_0 = 1 - Phi_P_0 = F_O/FM	Quantum yield (at t = 0) of energy dissipation

Phi_E_0 ≡ $ET_0/ABS = [1-(F_0/F_M)]_{\psi}E_0$ Quantum yield for electron transport from QA to PQ pool (ET)

PI_tot = Pi_ABS $[(RE_0/TE_0)^{*}/ (1-RE_0/TE_0)]$ Total performance index on absorption basis for energy conservation from exciton to the reduction of photosystem I acceptors

*where $RE_0/TE_0 = (Fm - F_I) / (Fm - F_J)$

F_0 is the minimum level of chlorophyll fluorescence (ChlF) emitted by the sample, after a darkening period. The PSII reaction centres (RCs) are all open, meaning that they are in a fully oxidized state, therefore ready to accept electrons.

Fm is the maximum peak reached by ChlF after a saturation pulse. The PSII reaction centres (RCs) are all closed, meaning that they are in a fully reduced state, then unable to accept electrons.

Fv is variable ChlF calculated as the difference between Fm and F_0. It indicates the ChlF which is variable during changing conditions; it gives indication of the reduction kinetics of Quinon a.

Fv/Fm gives the potential quantum yield of PSII in a given condition. It is always below 1 and it expresses the potential of PSII RCs to do photochemistry in the given condition. The higher its value, the more efficient is the photosynthetic machinery.

Fv/F_0 and Fm/F_0 are both ratio to minimal ChlF of maximum and variable ChlF. It has the same physiological meaning than the previous ones.

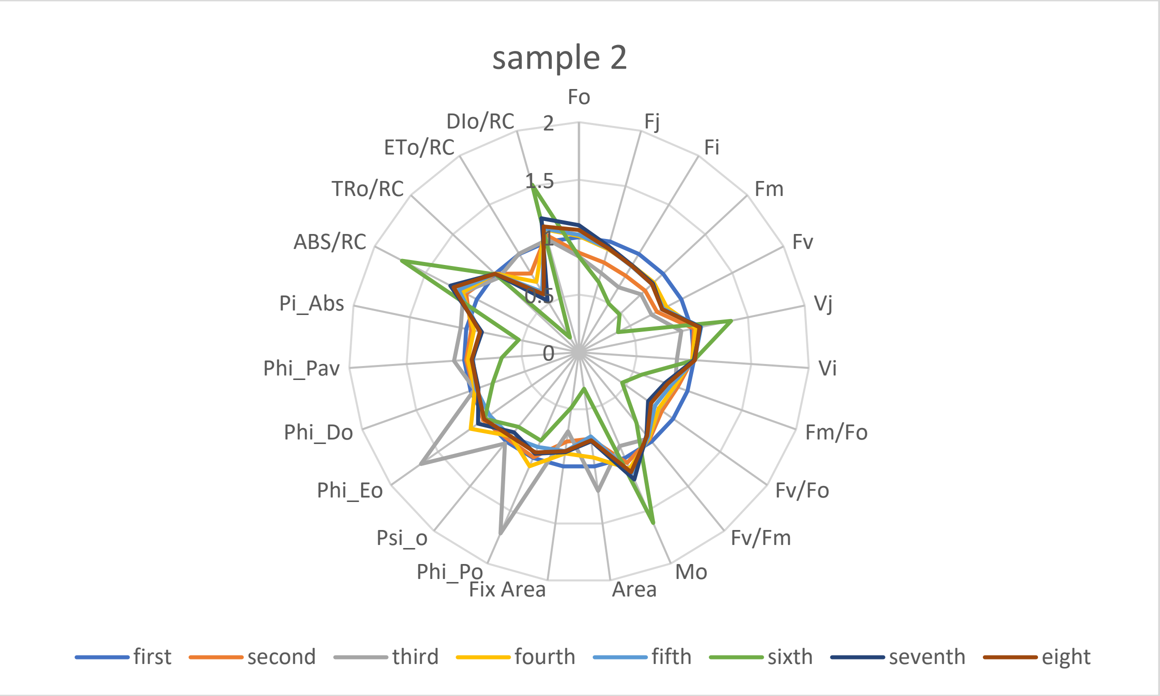


Fig. 6.4 Radar plot from a field measurement (sample two of *C. conicum*, first day of measurement; coloured lines with ordinal numbers indicate the time sequence of measurements, every 20 minutes, started at 9.00 a.m.)

In the presented example of Radar plot of OJIP-derived parameters, those most deviating ones from 1 (ABS/RC, DIo/RC, Mo) could be considered sensitive enough to reflect rapid perturbations in physical factors of environment, light availability in particular.

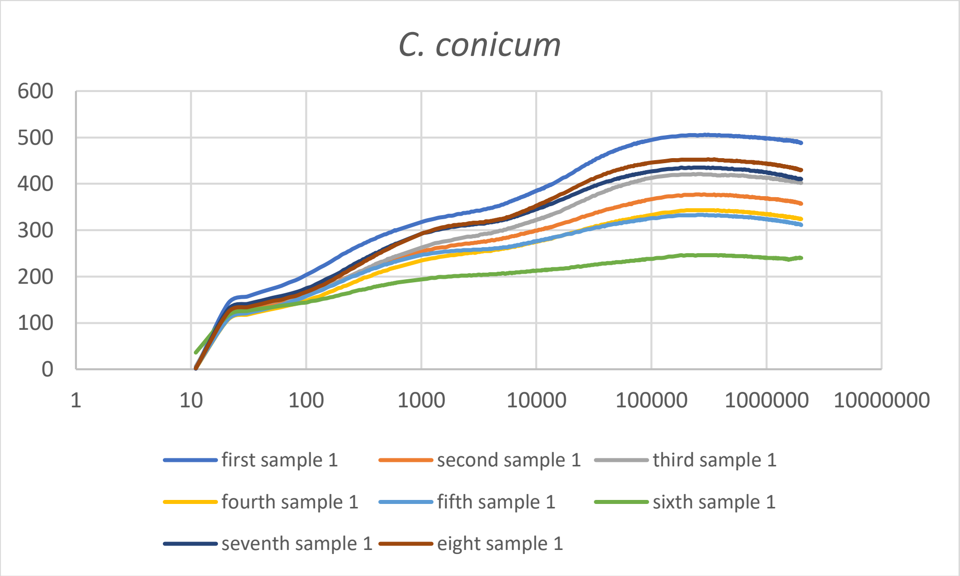


Fig. 6.5 *C. conicum*, first day OJIP daily course (absolute values). Ordinal numbers indicate the time sequence of measurements, every 20 minutes, started at 9.00 a.m.

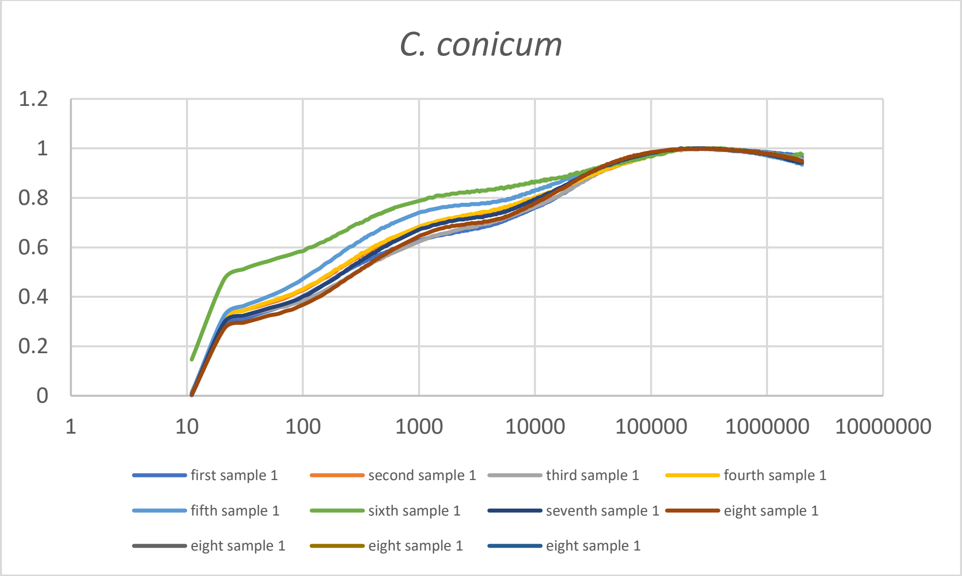


Fig. 6.6 *C. conicum*, first day OJIP daily course (normalized). Ordinal numbers indicate the time sequence of measurements, every 20 minutes, started at 9.00 a.m.

6.5. DISCUSSION

OJIP kinetics recorded for *C. conicum* and *W. radicans* showed increased chlorophyll fluorescence signal in a course of day (fig. 6.5). However, the overall shape of OJIPs seemed to be only slightly changed within a day. When the OJIPs are normalized to maximum (Fp) value, they show an increase in the chlorophyll fluorescence level at point J (fig. 6.6). This is indicative of limitation of redox state of Q_A^-, Q_B^-, and turnover of plastoquinone pool. Since the chlorophyll fluorescence level at J point stands for the part of reaction centres PSII that close during single charge separation in core of PSII (Strasser et al., 2004) the increase at J point is a consequence of restricted re-oxidation of Q_A^-. The phenomenon of an increase of chlorophyll fluorescence signal in point J in a course of a day is well described for many species (see e.g. Mlinaric et al. 2017 - *Ficus carica*, Bacari et al. 2016 - *Gallesia integrifolia*) and attributed to light, especially to daily courses of photosynthetically active radiation. In some case, high air/leaf temperature may interact and cause the chlorophyll fluorescence increase in J point as well.

Daily courses of Fv/Fm and PI_{ABS} revealed, that capacity of photosynthetic processes in PSII slightly declined in both species in a course of day. This is consistent with integral light dose absorbed by the plants within a day, since the slight decline in Fv/Fm and PI_{ABS} values reflects regulative processes activated during a light period of a day. In higher plants, Fv/Fm correlates with the number of functional PSII complexes. Therefore, the decrease of Fv/Fm below 0.83 is suggested to indicate photo-regulation of photosynthesis due to the inactivation of some PSII reaction centres by suboptimal environmental factors (Öquist et al., 1992). In *C. conicum* and *W. radicans* from the Ferriere Valley, however, the daily change of Fv/Fm was rather small suggesting that the two species were limited in primary photosynthetic processes to a very small extent. Indeed, incident light and air temperature were perfectly fitting physiological range for photosynthesis (fig. 6.3). Some Fv/Fm data points in *C. conicum* time course (fig. 6.7), however, showed substantial short-term decrease caused by previous sunfleck (high light spot on the upper leaf surface lasting from tens of seconds to several minutes).

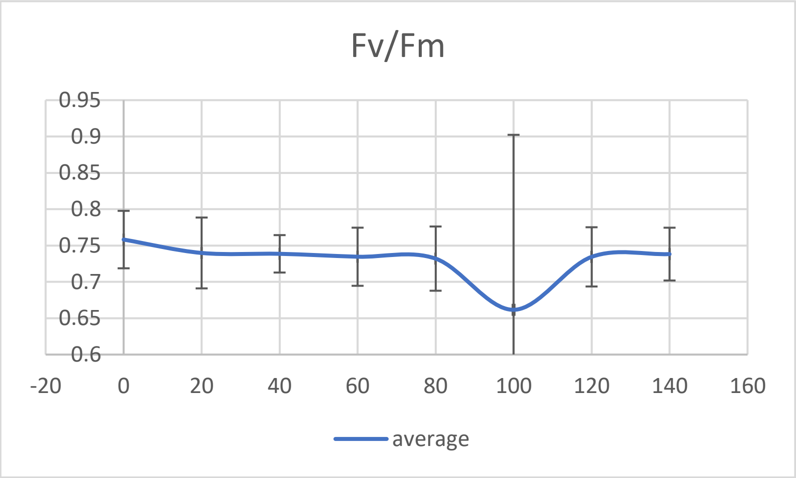


Fig. 6.7 Fv/Fm (mean of all areas)time course in *C. conicum*

Under such circumstances, Fv/Fm was downregulated rapidly. High light available for a short period of time increased the rate of photosynthesis. Such short-term high light-induced increase in photosynthesis might be documented by the increase of ABS/RC, TR/RC, ETo/RC and thermal dissipation of excess (absorbed) energy by DIo/RC. This indicate short-term increase in primary photosynthetic processes related to PSII as documented in other species.

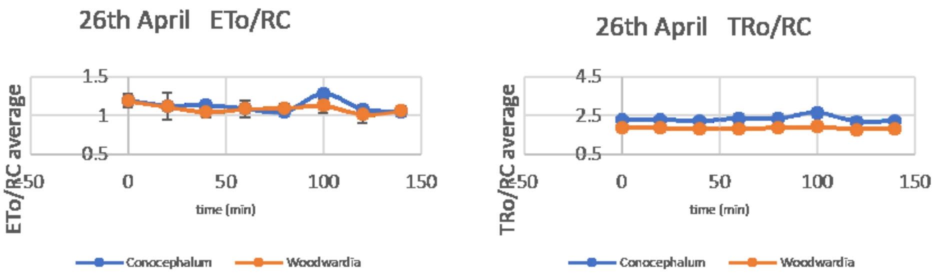


Fig. 6.8 ETo/RC and TR_0/RC daily course in both species on two days of measurements

Detailed analysis of a short-term increase of ETo/RC supported the idea of a short-term sunfleck-induced increase in photosynthesis in *C. conicum* since the change was accompanied by the increase in TR_0/RC. Moreover, the TR value was much higher than 0.75 (fig. 6.8) which is considered to be bordering value PSII functionality (BolharNordenkampf et al., 1989). Therefore, photoinhibition, even at initial phase, can be excluded in *C. conicum,* since it is typically accompanied by a decrease in TR/RC and specific fluxes per RC (Force et al. 2003), which was not the case of *C. conicum*. The short-term increase in ETo/RC was accompanied by the increase in DIo/RC values indicating that increased proportion of the energy absorbed by PSII was dissipated as heat. However, simultaneous increase in TR/RC, i.e. the percentage of absorbed light energy channelled to reaction centres indicated, that no photoinhibition happened during such a short period of high light. Photoinhibitory changes are typically induced by high light period lasting at least tens of minutes and generally known as midday depression of photosynthesis. Recently, photoinhibition caused by a 1- and 2-day-lasting high light treatment was documented and monitored by OJIPs for tropical fern *Platycerium bifurcatum* (Oliwa and Skoczkowski 2019). Such photoinhibition can be sensed by the changes in OJIP-derived parameters, i.e. simultaneous decrease in ETo/RC and PI_{ABS} values together by an increase in DIo/RC as shown by e.g. Panda (2011) for rice. In *C. conicum* and *W. radicans* such changes were not observed, which, once again supports the conclusion of no photoinhibition during a short-term sun fleck periods. Since OJIP and OJIP-derived parameters represents an effective tool in the analysis of particular processes in PSII, a radar-plot provides an insight into their sensitivity to particular environmental factor and/or microclimate. From data presented for *C, conicum* and *W. radicans* follows that, in spite of the fact that absolute values did not differed much (fig. 6.6) relative change is rather big (fig. 6.5). It is clear that ETo/RC decreases with daytime indicating negative adjustment of primary photosynthetic processes, photosynthetic linear electron transport in particular. In a course of daytime, activation of the mechanisms protecting PSII happened as indicated by the increase in DIo/RC. The increase in ABS/RC may indicate lowering of number of active RC in a course of daytime. Such inactivation of a certain part of RCs is, in majority of cases, caused by OEC inactivation as well as the transformation of active RCs to inactive, i.e. Q_A-non-reducing ones. Such Q_A-non-reducing centres can efficiently absorb excitation energy, but they are unable to reduce Q_A (Strasser et al., 2004).

In order to find out some functional interrelationship between OJIP-derived chlorophyll fluorescence, maximum quantum efficiency (Fv/Fm) was plotted against Performance Index (PI_{ABS}) of the two experimental species (data not shown). The analysis revealed that similarly to the findings of Liepina et Ievinsh (2013), PI_{ABS} was well related to Fv/Fm exhibiting a linear relationship. In general, PI_{ABS} shows a triphasic response in response to Fv/Fm in bryophytes exposed to stress as shown for variety of mosses (e.g. Folgar-Camean et Bartak, 2018). Typically, no photochemical performance of PS II is seen at the FV/FM values below 0.4 (phase 1, PI close to 0). Then, an exponential increase of the PI values is apparent at the FV/FM values ranging 0.4 to 0.7 (phase 2). Finally, a steep linear increase of PI_{ABS} values is found at the FV/FM values above 0.7 (phase 3). The Fv/Fm and PI_{ABS} data for *C. conicum* and *W. radicans* from the Ferriere Valley indicated no limitation of PSII and high photosynthetic performance since they belonged to the above-specified phase 3. Therefore, *C. conicum* and *W. radicans* may be considered not limited in photosynthetic performance by environmental factor(s) typical for the two experimental days in the Ferriere Valley. The data rank to the phase 3 of the PI_{ABS} to Fv/Fm relationship, therefore, the conclusion could be drawn for *C. conicum* and *W. radicans* that microclimate of the site and the two experimental days did not limit photosynthetic performance.

6.6. REFERENCES

Bacarin, M.A., Martinazzo, E.G., Cassol, D., Falqueto, A.R., Silva, D.M. (2016): Daytime variations of chlorophyll fluorescence in Pau d´Alho seedlings. Revista Árvore, Viçosa-MG, 40:1023-1030.

Boisvert S, Joly D, Carpentie R (2006) Quantitative analysis of the experimental O-J-I-P chlorophyll fluorescence induc- tion kinetics. FEBS J 273:4770–4777.
https ://doi.org/10.111 1/j.1742-4658.2006.05475.x

Brestič M, Živčák M, Olšovská K, Řepková J (2013) Involvement of chlorophyll a fluorescence analyses for identification of sensitiveness of the photosynthetic apparatus to high temperature in selected wheat genotypes. Springer, Berlin, Heidelberg, pp 510–513

EC - DG Environment 2013. Interpretation Manual of European Union Habitats, version EUR 28

Folgar-Cameán, Y., Barták, M. (2018): Limitation of photosynthetic processes in photosystem II in alpine mosses exposed to low temperatures: Response of chlorophyll fluorescence parameters. Czech Polar Reports. 8: 218-229.

Force, L., Critchley, C., van Rensen, J.J. (2003): New fluorescence parameters for monitoring photosynthesis in plants. Photosynth. Res. 78: 17–33.

Gargano D., M. Vena, L. Bernardo, N.G. Passalacqua, A. Santangelo., 2016. *Woodwardia radicans* (L.) Sm. In: Ercole S., Giacanelli V., Bacchetta G., Fenu G., Genovesi P. (ed.). Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie vegetali. ISPRA, Serie Manuali e linee guida, 140/2016.
https://doi.org/10.1016/j.jphotobiol.2015.08.024.

Kalaji HM, Bosa K, Koscielniak J, Hossain Z, 2011. Chlorophyll a Fluorescence – A usefull tool for the early detection of temperature stress in spring barley (Hordeum vulgare L.). Journal of Integrative Biology, 15: 925-934.

Kalaji HM, Jajoo A, Oukarroum A, Brestič M, Živčák M, Samborská IA, Cetner MD, Lukasik I, Golstev V, Ladle RJ (2016) Chlorophyll a fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. Acta Physiol Plant 38:102. https ://doi.org/10.1007/s1173 8-016-2113-y

Liepina, L., Ievinsh, G. (2013): Potential for fast chlorophyll a fluorescence measurement in bryophyte ecophysiology. Estonian Journal of Ecology, 62: 137-149.

Marečková M, Barták M, Hájek J (2019) Temperature effects on photosynthetic performance of Antarctic lichen Dermatocarpon polyphyllizum: a chlorophyll fluorescence study. Polar Biol 42:685–701. doi: 10.1007/s00300-019-02464-w

Mlinaric, S., Atunovic-Dunic, J., Skendrovic Babojelic, M., Cesar, V., Lepedus, H. (2017): Differential accumulation of photosynthetic proteins regulates diurnal photochemical adjustments of PSII in common fig (Ficus carica L.) leaves. Journal of Plant Physiology 209: 1–10.

NATURA 2000 - Standard Data Form2019

Oliwa J, Skoczkowski A. (2019): Different response of photosynthetic apparatus to high–light stress in sporotrophophyll and nest leaves of Platycerium bifurcatum. Photosynthetica, 57: 147-159.

Öquist, G., Chow, W. S., Anderson, J. M. (1992): Photoinhibition of photosynthesis represents a mechanism for the long-term regulation of photosystem II. Planta, 186, 450-460.

Panda, D. (2011): Diurnal variations in gas exchange and chlorophyll fluorescence in rice leaves: the cause for midday depression in CO2 photosynthetic rate. Journal of Stress Physiology and Biochemistry, 7: 175-186.

Rossi G., Montagnani C., Gargano D., Peruzzi L., Abeli T., Ravera S., Cogoni A., Fenu G., Magrini S., Gennai M., Foggi B., Wagensommer R.P., Venturella G., Blasi C., Raimondo F.M., Orsenigo S. (Eds.), 2013. Lista Rossa della Flora Italiana. 1. Policy Species e altre specie minacciate. Comitato Italiano IUCN e Ministero dell’Ambiente e della Tutela del Territorio e del Mare

Schansker G, Tóth SZ, Strasser RJ (2005) Methylviologen and dibromothymoquinone treatments of pea leaves reveal the role of photosystem I in the Chl a fluorescence rise OJIP. Biochim Biophys Acta - Bioenerg 1706:250–261. doi: 10.1016/j.bbabio.2004.11.006

Spampinato G, Cameriere P, Crisafulli A, Gangale C, Picone R. M, Santangelo A, Uzunov D. *Woodwardia radicans* (L.) Sm. Informatore botanico italiano 40(S1), 2008, pp. 132-134

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A. (2004): Analysis of the chlorophyll a fluorescence transient. In: Govinjee, G.C. (Ed.), Chlorophyll a Fluorescence: A Signature of Photosynthesis. Springer, Dordrecht, pp. 321–362.

Živčák M, Brestič M, Olšovská K, Slamka P (2008) Performance index as a sensitive indicator of water stress in Triticum aestivum L. Plant soil Environ, 2008:133–139

Zivcak, M., Brestic, M., Kunderlikova, K., Olsovska, K., Allakhverdiev, S.I., 2015. Effect ofphotosystem nactivation on chlorophyll a fluorescence induction in wheat leaves:does activity of photosystem I play any role in OJIP rise? J. Photochem. Photobiol. B:Biol. 152, 318– 4.

Chapter VII

7. COPING WITH STRESS: A COMPARATIVE STUDY ON DRIVING SPECIES OF *WOODWARDIA RADICANS* COMMUNITY IN THE FERRIERE VALLEY

7.1. ABSTRACT

An ecological study was carried out on the effect of light stress on some species sharing the same moist habitat in the Ferriere Valley (Campania, Italy) at species-specific and ecological level. At intraspecific level, juvenile stages (gametophyte, newly developed sporophyte, young sporophyte) and mature sporophyte were tested in two ferns (*Woodwardia radicans* and *Adiantum capillus-veneris*).

Interspecific differences in response to light were tested in mature stages of three Bryophites, one moss and two hepatics, (*Palustriella commutata, Pellia endiviifolia, Conocephalum conicum*) and three ferns (*Woodwardia radicans*, *Adiantum capillus-veneris* and *Polypodium vulgare*).

An additional experiment was added on resistance to desiccation in one hepatic (*Conocephalum conicum*) and one fern gametophyte (*W. radicans*).

Light stress was measured through Chlorophyll fluorescence (ChlF) parameters after lab-induced light stress. We considered Fv/Fm, related to capacity of LHC, and effective quantum yield (Φ_{PSII}), indicating the amount of photochemistry following the absorption of light. Both parameters are usually affected by excessive radiation. Light treatments, increasing in PPFD and/or time, were used to induce photoinhibition (PI) in the samples; chlorophyll fluorescence measurements were made immediately before and after the induction and then every 20 minutes until 3 hours after the PI treatment. ChlF parameters were plotted as time series and analysed, along with quenching parameters, to compare light stress sensitivity and protective mechanisms, through life stages and between species. Young stages in both ferns showed sensitivity to light and light duration, being more sensitive to a 1200 µmol m^{-2} sec^{-1} PAR treatment for 90 min, than to 1500 µmol m^{-2} sec^{-1} PAR for 60 min. In the mature leaves *A. capillus-veneris* showed the highest stress while *P. vulgare* was the more resistant. Among the Bryophites the most sensitive to light stress appeared to be *C. conicum*.

Tolerance to desiccation in *C. conicum* and *W. radicans* gametophyte, was tested by continuous measuring of ChlF parameters during desiccation, relating them to the declining relative water content (RWC) of the samples. Steady-state chlorophyll fluorescence (F_S), qN (non-photochemical quenching) and Φ_{PSII} (effective quantum yield) were analysed. In both species, during desiccation, the decrease in Φ_{PSII} was accompanied by an increase in F_S. The RWC at which a decline in Φ_{PSII} was observed (around 30 – 40%) may indicate that the species could be ranked among desiccation semi-tolerant. Increase in qN with gradual desiccation was gradual and constant in *C. conicum* but appeared later and steeper in *W. radicans*. Generally, both species demonstrated to possess effective protective mechanisms against desiccation, which however was found insufficient to cope with a short-term severe drought.

Key words: Chlorophyll fluorescence parameters, non-photochemical quenching, fern, desiccation

7.2. INTRODUCTION

Light has a very wide range of intensity, making it difficult for autotrophs to cope with high, and often sudden, variations. When high light exposure occurs, the photosynthetic apparatus could undergo temporary or permanent loss of efficiency; this reduction of photosynthetic productivity is called photoinhibition (PI). In order to protect photosynthetic apparatus from energy fluctuations, photosynthetic organisms have developed, through evolution, a variety of mechanisms, including limitation of absorption, dissipation of adsorbed energy, adaptation to light energy input. Traditionally adaptation to control high light are classified, and studied, as: long-term adaptations (acclimation) and short-term adaptation. The first is depending on genetic structure and expression, takes long time to be activated and is not effective in cases of quick increase of light energy.

Short-term mechanisms provide photoprotection in environments subject to sudden changes in light radiation. Among these, non-photochemical quenching (NPQ) is one of the most investigated processes of photoprotection in plants. NPQ is a non-radiative, non-photochemical mechanism related to light harvesting complexes (LHC) structure and activity, energy transfer to reaction centres (RC), electron transport chain and protonation of the chloroplast stroma. NPQ is studied through chlorophyll fluorescence quenching analysis in a variety of methods; we used slow Kautsky kinetics supplemented with quenching analysis. Photoinhibition is measured by the ratio variable/maximum Chlorophyll fluorescence Fv/Fm, related to capacity of LHC, and effective quantum yield, indicating the amount of photochemistry following the absorption of light. Both parameters are usually affected by excessive radiation, denoting negative interferences at different levels; in the first case LHC are affected by either chlorophyll and protein complexes destruction, or conformation changes. In the second protective mechanisms such as the carotenoids epoxidation, the xanthophylls cycle and non-photochemical quenchers are involved in the reduction. In or study we analysed the effect of light treatment differing in intensities and duration on selected group of plants to determine the interspecific or age-related response to light stress. An additional stress, water deficiency, was analysed in the association *Conocephalo-Woodwardietum radicantis.*

7.3. MATERIALS AND METHODS

7.3.1. Area of study

The Ferriere Valley is a deep valley rich in water and waterfalls, with a permanent stream at its bottom (Canneto) located in the southern side of the Sorrento peninsula, it is surrounded by mountains and peaks, the climate is warm temperate (20°C to 5°C). The average annual rainfall of 1 500 (mountain) to 1 000 (sea level) mm, hits its maximum in the autumn - winter period. The proximity of the mountains to the coast is the cause of the high rainfall and mists because of condensation of humid air brought from the sea by the wind. Thanks to many microclimatic conditions, the valley is a hotspot of biodiversity, and as such, a protected area.

7.3.2. Plant material

The choice of species has been determined by the community characteristics: *Palustriella commutata*, growing under *W. radicans* fronds, is the species of the EU priority habitat 7220 *Cratoneurion* "Petrifying springs with tufa formation habitat", *Pellia endiviifolia* was always found associated with the previous one in our sampling area, *Conocephalum conicum* is the liverwort of the association *Conocephalo-Woodwardietum radicantis*, typical for *W. radicans*; *Adiantum capillus-veneris* and *Polypodium vulgare* are two ferns found in the same site in a gradient of increasing light.

Pellia endiviifolia is a thallose liverwort with green or blackish-green thalli up to about 1 cm wide. It is a dioicous species found in shaded and moist environment, underwood, watercourses, dripping rocks (Atherton et al. 2010).

Palustriella commutata is a moss with green or yellowish-brown shoots, 4-6 cm long or more, with a densely and regularly pinnate pattern of branching. This type of branching looks rather feather-like, but calcareous deposits on the shoots make them stiff and rough. The stem is covered by tiny leaf-like structures and red-brown rhizoids. The species has triangularly heart shaped leaves. It is found in all kinds of wet base-rich soil (fig. 7.1).

Fig. 7.1 *P. commutata* and *P. endiviifolia*

Conocephalum conicum is a liverwort whose thallus, ribbonlike, deep green to dark green, is 5–20 cm long and 1–2.5 cm. wide, forked, broadly lobed, shows characteristic hexagonal patches bearing in the middle, above the respiratory cavities, slightly raised hyaline airway openings. Unicellular rhizoids are numerous on the underside, anchoring the thallus to substrate. The species is dioecious, with male gametes produced in antheridia submerged in flask-like cavities that open into conical papillae. The male gametes swim to the archegonia to fertilize the eggs inside. The spores are released by the rupture of sporangium produced at the end of the sporophyte (fig. 7.2).

Fig.7.2 *C. conicum*

Woodwardia radicans is a rhizomatous fern with fronds long up to more than 2 meters, characteristic elongated sori long the middle rib of the bipinnatifid leaf. This chain-like arrangement of the sori gives the fern its common name chain fern. It is homosporous, fertile leaves producing one kind of spores whose germination produces gametophytes with all combination of gametes. Vegetative reproduction is realized through rhizome dividing and by apical bulbils on the underside of the fronds, which can form independent individuals when touching the moist soil.

Adiantum capillus-veneris is a fern with fronds bearing numerous small leaflets in clusters. Leaves are delicate, pale green with spore on the underside protected by indusia. The frond rachis is black (fig. 7.3).

Fig. 7.3 *A. capillus-veneris*

Polypodium vulgare is a rhizomatous fern, with fronds 10 to 50 cm. long, bearing triangular leaflets, disposed in pairs (up to 18 pairs) gradually shortening at the tip of the frond. The leaflets become much shorter at the end of the frond, slightly denticulated and wider at their base, where they often touch each other. They have an alternating arrangement, those on one side being slightly offset from those on the other side. Sori are on the lower side of the fronds and range in colour from bright yellow to orange, brownish at maturity.

All plant material, with the exception of *W. radicans*, was proceeding from the Ferriere valley and cultivated in greenhouse with the average conditions of the area of origin. *W. radicans* plants and spores were collected in Botanical garden of Naples – Italy. Spores were cultivated in growing chamber.

7.3.3. Cultivation

W. radicans and *A. capillus-veneris* gametophytes and sporophytes were obtained by cultivation of spores in black peat. Freshly collected fronds were soaked for 10 minutes in a 10% solution of commercial sodium hypochlorite (5%). They were dried by gently blotting them with sterilized filter paper. Sporangia where removed from sori still covered by indusia in flow chamber and put in sterilized Petri dish on Whatman lens cleaning tissue 105 and left to open and release the spores. Spores separated by their sporangia were sowed in 22 mm sterilized Petri dishes with black peat. Sterilized water was filling the soil to maximum capacity. Sealed Petri dishes were put in growing chamber at 25±5 °C and 45 µmol m^{-2} sec^{-1} PAR L/D photoperiod 16/8. After 8 - 20 weeks fertilization produced new sporophytes. Juvenile sporophytes were defined as 2, 3 leafed sporophytes, whose leaves were still round and did not initiate the leaf indenting typical of mature stages (fig. 7.4, 7.6). Young sporophytes were defined as 4-5 leafed sporophytes whose leaves were clearly presenting the leaf indentation of mature leaves (fig. 7.5).

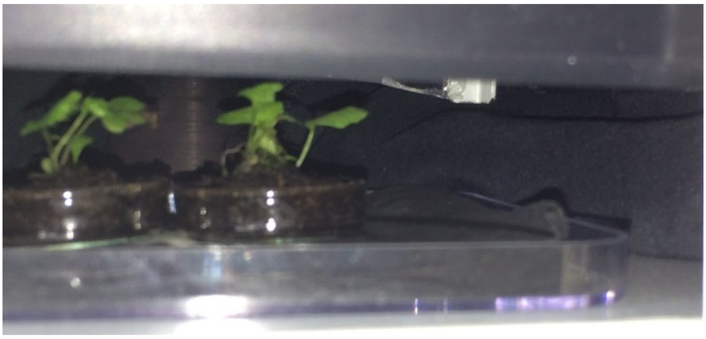


Fig. 7.4 juvenile sporophytes under the measuring hood.

Fig. 7.5 young sporophyte

Fig. 7.6 newly emerged sporophytes (*W. radicans*)

7.3.4. Plant handling and treatments

Samples were acclimated to lab condition for two hours at 21,5 ± 0,7 °C and 20 µmol m^{-2} sec^{-1} PAR before treatments.

Light treatments (LED source PSI SL3500-498) PAR and time as follows: 700 µmol m^{-2} sec^{-1} 30 min, 1200 µmol m^{-2} sec^{-1} 30 min, 1200 µmol m^{-2} sec^{-1} 60 min, 1200 µmol m^{-2} sec^{-1} 90 min, 1500 µmol m^{-2} sec^{-1} 60 min.

All Kautsky kinetic measurements were carried out on pre-darkened (5 min) samples with a PSI Handy Fluorcam HFC-010 (Photon Systems Instruments, Czech Republic). They were recorded immediately before and after the photoinhibitory (PI) treatment and every 20 min for three hours after the end of PI. Recovery after PI was in dim light, 10-15 µmol m^{-2} sec^{-1} PAR. Time series were plotted for every significant parameter and examined.

7.3.5. Desiccation measurements

Measurements of chlorophyll fluorescence (ChlF) parameters were recorded continuously on samples desiccating from fully wet state to dry state, they were then related to relative water content (RWC) calculated as RWC (%) = $[(F_M-D_M)/(F_W-D_M)]*100$ where F_M is the fresh weight, D_M is the completely dry weight and F_W is the fully wet weight. Samples weight was continuously measured on an analytical Mettler scale (Mettler AS100, Germany), while (ChlF) parameters were recorded with a PAM-2000 fluorometer (H. Walz, Germany); pulses were applied on the samples at light-acclimated state (exposed to 20 µmol m^{-2} sec^{-1} PAR) at 10 min. intervals. Each recorded parameter was related to the corresponding RWC.

7.4. RESULTS

7.4.1. Photoinhibition

From the Fv/Fm and Φ_{PSII} time courses (fig 7.9, 7.11, 7.15) in gametophyte and new sporophytes of *W. radicans*, it follows that the most pronounced decrease was found in the treatment 1200 µmol m^{-1} sec^{-1} for 90 min. Apparently, the treatment of 1500 µmol m^{-1} sec^{-1} for 60 min led to a smaller decrease in Fv/Fm and Φ_{PSII}. Similarly, the photo-inhibitory treatment of 1500 µmol m^{-1} sec^{-1} for 60 min brought faster recovery than treatment of 1200 µmol m^{-1} sec^{-1} for 90 min. This indicates that rather duration of high light stress than high light intensity has a dominant role in photo-inhibitory changes in PSII. Such conclusion might be supported by the fact that both Fv/Fm and Φ_{PSII} recovered to about 80% in the 1200 µmol m^{-1} sec^{-1} for 90 min treatment while the recovery was above 80% in all the other treatments. Such effect, however less pronounced, is apparent in *W. radicans* young sporophyte, the only difference being the levels of NPQ parameter (fig. 7.13, 7.15, 7.17). In juvenile forms it was at the maximum in the longest treatment, while in the young sporophyte NPQ parameter was light-dependent. Furthermore, in young sporophytes NPQ component were equally concurring, while in the juvenile form they were slightly differently distributed. Juvenile and young sporophytes of *A.* capillus-*veneris* showed different behaviours, the first being light duration-affected, while the latter sensitivity was light intensity-dependent (fig. 7.18, 7.20). However protective mechanisms were activated proportionally to the intensity of light (fig. 7.21, 7.22).

On leaf level, *W. radicans,* and *A. capillus-veneris*, showed similar time courses of chlorophyll fluorescence parameters in responses to high light stress and during consequent recovery. They showed similar initial values for Fv/Fm, and ΦPSII. Immediately after photoinhibitory treatment the declines in Fv/Fm, and ΦPSII were in similar order, i.e. reflecting increasing doses but higher decline in the longest treatment (fig. 7.33, 7.35); Polypodium (fig. 7.39), on the contrary, was responding only to doses. Recovery was almost completed after 3h, especially in the treatments (1 200 μmol m^{-2} s^{-1} for 30 and 60 min). Photoinhibition-induced NPQ was apparent in all species and, light dose dependent. In majority of cases, maximum NPQ values were reached in the most severe treatments, i.e. 1 200 μmol m^{-2} s^{-1} for 90 and 1 500 μmol m^{-2} s^{-1} for 60 min (fig, 7.37, 7.38, 7.41).

The accompanying species, *P. endiviifolia* and *P. commutata* showed similar behaviours in their responses to high light stress. In controlled photo-inhibition experiments, they have similar initial values for Fv/Fm, heavy drops in both Fv/Fm and Φ_{PSII} in the two most severe treatments, very distant from the other two treatments (fig. 7.25, 7.26, 7.30). The time courses of NPQ were similar in the two species (fig. 7.28, 7.29, 7.32), showing slow recovery even in the weakest photo-inhibition treatment (700 for 30 min). This may indicate insufficient pool of antioxidative substances in the species and, generally low capacity of protective mechanisms involved into non-photochemical quenching. Such physiological behaviour is typical for obligate shade plants and/or shade tolerant plants.

On the other hand, *C. conicum* shows lower initial values and more proportional drops to treatments especially if the drops are not absolute values but considered proportional to initial values. Apparently, they are more shade-adapted and the two most severe treatments are reaching more distressful conditions. This finding could be supported by the fact that the uncompleted recovery of Fv/Fm and Φ_{PSII} did not reach even 80% of initial values (fig. 7.32) while in the other two species it was found far above this value (fig. 7.25, 7.27). This might be considered as an indicator of particularly higher sensitivity of *C. conicum* to photo-inhibition than the other two species. Nevertheless, *C. conicum* showed a constant rate of response to PI, even in the most severe treatment which was not applied to the other ones.

7.4.2. Desiccation on *W. radicans* gametophyte and on thallus of *C. conicum*

Controlled desiccation led to a decrease in primary photosynthetic processes, which was well documented by Φ_{PSII} decline in the course of decreasing relative water content (RWC). RWC at which Φ_{PSII} showed the first sign of decline was found between 30 - 40% of RWC. With further decrease in RWC, Φ_{PSII} showed a rapid decline following a S-curve. Steady-state chlorophyll fluorescence (F_S) in *C. conicum*, showed slight increase starting around 10 - 20% of RWC (fig. 7.7), while in *W.*

radicans gametophyte the increase started around 40 until 20 % of RWC value (fig. 7.8). The response of F_S with desiccation was species-specific. However, the increase in Fs corresponded to the beginning of qN increase which is activated in low RWCs as the mechanism protecting PSII.

Non-photochemical quenching (qN) showed species-specific difference. It started to increase constantly and slowly from the very beginning in *C. conicum*, and with substantial increases from (40 % RWC), in *W. radicans* gametophyte. 50% of Φ_{PSII} initial values were reached at RWC 18% in *C. conicum* and 22% in *W. radicans* gametophyte.

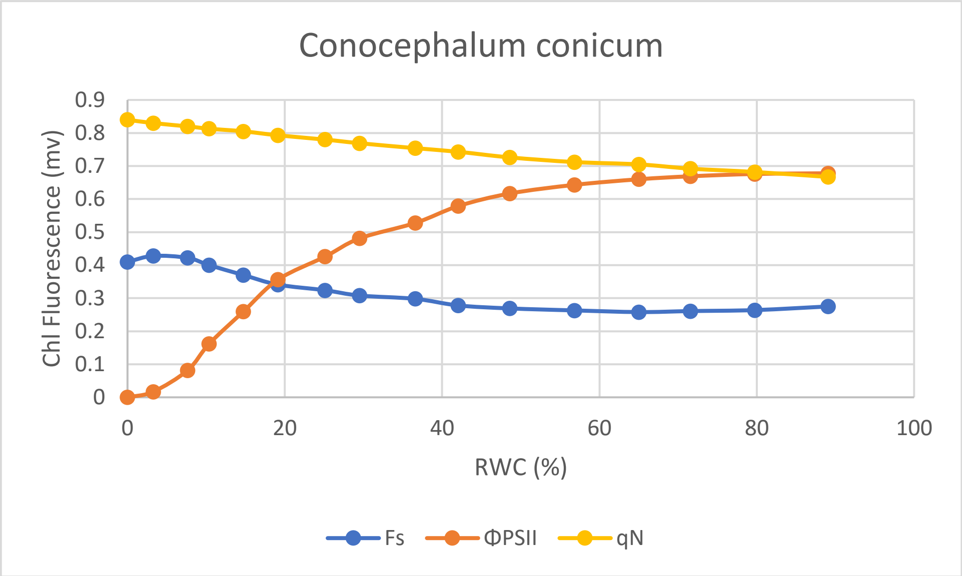


Fig. 7.7 ChlF parameters course during desiccation in *C. conicum*

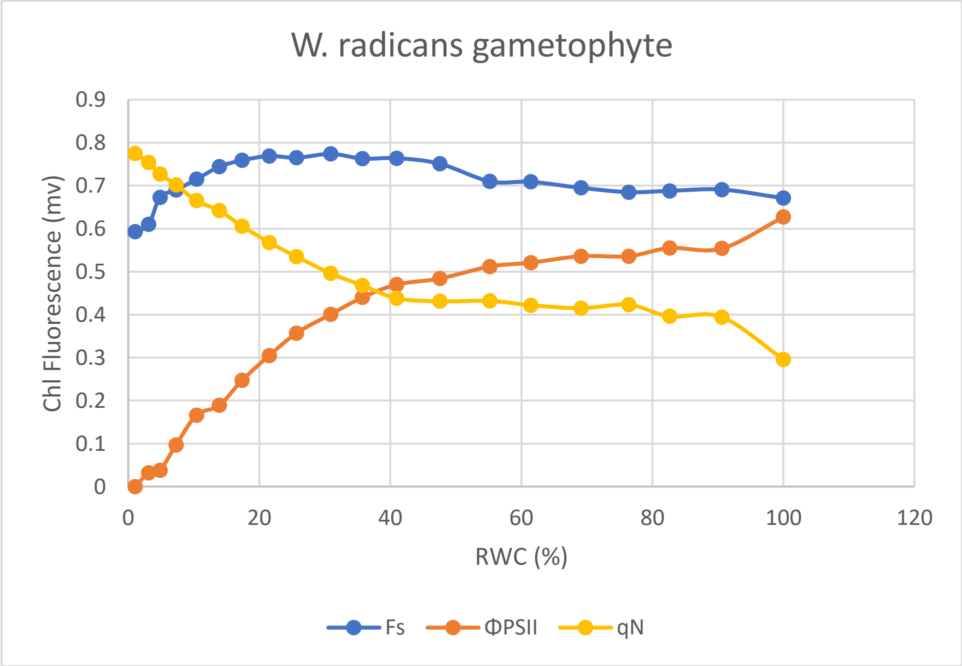


Fig. 7.8 ChlF parameters course during desiccation in *W. radicans*

7.5. DISCUSSION

7.5.1. Photoinhibition

Intraspecific and interspecific comparison of young stages in two ferns

The noticeable inability of juvenile sporophyte of *W. radicans* and *A. capillus-veneris* to achieve full recovery in Fv/Fm over short period (in term of hours) may be interpreted as evidence for PS II photodamage. In PSII the damage is associated with D1 protein degradation. Restoration of PSII activity requires the synthesis of new D1 protein for regenerating the inactivated one. Contrastingly, the complete recovery of Fv/Fm in plants exposed to high light stress is attributed to PSII downregulation or dynamic photoinhibition (Osmond 1994). These results are in accordance with those reported in the literature (e.g. Hu et al. 2007). The Fv/Fm and Φ_{PSII} time courses in juvenile sporophyte of *W. radicans,* however, did not show such recovery, which can be interpreted as evidence for a certain degree of irreversible loss in PSII photochemical efficiency as a consequence of photo-inhibitory treatment.

Uncompleted recovery is typical for shade-grown species or obligatory shade tolerant, when exposed to a short-term high light event as shown e.g. for *Rhododendron ponticum* (Osório et al. 2010).

Typically, such response is associated with the early stages of chronic photoinhibition (Björkman and Demmig, 1987). Similar results, i.e. uncompleted recovery, are reported even for plants grown in lit environments if the high light dose is too high and causing PSII damage, sometimes in interaction with other stress factors (Olsson et al. 2000). *A. capillus-veneris* showed the same courses but with lower recovery in the young sporophytes.

The finding that lower high light dose applied for longer period (90 instead of 60 min) caused more pronounced photoinhibition than higher dose applied for 60 min is in a good agreement with earlier observation made in lichens. It was shown for *Lasallia pustulata*, that duration of photo-inhibitory treatment rather than quantity and frequency of high light inhibits primary photosynthetic processes (Barták et al. 2008). If partial desiccation co-act with a high light episode, negative effects on Fv/Fm and Φ_{PSII} time courses are more pronounced and typically demonstrated as a more substantial decrease of Fv/Fm and Φ_{PSII} values immediately after photo-inhibitory treatment and slowed down or diminished recovery.

Finally, NPQ time courses in juvenile and young sporophytes showed that, even if protective mechanisms were activated, these were not sufficient in the prolonged treatments; at the same time the direct proportion to light intensity in the young stage shows that the protective pattern which was maybe still underdeveloped in the juvenile form, took full shape in the young form. Resistance to brief intense light, but sensitivity to prolonged light stress, could be interpreted as adaptation to under canopy conditions, where medium-low light could be modified by sunflecks.

Mature leaves on the three ferns

Partially uncompleted recovery of NPQ and particular components of non-photochemical quenching (qI, qE) was apparent in *Woodwardia,* and *Polypodium* indicating involvement of photoprotective mechanisms during high light treatment and their relaxation during recovery period. Contrastingly, *Adiantum* showed only limited recovery of NPQ, qI and qE. This behaviour, in combination with less pronounced recovery of ΦPSII suggest that *Adiantum* was more susceptible to photoinhibition.

Involvement of NPQ into the response of *Woodwardia, Polypodium*, and A*diantum* to high light stress suggests that both dissipation of excess absorbed energy into heat as well as activation of particular dissipative pathways, i.e. the energy-dependent quenching (qE), and photoinhibitory quenching (qI) (Derks et al., 2015) worked efficiently in all three species. However, *Adiantum* showed less pronounced recovery, indicating that the protective mechanisms remained active for longer time than in the other two species. It is well established that zeaxanthin, in particular increase the sensitivity of LHCII to the lumen protons inducing qE (Horton et al., 2000, Ruban et al., 2012). The qI coefficient is the result of photoinhibition and is caused predominantly by inactivation and/or degradation of D1 protein. Nevertheless, qI depends only partially on D1 degradation and this independent portion of quenching has been recently named qH (Malnoë et al., 2017) that encompasses different processes. Some of which aimed at photo-protecting, occur in the antenna possibly with similar mechanisms involved in qE. However, these components act in a different way to dissipate the excess of excitation energy and are involved in the adaptive response to environmental constraints (Malnoë et al., 2017). The only dose-dependent response of *P. vulgare* is consistent with sun-adapted species. For *Woodwardia, Polypodium*, and A*diantum*, however proportion of qH have not yet been evaluated and remains a challenge for future studies.

Coexisting species, Bryophytes

In the coexisting species inter-specific comparison of Fv/Fm and Φ_{PSII} time courses suggested that *C. conicum* was more susceptible to photoinhibition than *P. endiviifolia* and *P. commutata*. It is likely because of lower capacity of photosynthetic processes associated with PSII, i.e. lower Fv/Fm found in *C. conicum* before the photo-inhibition experiment could indicate low level of photoinhibition of PSII of the species even before the photo-inhibition treatment. In such cases, a plant grows in higher than optimum light environment, which leads to cumulative (long-term) photoinhibition demonstrated as a Fv/Fm decline.

The parameter Φ_{PSII} provides information on the electron transport rate and, differently to the Fv/Fm ratio (determined in dark-adapted conditions) on the nature of photoinhibition. The decline in Φ_{PSII} found in the three experimental species is due to the inactivation of PSII reaction centres aimed at photoprotection (Krause et al., 1990) or may be a mechanism which adjusts the efficiency of PSII to photosynthetic photon flux density (Critchley, 1994). Besides, under light, Φ_{PSII} depends on the activity of energy-consuming biochemical reactions of CO_2 assimilation. Therefore, we may expect that *P. endiviifolia, P. commutata* and *C. conicum* decreased their fixation after photo-inhibition treatment and re-adjusted during recovery. Activation of photoprotective mechanism in all three species is well documented by NPQ.

The general conclusions are:

- all tested species (excepted *P. vulgare*) showed adaptation to low light conditions
- all of them showed protective mechanisms with huge differences in effectiveness, functioning and time of appearance during life cycle

- Some responses were consistent with evolutionary adaptation to sunflecks, suggesting under canopy habitat adaptation
- the effects of light stress should be further investigated in its long-term effects on individual development

7.5.2. Desiccation

In both species, decrease in Φ_{PSII} was accompanied by an increase in F_S. Generally, the F_S responses found in *W. radicans* gametophyte and *C. conicum* differed from lichens that show a rapid F_S decline with RWC decrease from 100 to 0% (Barták , unpublished). The response of F_S, however, seems to be specific to different plant groups and ecotypes. Mosses and liverworts exhibit more or less constant Fs within a wide range of RWC as documented from slow Kautsky kinetics (Giudici et al. 2018). Contrastingly, Tuba et al. (1996) reported big differences in chlorophyll fluorescence signal in *Tortula ruralis*. Therefore, in follow-up studies, attention should be payed to the changes in F_S during desiccation and the relation of F_S to Fv/Fm, qN and Φ_{PSII}.

Increase in qN with gradual desiccation is indicative of the involvement of protective mechanisms helping the plants to cope with the initial phases of drought stress. The co-acting increase in qN is associated with protective mechanisms activated under drought stress. Such behaviour is well documented for a wide variety of higher plants. The qN increase is associated with the protective mechanisms such as e.g. involvement of xanthophyll cycle pigments interconversion, i.e. violaxanthin (V) to zeaxanthin (Z) via an intermediate antheraxanthin. Zeaxanthin is capable to accept energy from excited PSII centres (with pronounced desiccation stress, increased excitation of PSII happens due to decreased photosynthetic electron transport rate). Delivery of energy to Z in desiccating plant thus protect core of PSII from over-energization and resulting oxidative stress. This is an avoidance strategy to protect core of PSII from photooxidative damage and, finally destruction if unfavourable conditions last too long. Desiccation-induced conversion of V to Z affects de-epoxidation state of the xanthophyll cycle pigment (DEPS) which is considered to be a sensitive parameter evaluating involvement of protective mechanisms in a plant response to stress factors.

The RWC at which a decline in Φ_{PSII} was observed (around 30 – 40%) may indicate that the species could be ranked among desiccation semi-tolerant, with different sensitivity, *W. radicans* higher than *C. conicum*, considering the RWC at which Φ_{PSII} lowered to 50% of its initial value (22 and 18% of RWC). However, these values place them among the tolerant species. In *W. radicans.* the qN increase at a low RWC (20-30 %) and its rapid and high increase might be indicative of effective protection in *W. radicans* gametophyte activated during episodes typical by high air temperature and resulting in high water saturation deficit in the air.

Chlorophyll fluorescence study in *W. radicans* gametophyte and *C. conicum* brought several findings: (1) both species do possess effective protective mechanisms against desiccation, (2) capacity of the mechanism, however sufficient to cope with a short-term severe drought stress, should be further investigated in long-term effects, (3) more experimental evidence is needed to evaluate underlying mechanisms, the extent and rate of their activation in particular, and their involvement as related to the speed of desiccation. The latter topic seems to be crucial for understanding the survival of particular species in the field, especially in the area of interest (Ferriere valley).

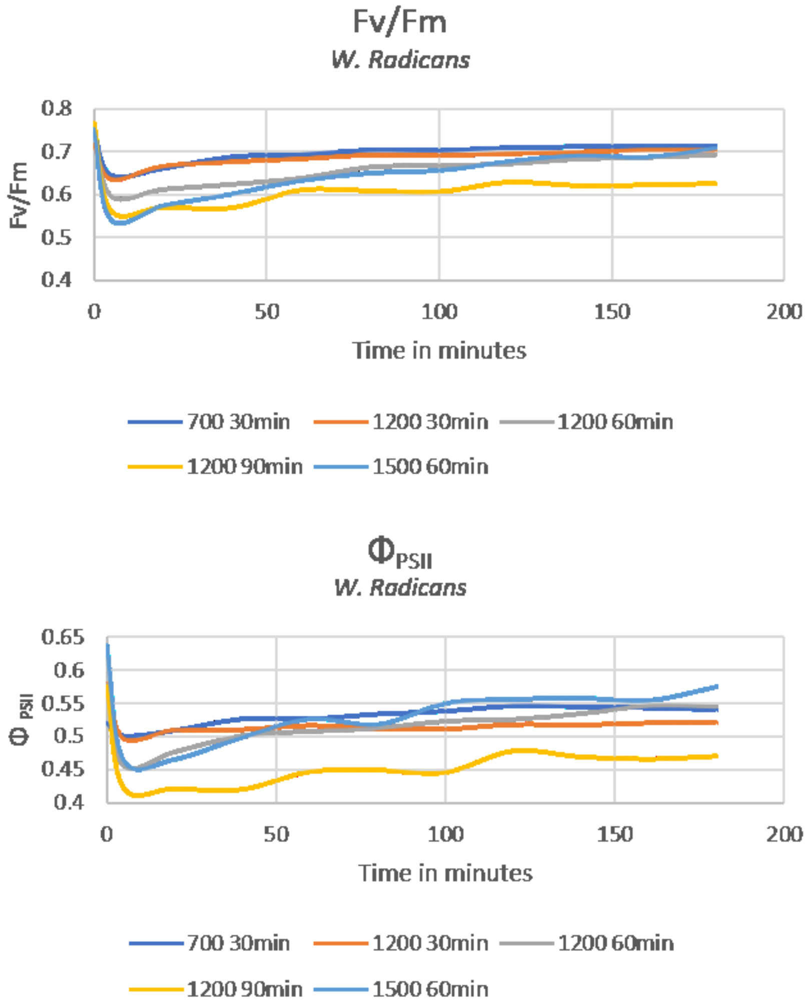


Fig. 7.9 Fv/Fm and Φ_{PSII} time courses of gametophyte of *W. radicans*

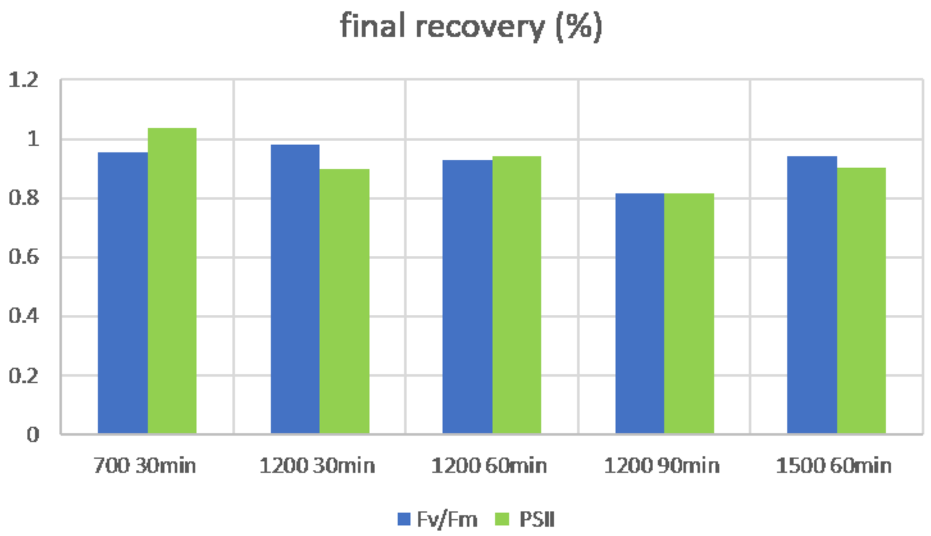


Fig. 7.10 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of gametophyte of *W. radicans*

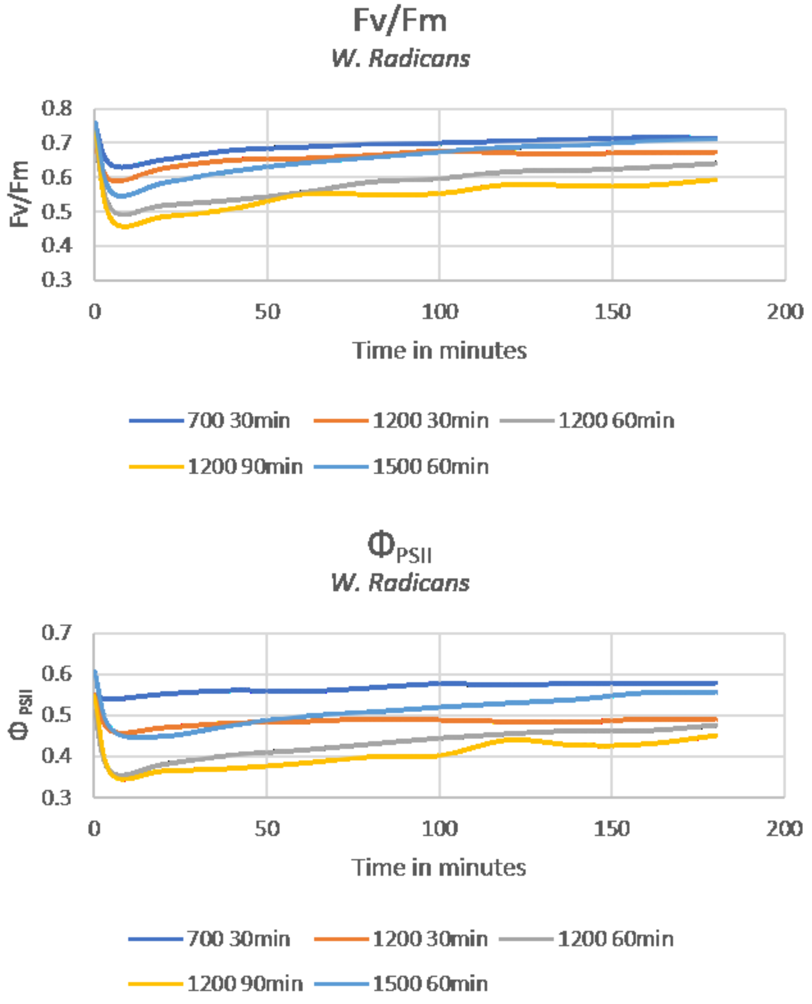
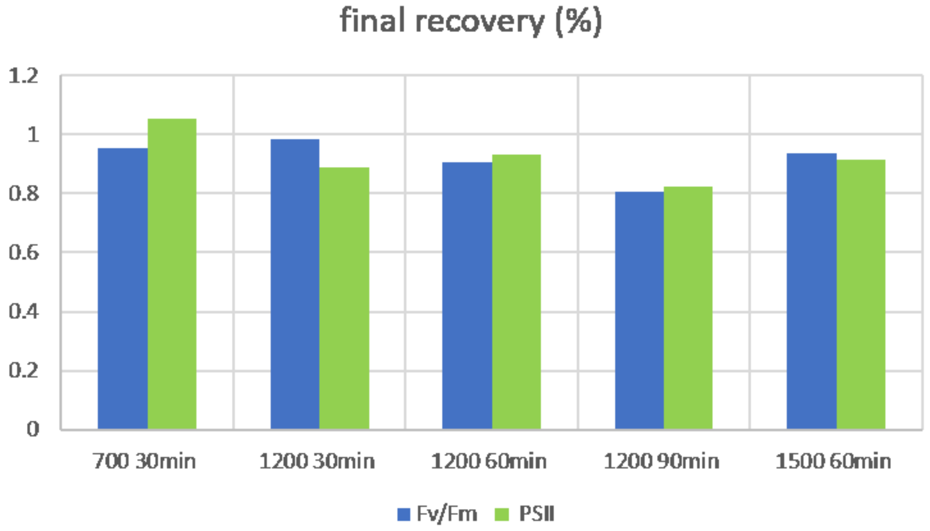


Fig. 7.11 Fv/Fm and Φ_{PSII} time courses of Juvenile sporophyte of *W. radicans*



F

sporophyte of *W. radicans*

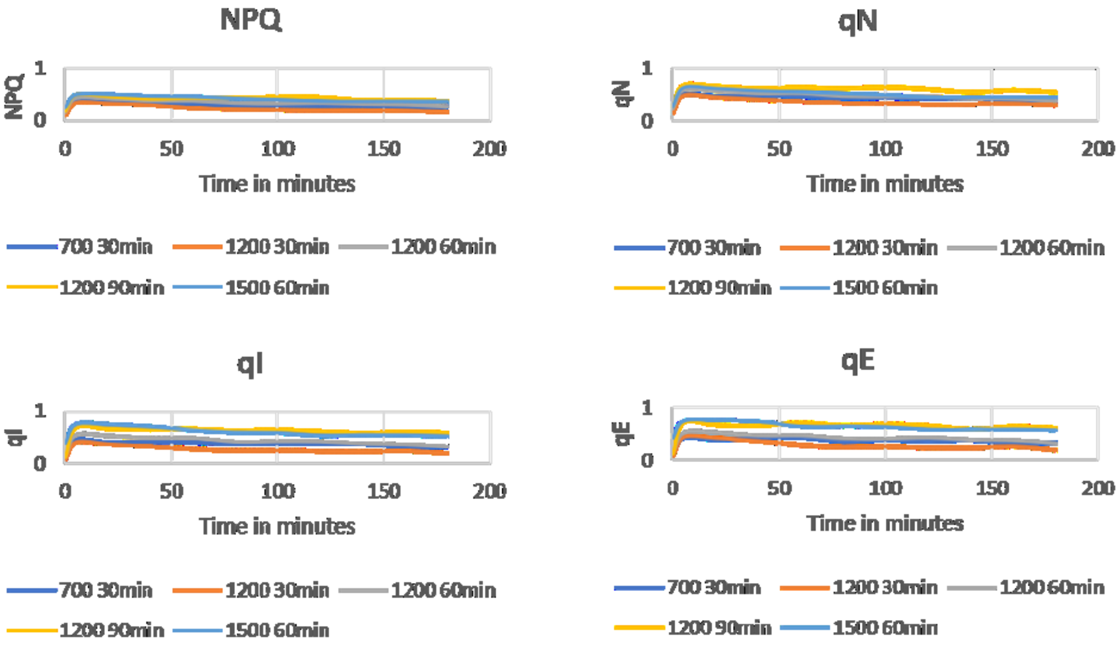


Fig 7.13 NPQ and its component time course in gametophyte of *W. radicans*

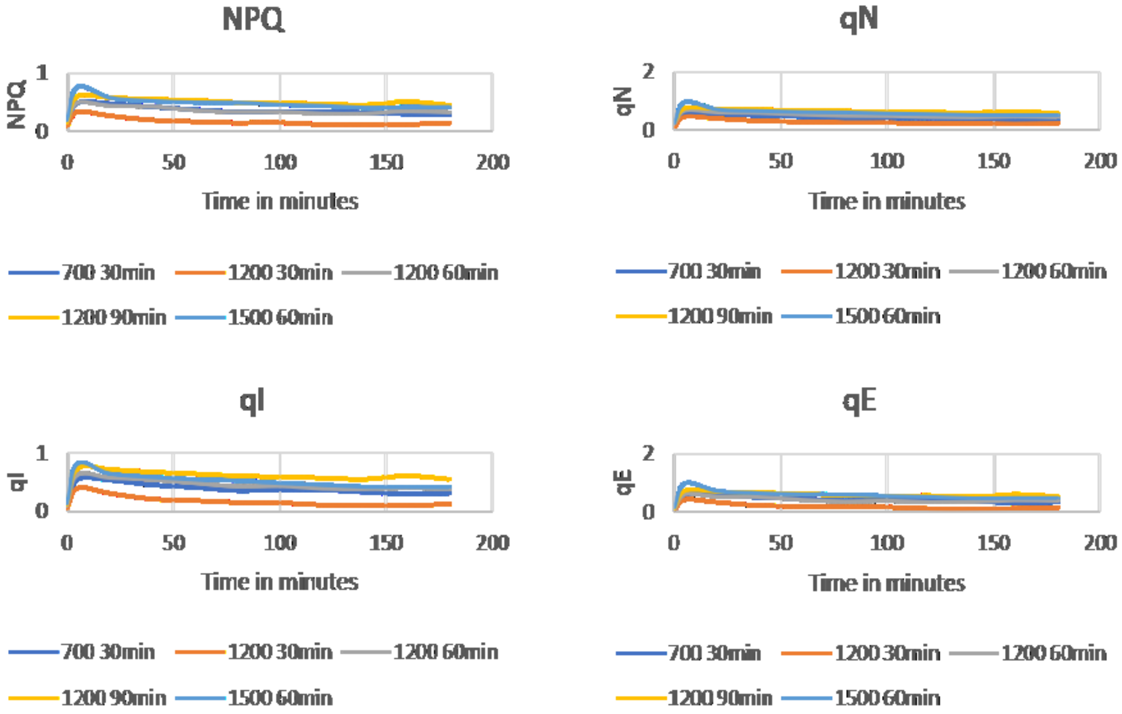


Fig 7.14 NPQ and its component time course in Juvenile sporophyte of *W. radicans*

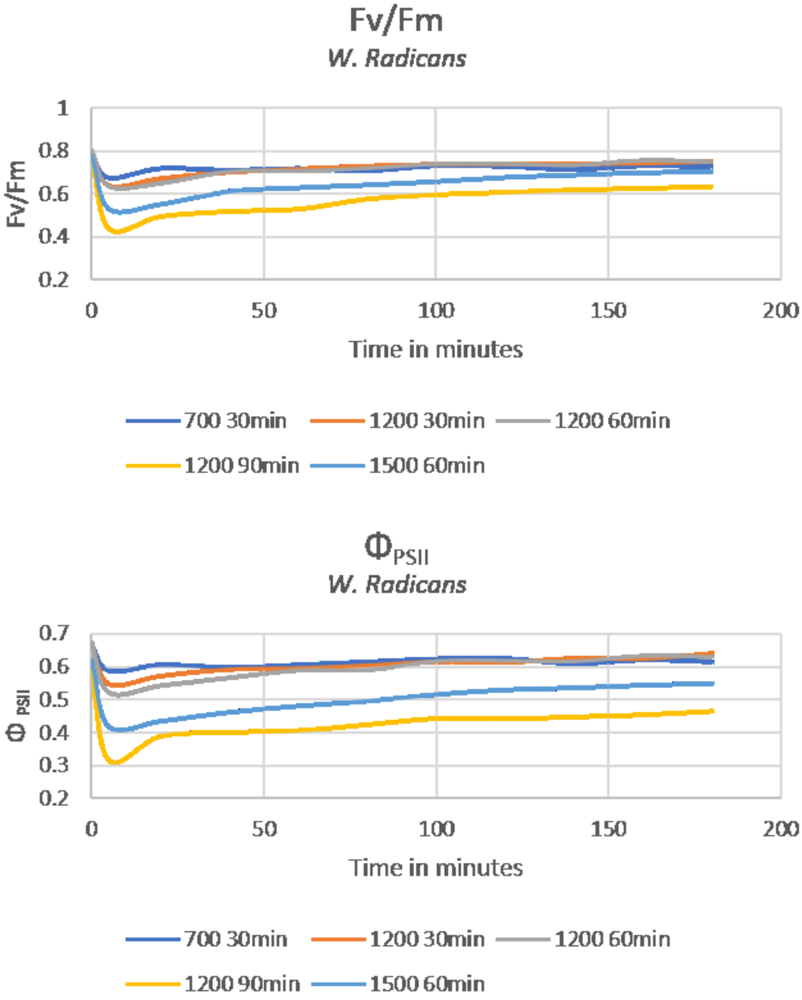


Fig. 7.15 Fv/Fm and Φ_{PSII} time courses of young sporophyte of *W. radicans*

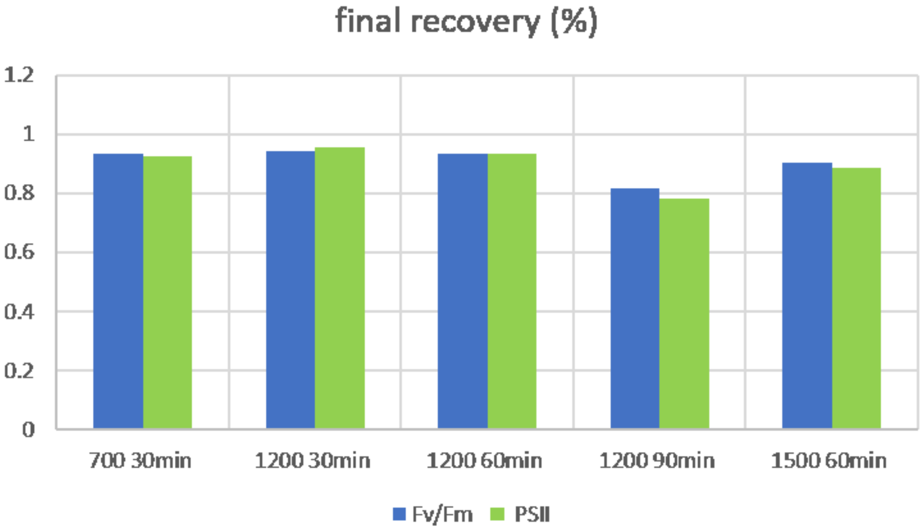


Fig. 7.16 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of young sporophyte of *W. radicans*

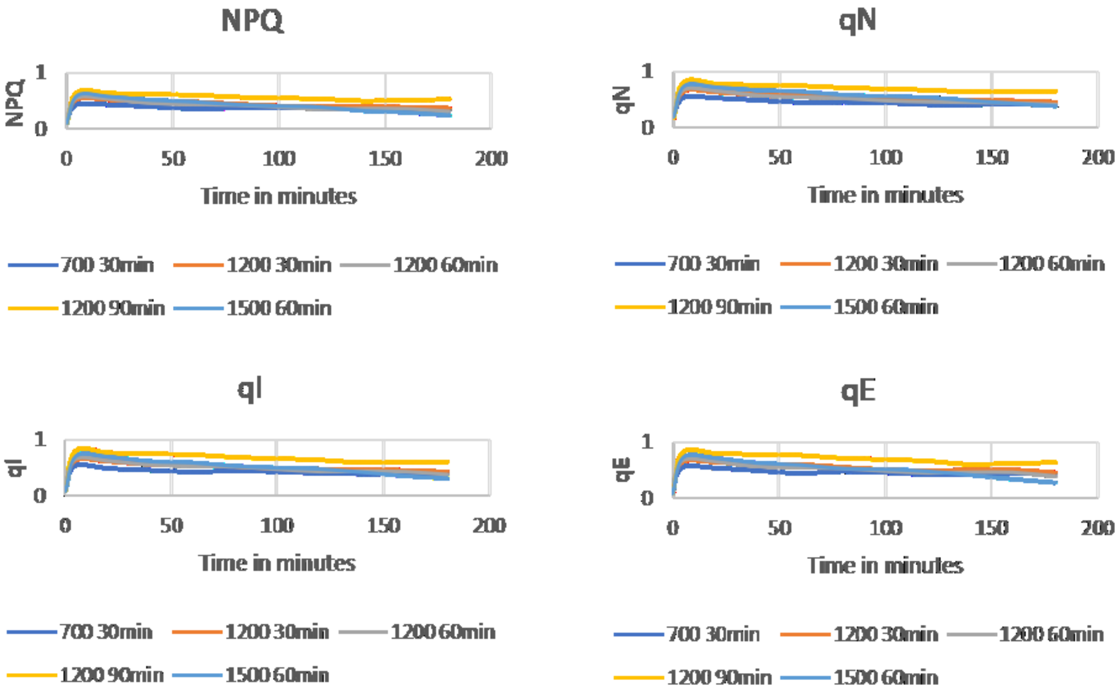


Fig 7.17 NPQ and its component time course in young sporophyte of *W. radicans*

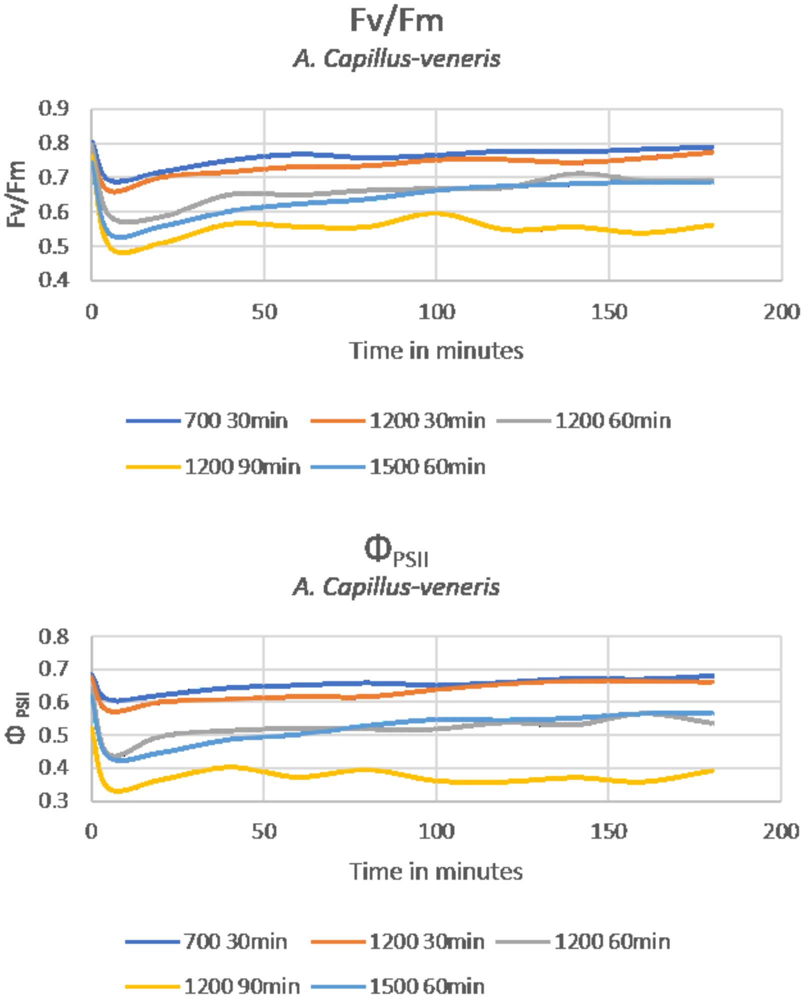


Fig. 7.18 Fv/Fm and Φ_{PSII} time courses of *A. capillus-veneris* juvenile sporophytes

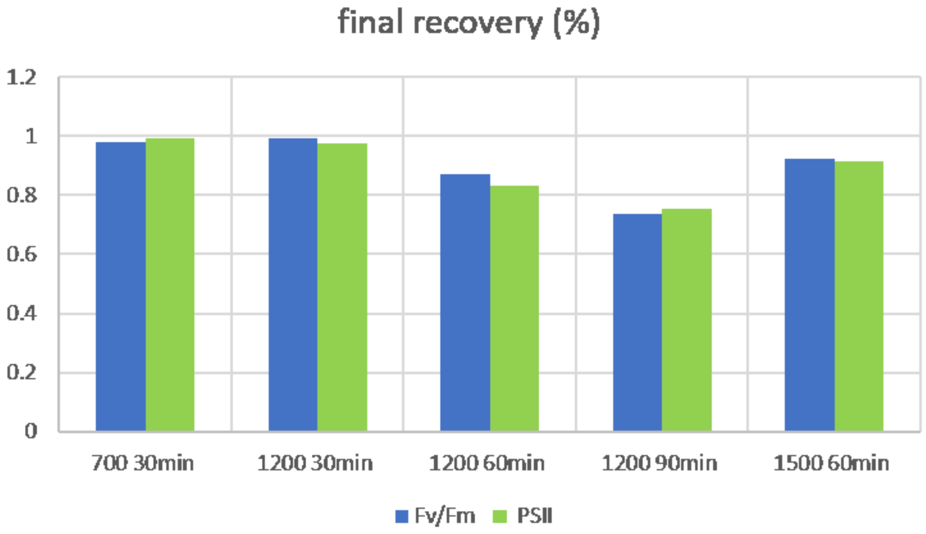


Fig. 7.19 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *A. capillus-veneris* juvenile sporophytes

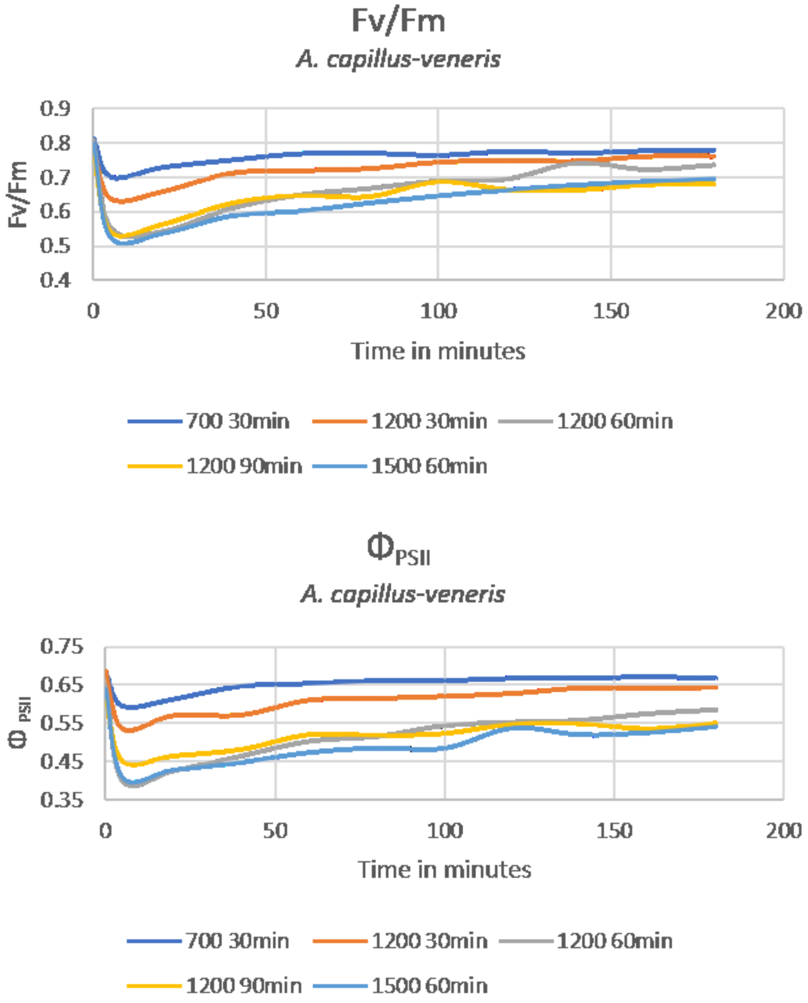


Fig. 7.20 Fv/Fm and Φ_{PSII} time courses of *A. capillus-veneris* young sporophytes

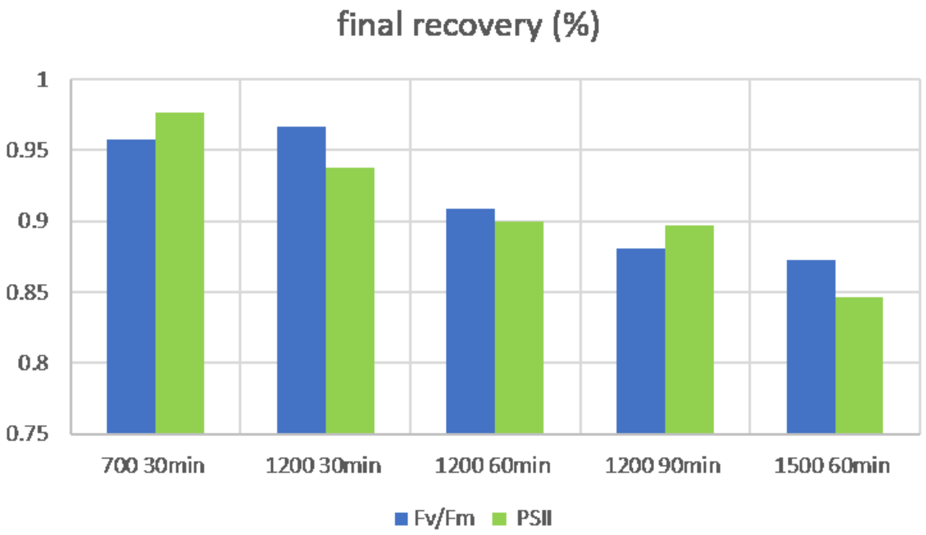


Fig. 7.21 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *A. capillus-veneris* young sporophytes

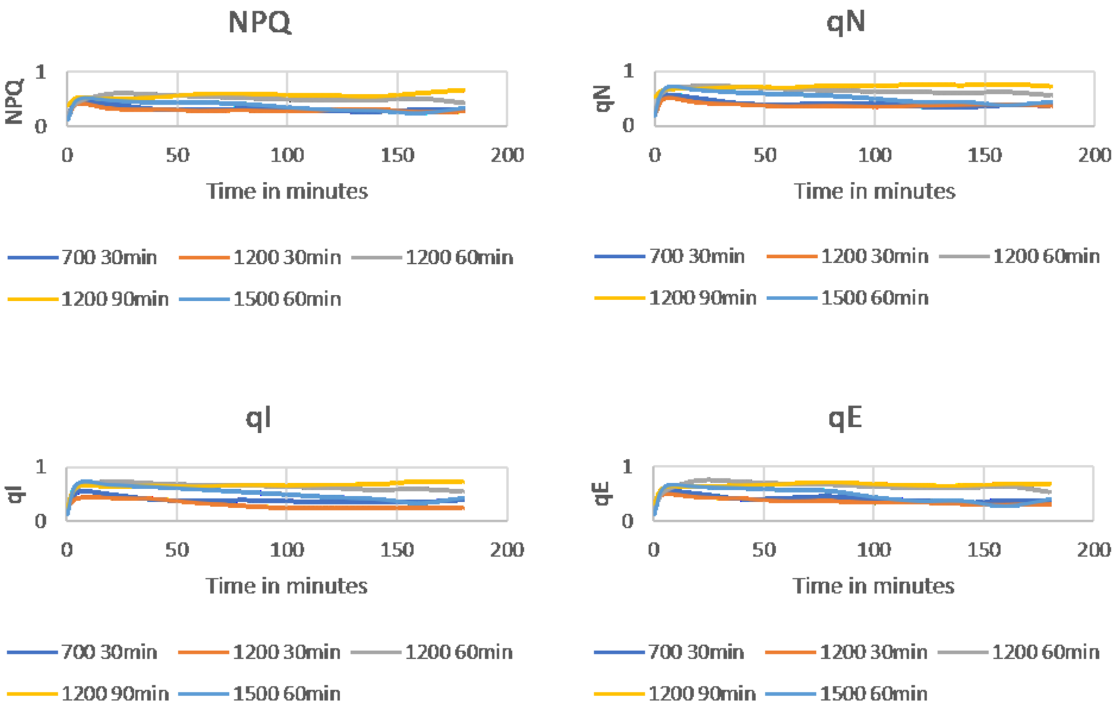


Fig 7.22 NPQ and its component time course in Juvenile sporophyte of *A. capillus-veneris*

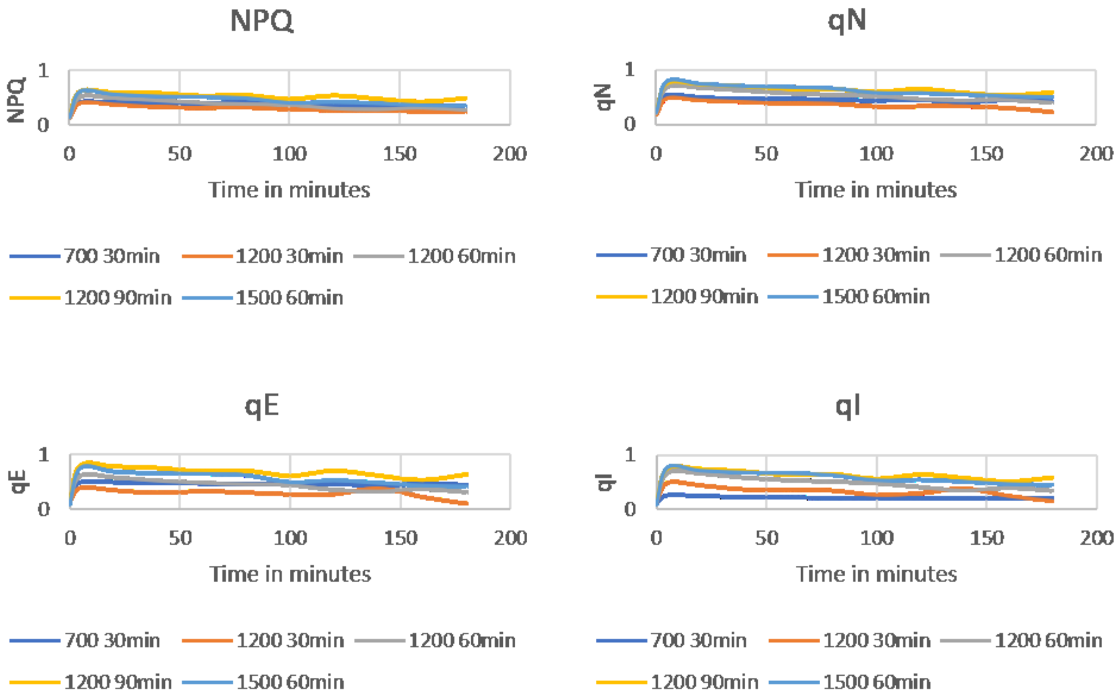


Fig 7.23 NPQ and its component time course in young sporophyte of *A. capillus-veneris*

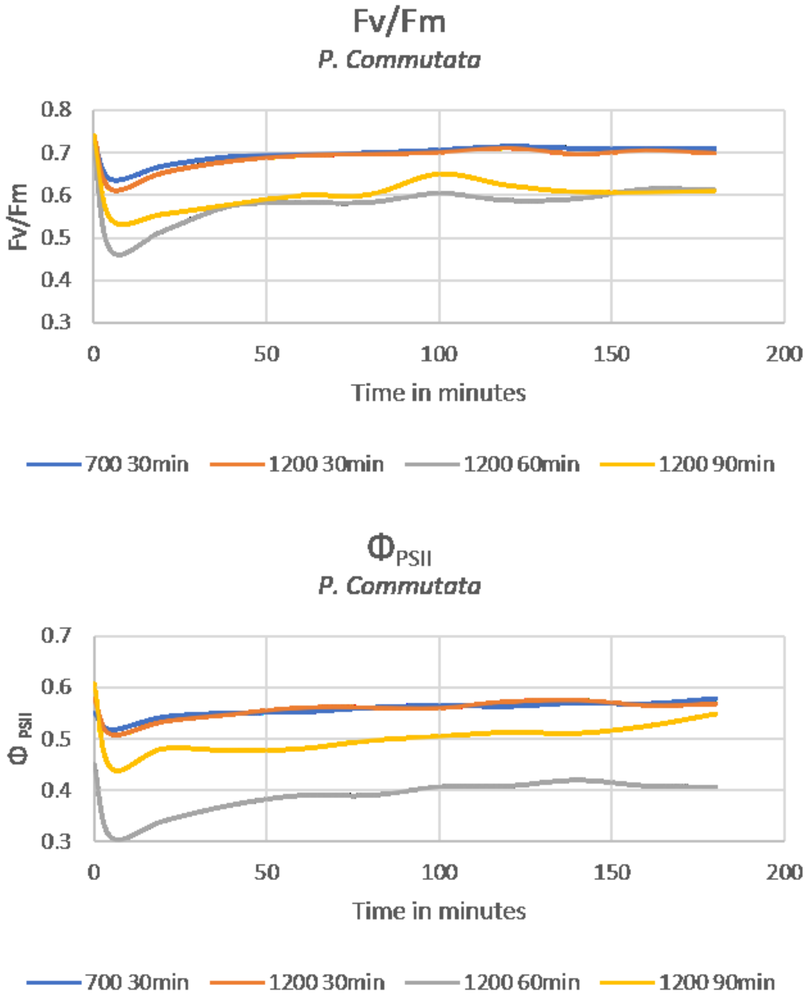


Fig. 7.24 Fv/Fm and Φ_{PSII} time courses of *P. commutata*

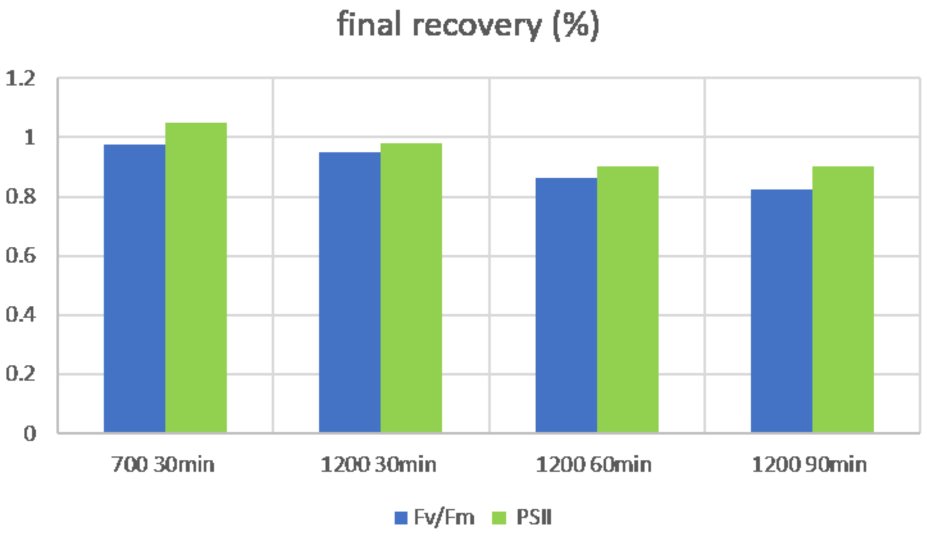


Fig. 7.25 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *P. commutata*

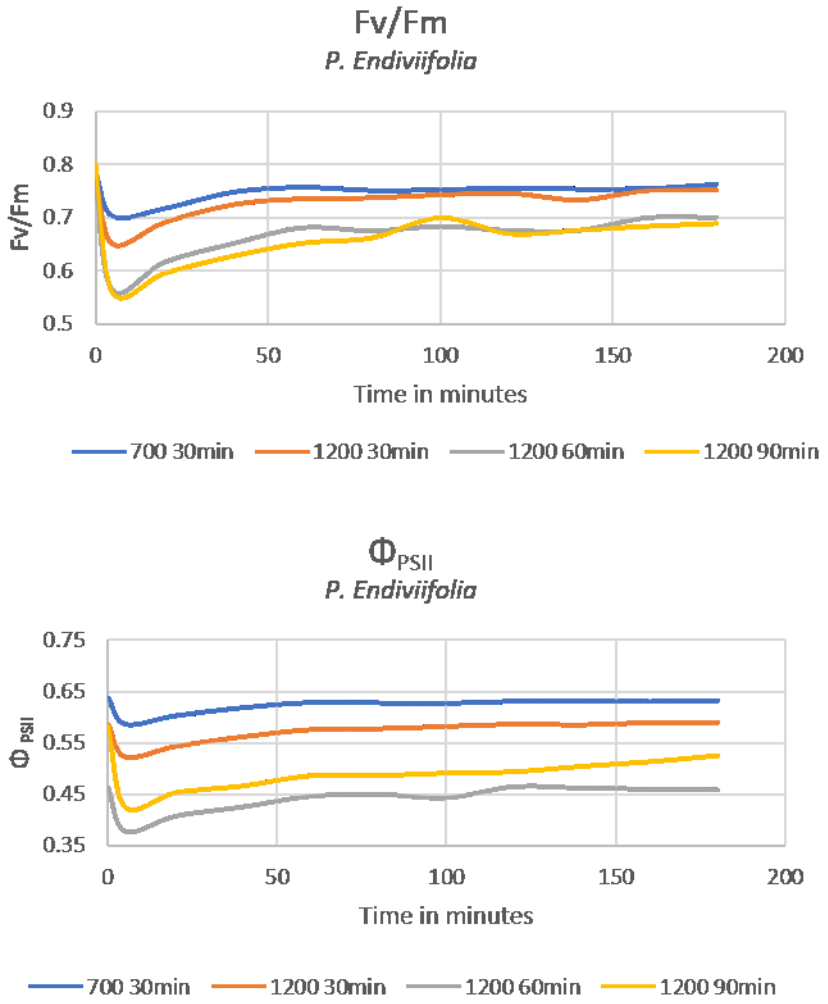


Fig. 7.26 Fv/Fm and Φ_{PSII} time courses of *P. endiviifolia*

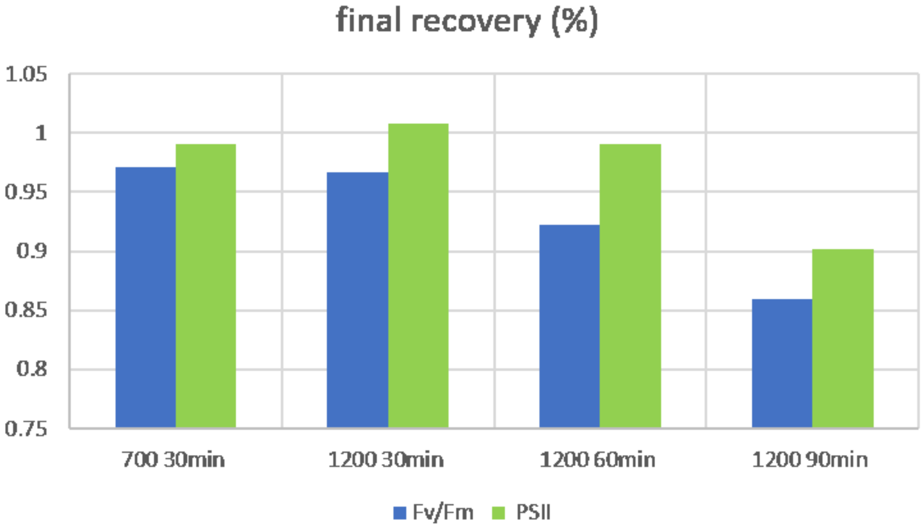


Fig. 7.27 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *P. endiviifolia*

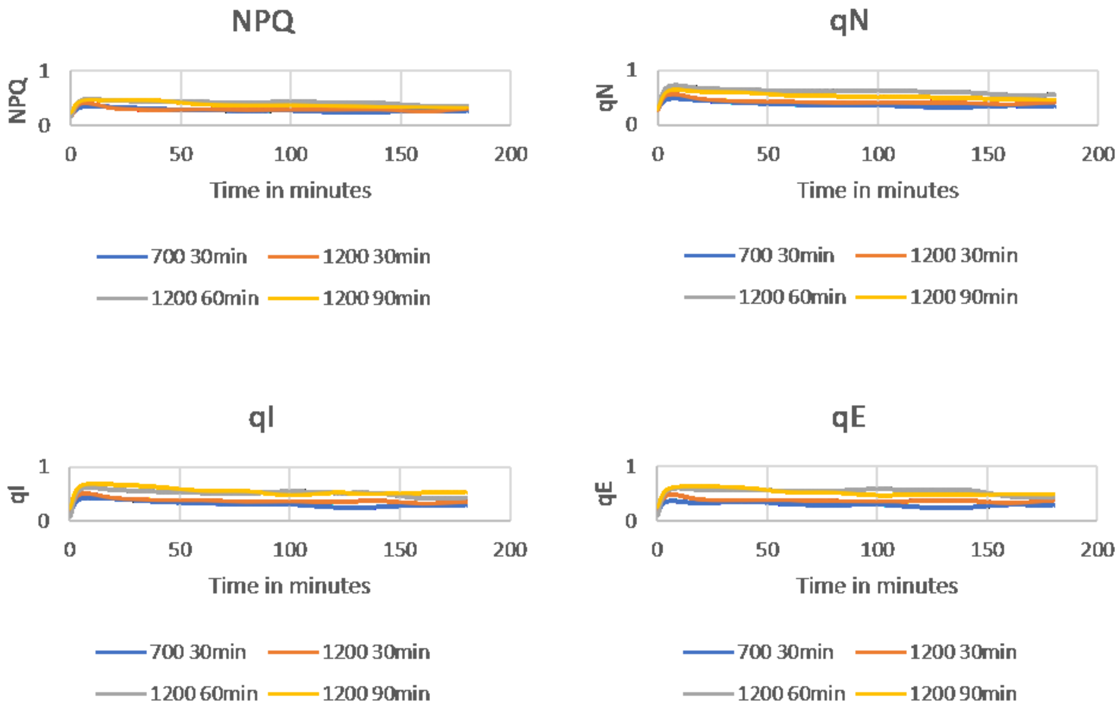


Fig 7.28 NPQ and its component time course in *P. commutata*

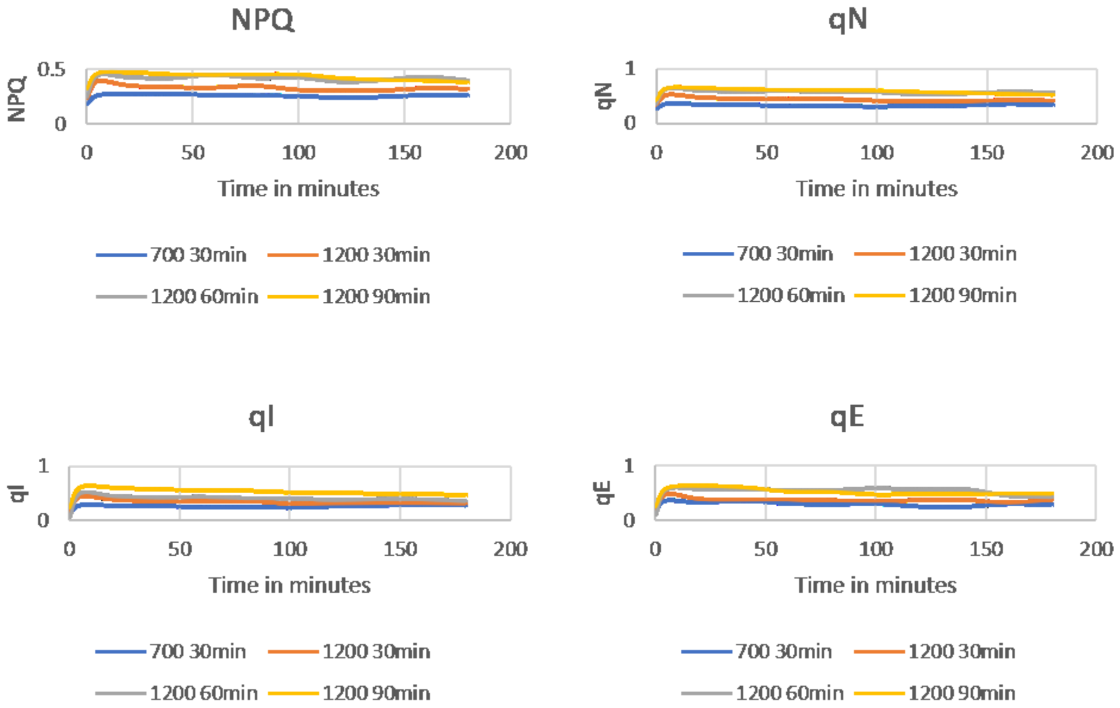


Fig 7.29 NPQ and its component time course in *P. endiviifolia*

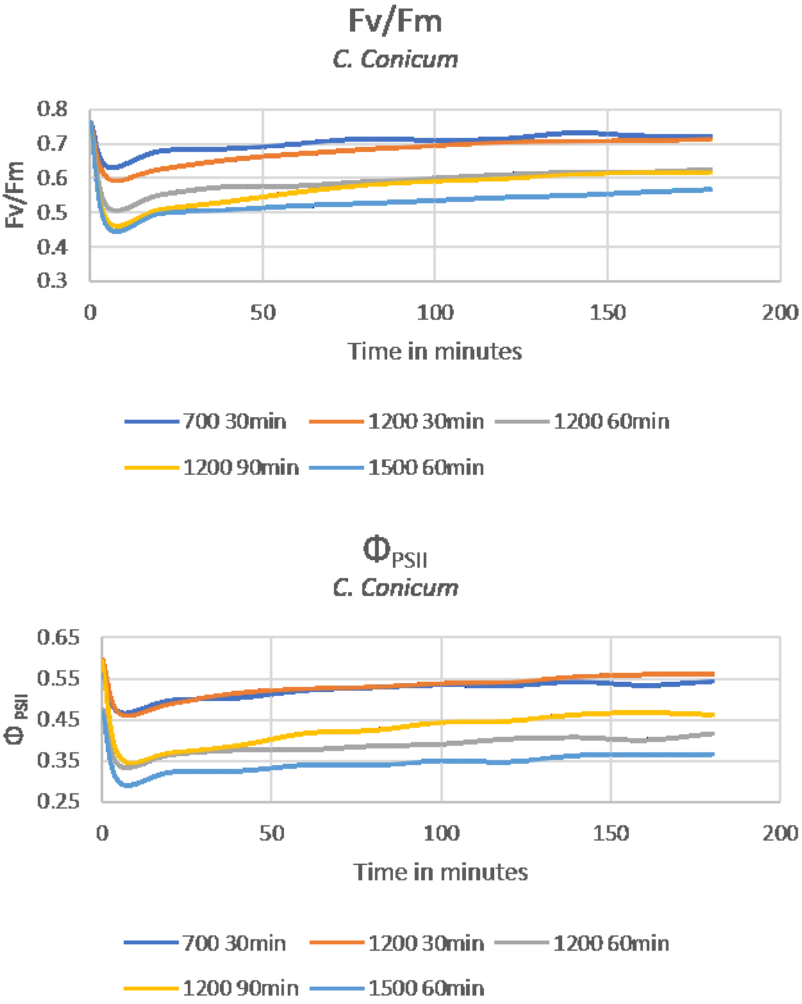


Fig. 7.30 Fv/Fm and Φ_{PSII} time courses of *C. conicum*

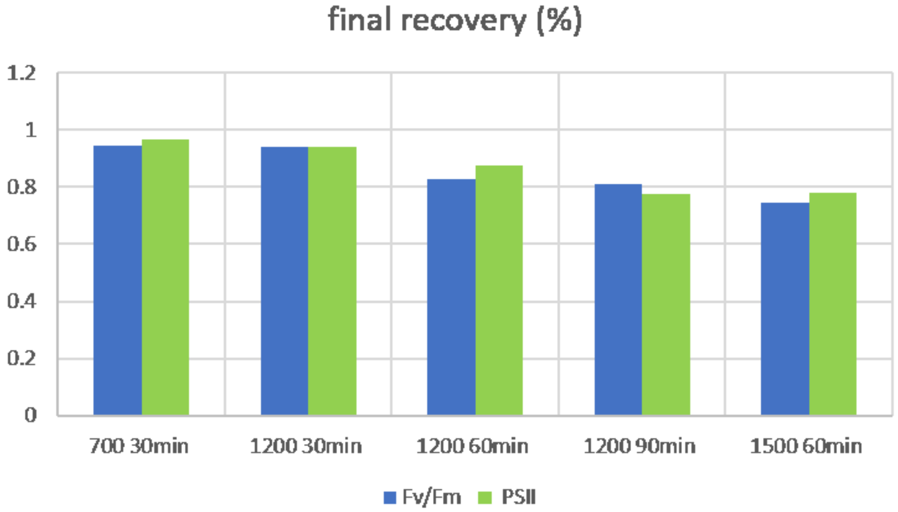


Fig. 7.31 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *C. conicum*

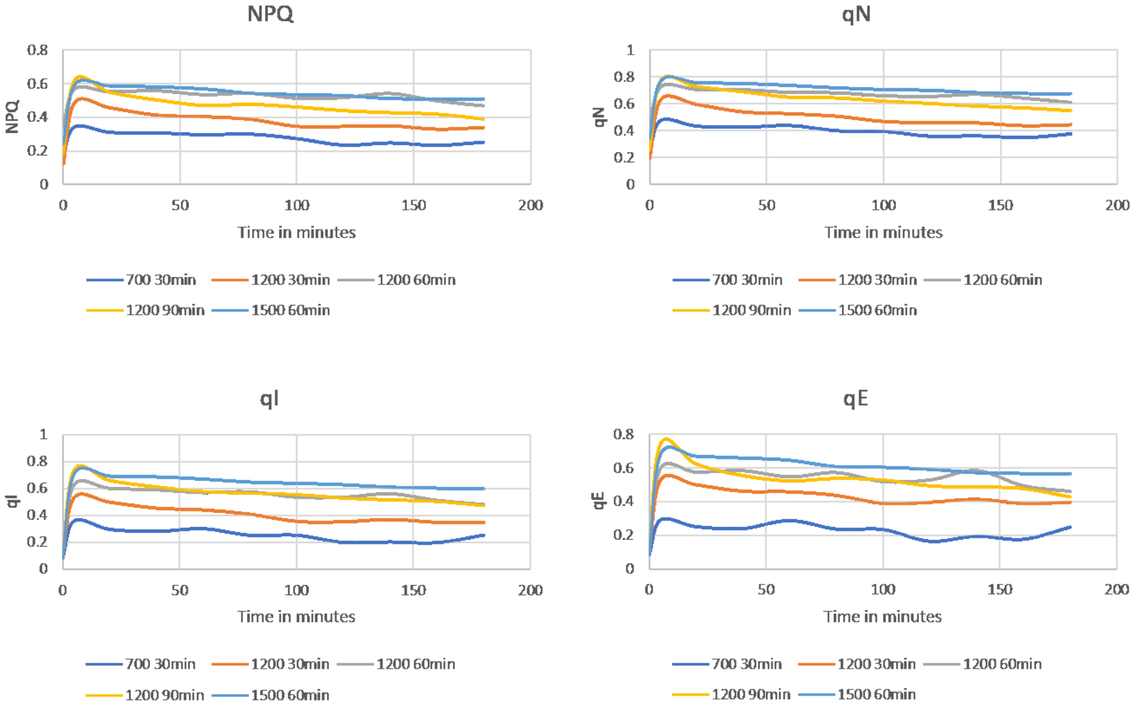
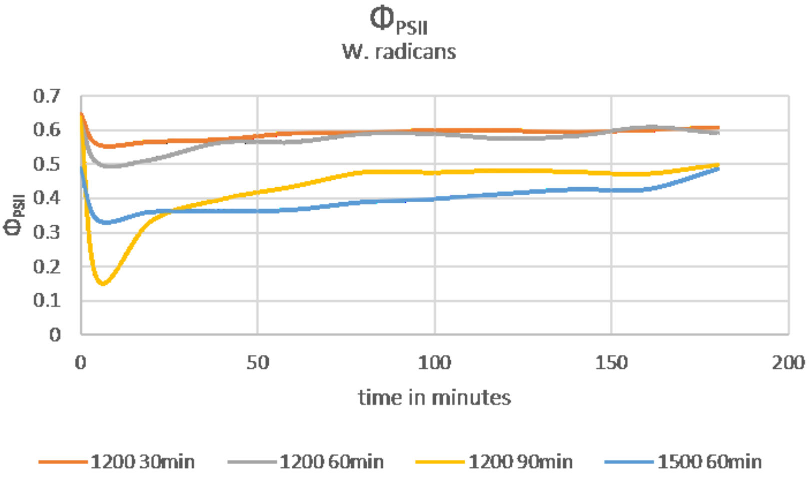


Fig 7.32 NPQ and its component time course in *C. conicum*



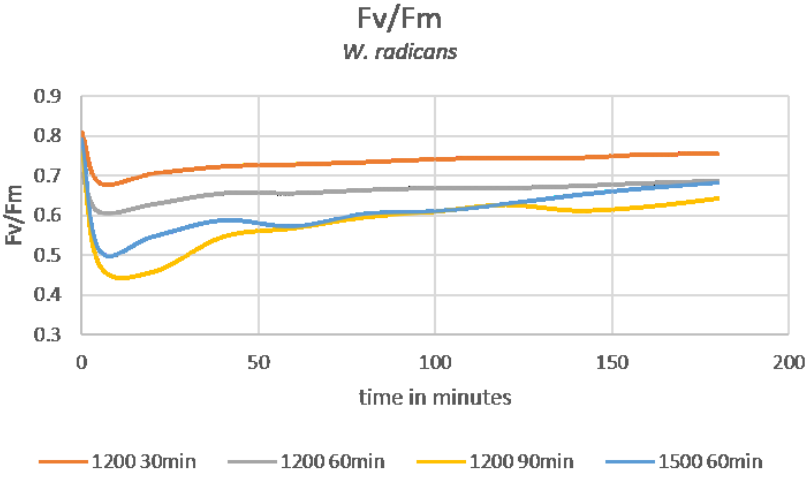


Fig. 7.33 Fv/Fm and Φ_{PSII} time courses of *W. radicans* leaves

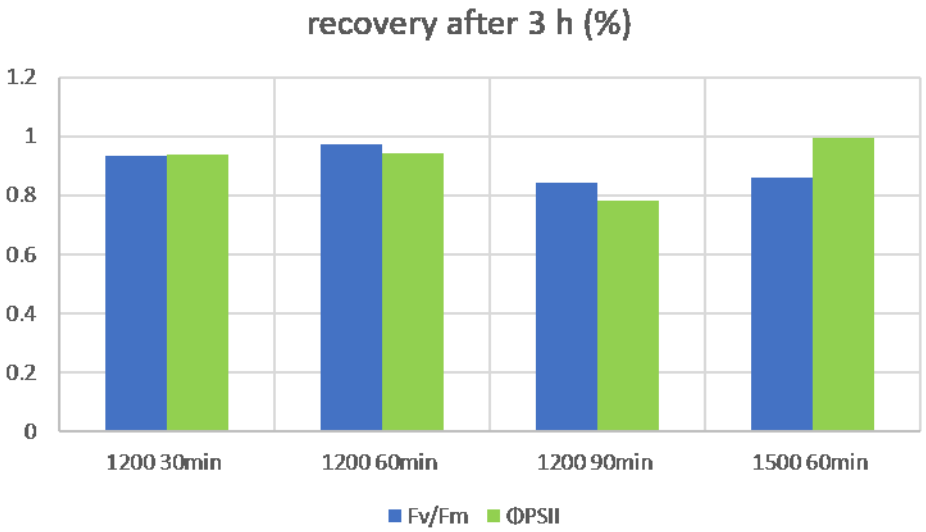
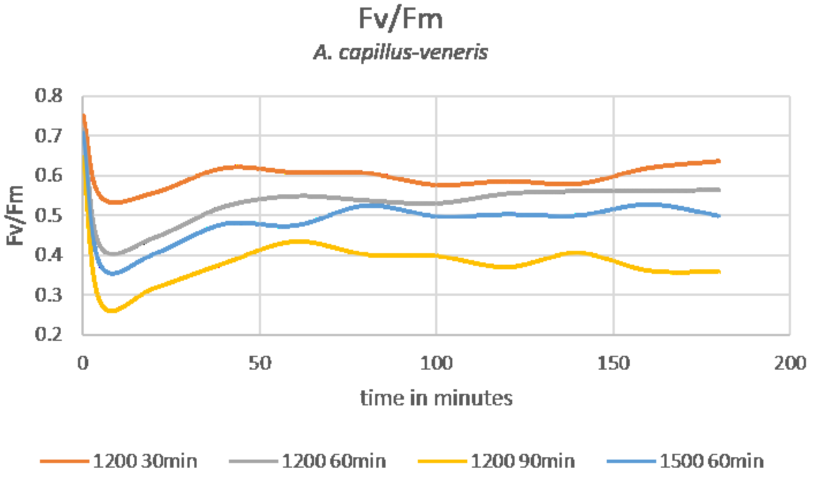


Fig. 7.34 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *W. radicans* leaves



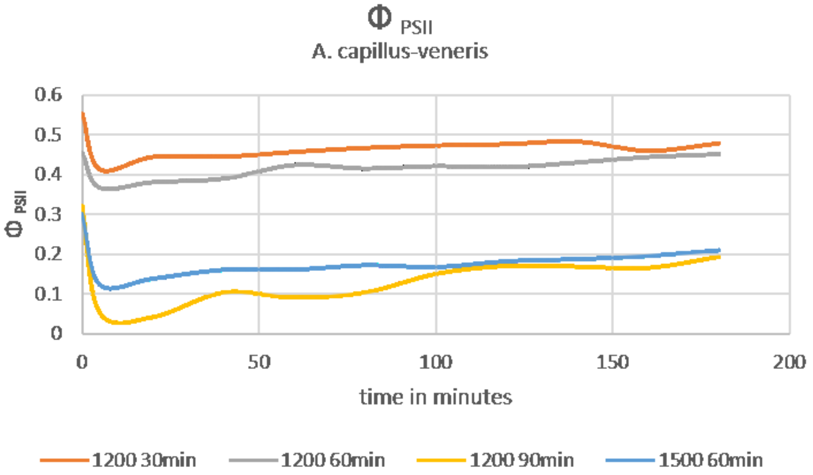


Fig. 7.35 Fv/Fm and Φ_{PSII} time courses of *A. capillus-veneris* leaves

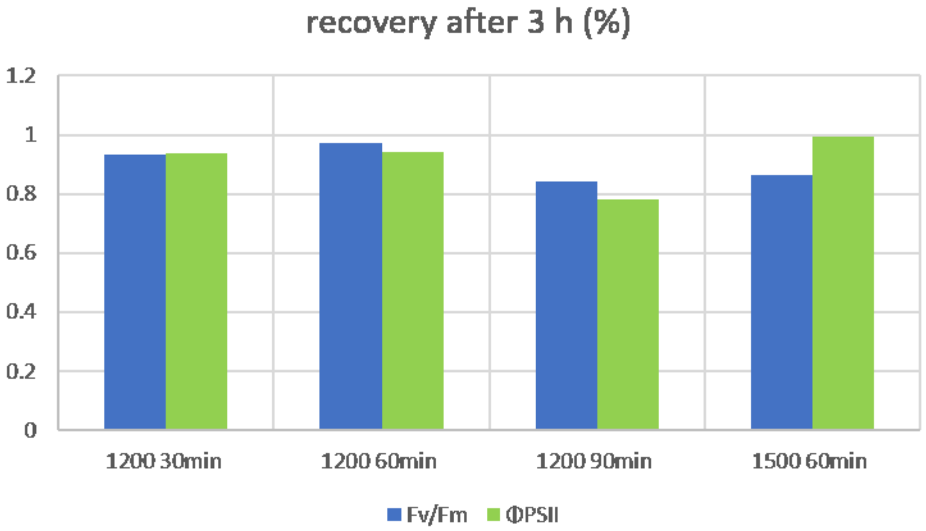


Fig. 7.36 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of *A. capillus-veneris* leaves

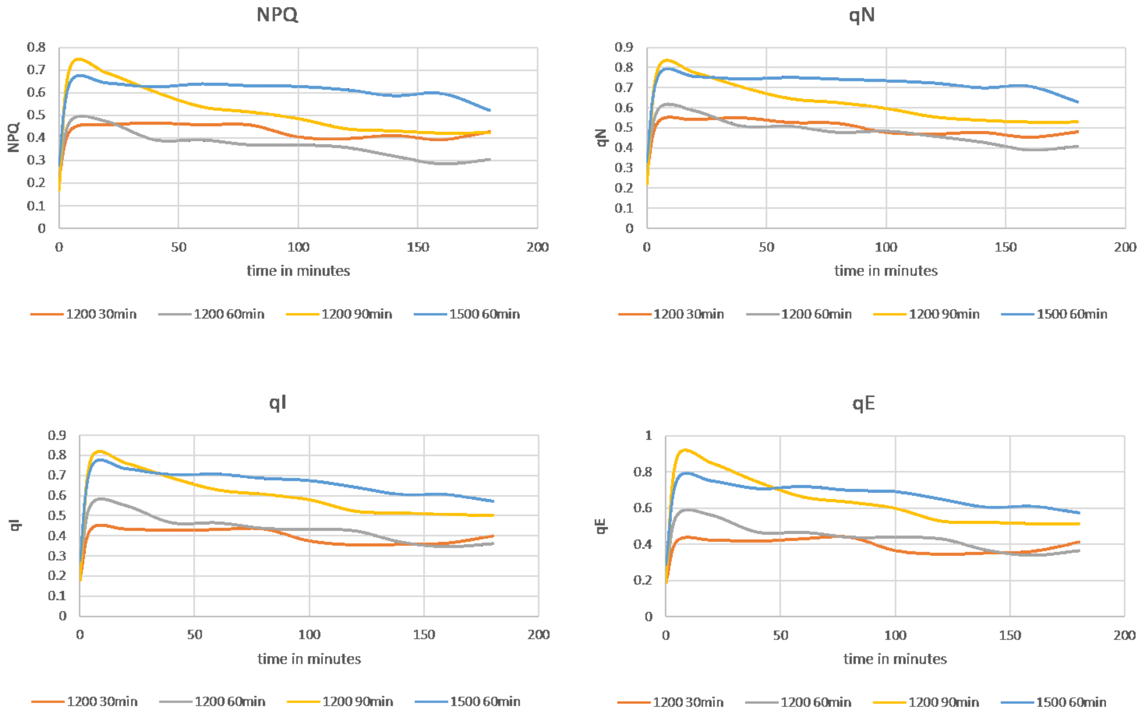
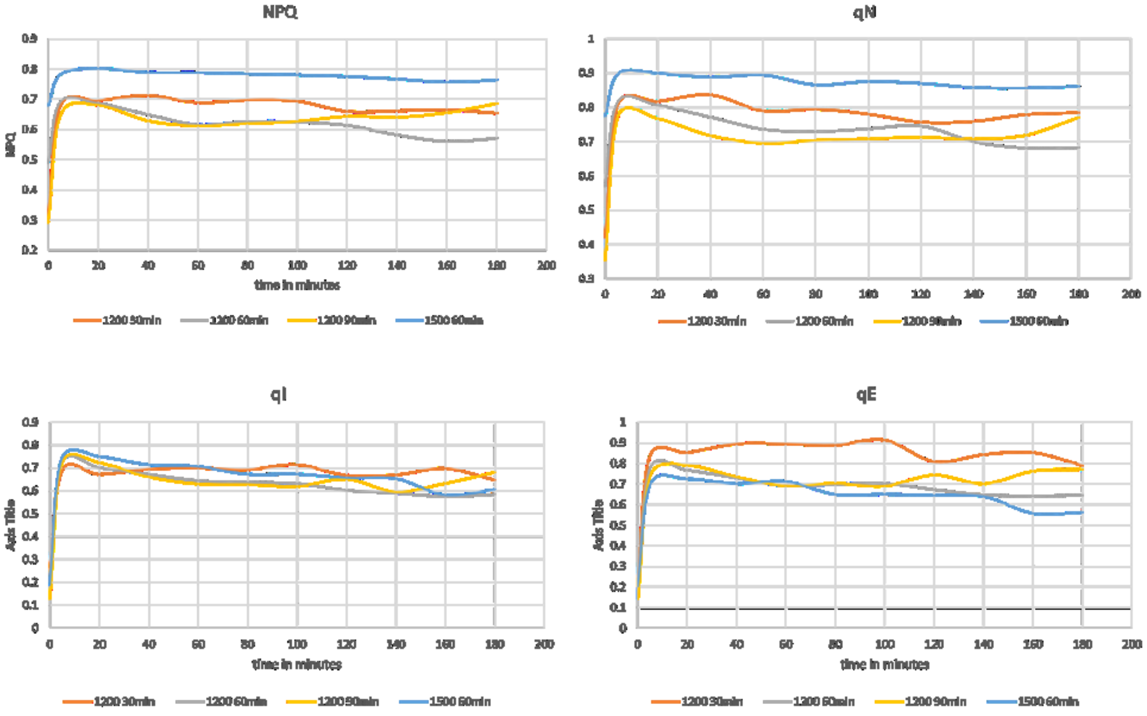


Fig 7.37 NPQ and its component time course in *W. radicans* leaves



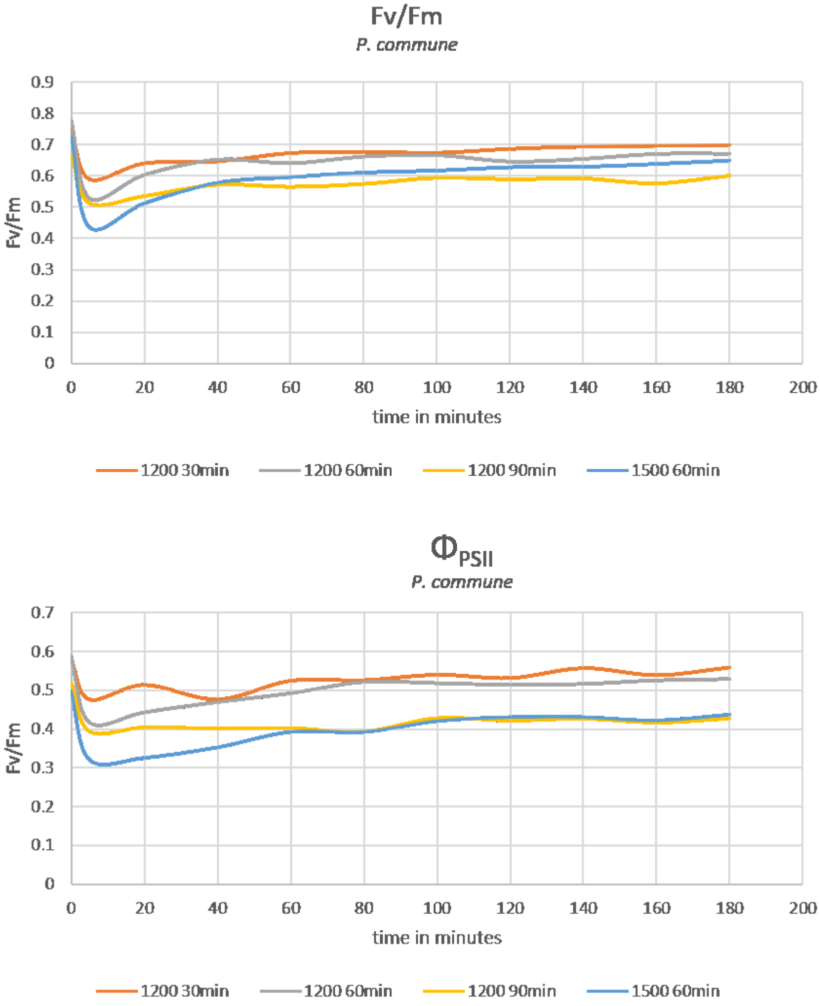


Fig. 7.39 Fv/Fm and Φ_{PSII} time courses of *P. vulgare* leaves

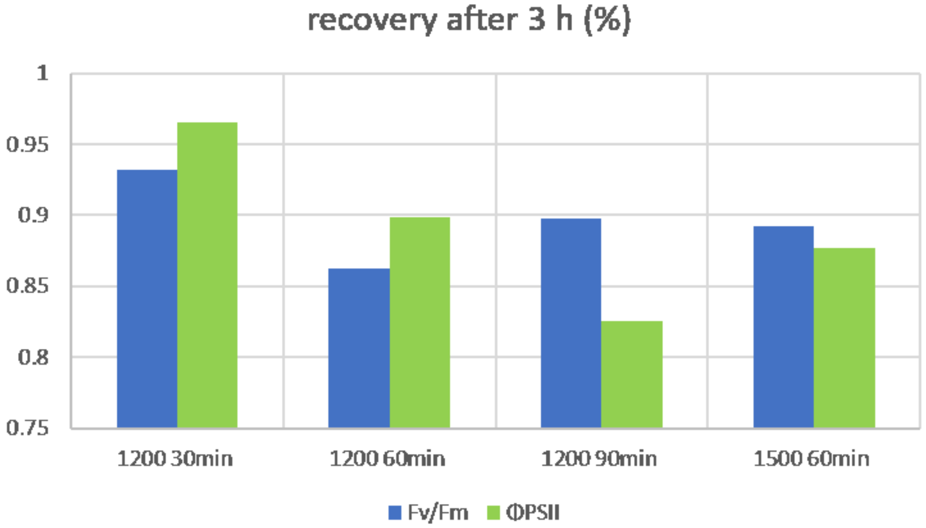


Fig. 7.40 final percentual recovery of initial Fv/Fm and Φ_{PSII} values of

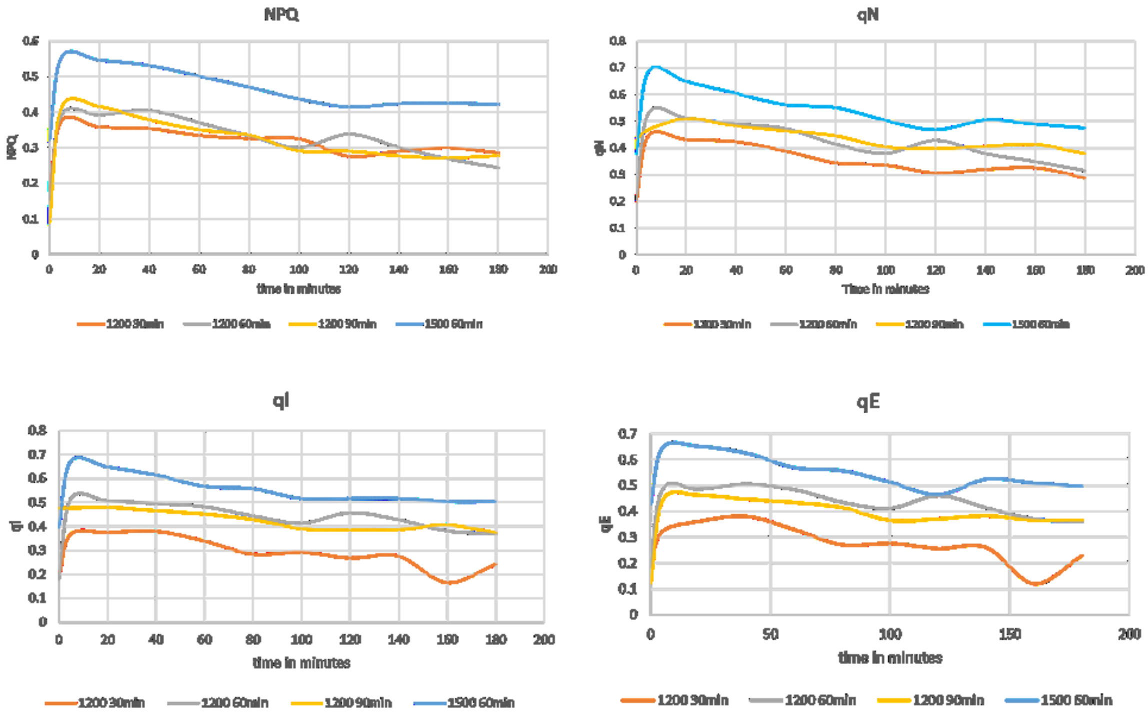


Fig 7.41 NPQ and its component time course in *P. vulgare* leaves

7.6. REFERENCES

Atherton , I., Bosanquer, S. and Lawley, M. (Eds.) (2010). Mosses and liverworts of Britain and Ireland – a field guide. British Bryological Society, Plymouth, 848 p.

Barták, M., Vráblíková-Cempírková, H., Štěpigová, J., Hájek, J., Váczi, P., večeřová, K. (2008): Duration of irradiation rather than quantity and frequency of high irradiance inhibits photosynthetic processes in the lichen *Lasallia pustulata*. Photosynthetica 46, 161-169.

Björkman, O., Demmig, B. (1987): Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins. Planta 170: 489-504.

Critchley, C. (1994): “D1 protein turnover: response to photodamage or regulatory mechanism in Photoinhibition of Photosynthesis, eds N. R. Baker and J. R. Bowyer Jr. (Oxford: Bios Scientific), 195–203.

Derks, A., Schaven, K., and Bruce, D. (2015). Diverse mechanisms for photoprotection in photosynthesis. Dynamic regulation of photosystem II excitation in response to rapid environmental change. Biochim. Biophys. Acta 1847, 468–485. doi: 10.1016/j.bbabio.2015.02.008

Giudici, G.N.M., Hájek, J., Barták, M., Kubešová, S. (2018): Comparative research of photosynthetic processes in selected poikilohydric organisms from Mediterranean and Central European alpine habitats. Czech Polar Reports, 8: 286-298.

Horton, P., Ruban, A. V., and Wentworth, M. (2000). Allosteric regulation of the light harvesting system of photosystem II. Philos. Trans. R. Soc. B 355, 1361–1370. doi: 10.1098/rstb.2000.0698

Hu, Y.-A., Sun, G.-Y., Wang, X.-C. (2007): : Induction characteristics and response of photosynthetic quantum conversion to changes in irradiance in mulberry plants. J. Plant Physiol., 164: 959-968.

Krause, G. H., Somersalo, S., Zumbusch, E., Weyers, B., and Laasch, H. (1990): On the mechanism of photoinhibition in chloroplasts. Relationship between changes in fluorescence and activity of Photosystem II. J. Plant Physiol. 136, 472–479. doi: 10.1016/S0176-1617(11)80038-6.

Malnoë, A., Schultink, A., Shahrasbi, S., Rumeau, D., Havaux, M., and Niyogi, K. K. (2017). The plastid lipocalin LCNP is required for sustained photoprotective energy dissipation in arabidopsis. Plant Cell 30, 196–208. doi: 10.1105/tpc.17.00536.

Olsson, L.C., Fraysse, L., Bornman, J.F. (2000): Influence of high light and UV-B radiation on photosynthesis and D1 turnover in atrazine-tolerant and -sensitive cultivars of *Brassica napus*. Journal of Experimental Botany 51: 265-74.

Osmond, C.B. (1994): What is photoinhibition? Some insights for comparisons of shade and sun plants. - In: Baker, N.B., Bowyer, J.R. (ed.): Photoinhibition of Photosynthesis - from Molecular Mechanisms to the Field. Pp. 1-24. Bios Scientific Publishers, Oxford.

Osório, M.L., Osório, J., Romano, A. (2010): Chlorophyll fluorescence in micropropagated *Rhododendron ponticum* subsp. *baeticum* plants in response to different irradiances. Biologia Plantarum, 54: 415-422.

Ruban, A. V., Johnson, M. P., and Duffy, C. D. P. (2012). The photoprotective molecular switch in the photosystem II antenna. Biochim. Biophys. Acta 1817, 167–181.
doi: 10.1016/j.bbabio.2011.04.007

Tuba, Z., Csintalan, Z., Proctor, M. C. F. (1996): Photosynthetic responses of a moss, *Tortula ruralis* ssp. ruralis, and the lichens *Cladonia convoluta* and *C. furcata* to water deficit and short periods of desiccation, and their ecophysiological significance: A baseline study at present-day concentration. New Phytologist, 133: 353-361.

Chapter VIII

8. CONCLUSIONS

8.1. PERSONAL OBSERVATIONS

8.1.1. Cultivation

Cultivation conditions change a lot the final output. Preliminary tests showed that although cultivation in agar gives excellent results, these are not in the least related to real environment. It is not by chance that most of researchers declare that they rarely obtained new sporophytes. In cultivation on earth they are always produced, even though with a high amount of variability; on the other hand, cultivation on earth provided slower results.

8.1.2. Ecological traits

Microrganisms on *W. radicans* present in wild environment were studied because of the potential effect on the fern performance. Fungus *Talaromyces minioluteus*, syn. *Penicillium minioluteum* (fig 8.1), was always found on spore-producing leaves; differnt effects of growing fungi, in both agar-grown and soil-grown cultures were observed.

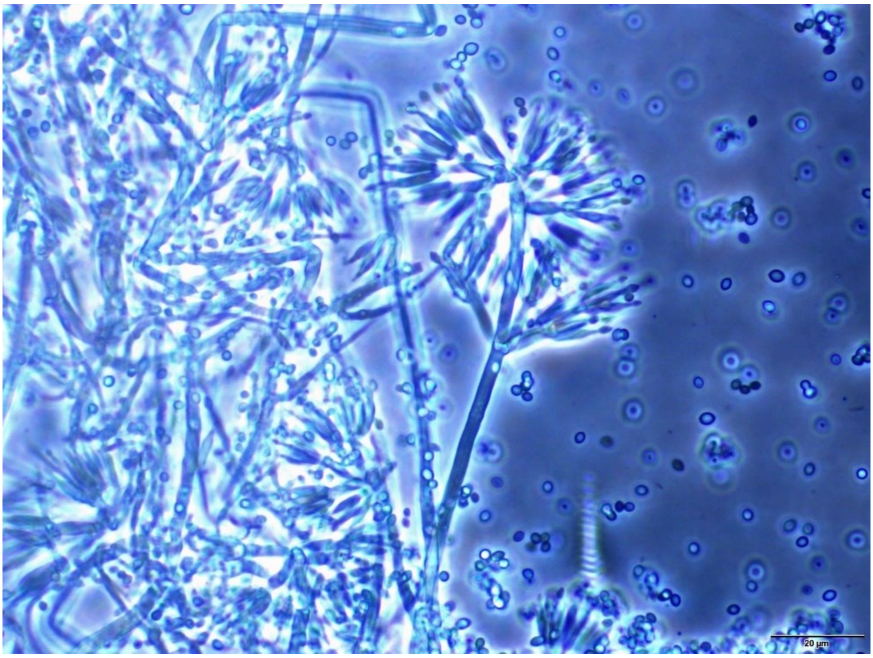


Fig. 8.1 *Talaromyces minioluteus* syn. *Penicillium minioluteum*; hyphae and spores (curtesy of Ing. Monika Laichmanová, Masaryk University Brno)

In agar-grown cultures the growth of mycelium affected newly germinated spores, while more advanced stadia apparently were not affected; however, when the growth of the fungus was substantial and fast the newly formed gametophytes were slowly decaying and died. On microscope analysis, gametophytes showed loss of turgor and chlorophyll. Apparently, the fungi were more affecting the developing gametophytes because of water competition than because of antibiotic effect; consistently with this are the observations of the same infection in soil-grown gametophytes. In this case the growth of even a thick and covering mycelium didn't influence the growth of the germinating spores, which, in some cases, emerged from a sort of "fur" of hyphae without any decline in growth rate (fig. 8.2).

Fig 8.2 A few-cell prothallus of *Woodwardia radicans* emerging from *Penicillium minioluteum* hyfae

In early stages of emerging sporophytes (observed only in soil-grown cultures) the presence of aerial threads of hyphae didn't affect the growth unless the individuals presented some injuries (i. e. any sort of discontinuity in the external cell layer). In this case the presence of the fungus was quickly leading to the death of the individual. In case of developed new sporophytes (first leaf already unfolded) sometimes the matured leaf decayed, but the newly formed pastoral was able to develop a new leaf. All these observations point to the fact that the presence of *P. minioluteum* is only accompanying the different stages of the fern without forming a parasitic association; which, on the contrary, could be observed in case of passive entering the gametophytes body, not having the fungus any active means of penetrating the host. In some cases the mycelium provided the newly germinated spores with a more constant microclimate in relation to temperature and humidity. The fungus appears to be a threat to young developmental stages only in case of water deficiency. It was also observed that when the fungi were present in more developed stages of the fern, collembola and other small arthropods, were present, cleaning their surfaces to obtain nutrition from the fungal spores.

8.2. GENERAL RESULTS

Our data confirmed that both light intensity and water availability are driving factors in affecting photosynthetic performance and that their effects are species-specific

8.2.1. Sensitivity to light, or photoinhibition (PI)

Under canopy level: three fern-species (*W. radicans*, *A. capillus-veneris, P. vulgare*)

The three fern-species, in the mature stages showed light intensity-related PI. Differently from *P. vulgare*, both *W. radicans* and *A. capillus-veneris* showed that duration of PI played a significant role as well as intensity. This is consistent with adaptation to low light understory environment and to sunflecks appearance, while *P. vulgare*, seemed to be more aadapted to lighted environments.

All species showed, 3 hours after PI, almost full recovery of previous value of Fv/Fm and Φ_{PSII} parameters. This, along with the involvement of NPQ into the response to high light stress, suggested that quenching mechanisms were effectively working for all of them, so that the reduced photosynthetic performance was due more to downregulation than to damages to photosynthetic apparatus. However, *A. capillus-veneris* lower Φ_{PSII} recovery value showed this species to be more sensitive to light than the other two. Furthermore, different proportion of quenching components indicates that the three species had different protective pathways. Further example of negative adjustment of primary photosynthetic processes in PSII was offered by the greenhouse experiment, in which downregulation was a consequence of adaptation, in this case, to low light of the environment.

On the forest floor: gametophytes and young stages of *A. capillus-veneris* and *W. radicans* and coexisting species (*P. commutata, P. endiviifolia, C. conicum*)

More sensitivity to high light duration than high light intensity was shown by *W. radicans* gametophytes and young sporophytes and both *W. radicans* and *A. capillus-veneris* juvenile sporophytes. In *W. radicans* photoprotective mechanisms were activated throughout the whole life cycle, but differently composed, suggesting that the pattern of the mature sporophyte was reached through adjustments during the young stages. In *A. capillus-veneris* photoprotective mechanisms were light intensity-dependent through all life stages. In these species and stages the lowered photosynthetic performances because of photoinhibition were due to downregulation of protective mechanisms more than to PSII damages, as was showed by the almost complete recovery to previous photosynthetic leves reached a few hours after the stress induction. *A. capillus-veneris* was an exception, because its lower recovery showed more sensitivity lo high light stress. Other species in the same layer, *P. commutata* and *P. endiviifolia,* showed increasing responses to higher doses, with better tolerance to low-medium light stress; contrastingly *C. conicum* showed linear relationship between light intensity and PI response. Inter-specific comparison of Fv/Fm and Φ_{PSII} time courses suggested that *C. conicum* was more susceptible to photoinhibition than *P. commutata* and *P. endiviifolia*. This is likely because of lower capacity of photosynthetic processes associated with PSII due to low light acclimatory downregulation of photosynthesis.

In the comparative analysis with *Polytrichum commune*, a moss species from alpine environment, time courses and final values showed only slight differences and these were: 1) final value after recovery were closer to initial values than in the Ferriere valley species, 2) time recovery was faster immediately after the PI and then slower, meaning that most of recovery in the parameter values was reached in the first 20 minutes after the induction, while recovery of the Mediterranean species was slower, more gradual and proportional to time, 3) In *P. commune* the main energy quenching mechanism was represented by xanthophyll pigments along with changes in PS II.

All observations showed that the alpine species had higher tolerance to light stress, while protecting mechanisms of Mediterranean species were more complex.

Response to light course in the Ferriere Valley (W. radicans, C. conicum)

In both analysed days *C. conicum* and *W. radicans* showed a daily change of Fv/Fm rather small, in contrast with expected data; in fact, usually day course shows a decline due to regulative adjustment to total adsorbed light or to high light increase in the central hours of day. However, the small change suggested that incident light and air temperature were perfectly fitting physiological range for photosynthesis in the days of measurements. On the other hand, in some Fv/Fm data points relating to *C. conicum*, it was showed a pronounced drop, a short-term decrease caused by preceding sunflecks. Because of this, Fv/Fm was downregulated rapidly, but at the same time, high light available for a short period of time increased the rate of photosynthesis. Such short-term, high light-induced increase in photosynthesis might be documented by the increase of ABS/RC, TR/RC, ETo/RC and thermal dissipation of excess (absorbed) energy by DIo/RC. It could be concluded that *C. conicum* is able to exploit short term high light in a low light environment, as reported for other species.

8.2.2. Sensitivity to desiccation

Desiccation (*P. commutata, P. endiviifolia, C. conicum* , *W. radicans* gametophyte)

All species showed an exponential course of Φ_{PSII} related to relative water content (RWC) except for *P. commutata* which had sigmoidal shape. In all species, desiccation was accompanied by an increase in qN, whose course was species-specific. The four species do possess effective protective mechanism against desiccation, but capacity of the mechanism, however, is insufficient to cope with a short-term severe drought stress. Tolerance to desiccation was showed in different degrees: 50% of Φ_{PSII} initial value, which is considered the initial phase of inhibition of primary photosynthetic processes, was found as follows: *C. conicum* at 18 % RWC, *W. radicans* gametophyte at 22% RWC, *P. endiviifolia* at 24% RWC, *P. commutata* at 53% RWC, which may indicate that all the species could be ranked among desiccation tolerant, except for *P. commutata* to be considered semitolerant or intolerant. However, more experimental evidence is needed to evaluate underlying mechanisms, the extent and rate of their activation, and their involvement as related to the speed of desiccation. The latter topic seems to be crucial for understanding the survival of species in the field.

8.3. METHOD EFFECTIVITY IN MEASURING PHOTOSYNTHETIC PERFORMANCE PARAMETERS

The method of Φ_{PSII} monitoring and subsequent ETR calculations proved to be sensitive enough to detect environmental-induced responses in ferns. Therefore, the method can be recommended for detailed analysis of *in situ* photosynthetic parameters

The OJIP measurements were showed useful to detect not only common photosynthetic parameters, but a whole set of correlated parameters necessary to discriminate among different causes for a single phenomenon (e.g. the increased ABS/RC, TR/RC, ETo/RC in relation to a decreased Fv/Fm). The OJIP techniques then is well apt to field measurements finalized to monitor the state of species in protected areas.

8.4. CONCLUDING REMARKS

- From the very initial stages *Woodwardia radicans* shows all the protecting mechanisms typical of the species (which only differ in degree), which is a strength point in survival strategy.
- However, environmental factors which are not dramatically affecting the individual could delay each life stage completion, which in turn could enhance probability of death (because of competition, predation, exposure to adverse conditions etc.).
- Experimental lab conditions, under which most of previous studies were carried out, are never met with in the field, then it is important to develop more field research and lab experiment based on field conditions.
- In light stress, induction time of exposure could, in some cases, be more important than level of irradiance. This is consistent with understory dim light adaptation and ability to exploit sunflecks energy.
- At the same time long-term exposure to high light could be either dangerous or hindering/ completion of life cycle.

8.5. CONSERVATION IMPLICATIONS

All the above considerations induce to consider likely that deforestation could be a threat to survival of *Woodwardia radicans* along with its coexistent species. Furthermore:

- Temporary slight medium drought, even if not affecting survival of reproductive forms, could slow down the growth rate because of the limitation of photosynthesis productivity and increase probability of disappearance. Some species, however, face critical conditions even in case of small decrease in watwer supply (*Palustriella commutata*)
- This is ground to protect from changes in water bodies conditions in the environment

- Measurements of other *Woodwardia* environmentally related species could be used to monitor the state of its habitat when topographical conditions make it difficult to reach the fern.

8.6. FUTURE PERSPECTIVES

- Any conservation strategy needs better knowledge of *Woodwardia radicans* physiology, especially in the field, under multiple variables
- Changes in phenological stages durations determined by environmental factors should be investigated, because they are likely to affect the final output of the multiplication/reproduction of the species

ACKNOWLEDGEMENTS

At the end of a three-year work there is a small crowd I feel grateful to and I would like to miss nobody. The order is, like in the movies, that of appearance, no strings or meaning attached to it.

I would like to thank my tutor, prof. Mazzoleni for the patience showed in dealing with my, sometimes erratic, projects; my thanks go as well to prof. D’urso for the kind guidance through the technical and administrative fulfillment of a doctoral thesis. Prof. Carafa was my kind mentor and guide into the knowledge of *Woodwardia*’s secrets. Not only an advisor, but a model to follow. Gratitude is due to all the operatives at the Ferriere Valley for their keen support during sampling and monitoring in the field. The same gratitude goes to directors and operatives of botanical gardens of Naples, Portici and Brno, with particular reference to Nello Marsilio and Bruno Chianese. A special thanks to prof. Milos Barták and staff of Lab of Photosynthetic Processes of Masaryk University in Brno (Czech Republic) whose continuous support and experience made my studies possible. Prof. Barták was not only generous in experience and knowledge but lavish in the most precious thing for everybody: his time. My training would have never been possible without his guidance and the silent help of his staff. Finally, I would like to thank all my close friends for coping with my down moments and with my needs during the abroad period. I don’t need to mention their names because they know.