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Ultraviolet radiation and aquatic bryophytes: physiological damage and protection mechanisms under laboratory and field conditions
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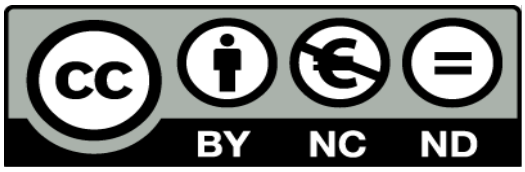

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DEPARTAMENTO DE AGRICULTURA Y ALIMENTACIÓN

Ultraviolet radiation and aquatic bryophytes: physiological damage and protection mechanisms under laboratory and field conditions

Radiación ultravioleta y briófitos acuáticos: daños fisiológicos y mecanismos de protección en condiciones de laboratorio y campo

Memoria presentada por:

GABRIEL FABÓN ANCHELERGUES

para optar al Grado de Doctor por la Universidad de La Rioja

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Fdo. GABRIEL FABÓN ANCHELERGUES

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DEPARTAMENTO DE AGRICULTURA Y ALIMENTACIÓN

LOS DOCTORES ENCARNACIÓN NÚÑEZ OLIVERA Y JAVIER MARTÍNEZ ABAIGAR, CATEDRÁTICOS DE FISIOLOGÍA VEGETAL Y BOTÁNICA DE LA UNIVERSIDAD DE LA RIOJA,

CERTIFICAN:

Que la Memoria titulada "**Ultraviolet radiation and aquatic bryophytes: physiological damage and protection mechanisms under laboratory and field conditions**" ha sido realizada en las Áreas de Fisiología Vegetal y Botánica de la Universidad de La Rioja bajo nuestra dirección por el Licenciado en Ciencias Biológicas y Bioquímica GABRIEL FABÓN ANCHELERGUES. Considerando que se encuentra concluida, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

Y para que conste, expedimos el presente certificado en Logroño, a 1 de Febrero de 2012.

Fdo. Encarnación Núñez Olivera Fdo. Javier Martínez Abaigar

A mis padres

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ABBREVIATIONS

$AUC_{280-400}$, area under the absorbance curve in the interval 280-400 nm

C1, *p*-coumaroylmalic acid

C2, caffeoylmalic acid

C3, feruloylmalic acid

C4, 5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid

C5, 5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid

C6, *p*-coumaric acid

C7, ferulic acid

Chl, chlorophyll

DM, dry mass

ETR, electron transport rate

F_m, maximum chl fluorescence level

F_0, minimum chl fluorescence level

F_v/F_m, maximum quantum yield of PSII

F_v, variable chl fluorescence

Φ_{PSII}, effective quantum yield of PSII

NPQ, non-photochemical quenching of chl fluorescence

PAR, photosynthetically active radiation

PFD, photon flux density

PI, performance index on absorption basis

PSII, photosystem II

SE, standard error

SUVAC, methanol-soluble UV-absorbing compounds

UV, ultraviolet radiation

UVAC, UV-absorbing compounds

WUVAC, methanol-insoluble UV-absorbing compounds

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ÍNDICE

CHAPTER 1

INTRODUCTION

1.1. ULTRAVIOLET RADIATION AND ITS EFFECTS ON PHOTOSYNTHETIC ORGANISMS

Ultraviolet radiation (UVR) is a minority component (about 6%) of solar radiation in comparison with the dominant visible/photosynthetic and infrared bands. However, UVR is a natural environmental factor that has been involved in the appearance of diverse adaptive changes in organisms through the development of life on Earth (Cockell & Knowland, 1999). UVR induces a number of biological processes in all living organisms, including humans, and many of them are harmful. In this respect, among the three wavelength categories in which UVR is divided by the CIE (Commission Internationale d'Eclairage), the most damaging UV-C (<280 nm) is not relevant at the present time because of its complete absorption by stratospheric oxygen and ozone, but both UV-B (280-315 nm) and UV-A (315-400 nm) penetrate the biosphere and have significant biological effects. These effects are highly dependent on wavelength, and different biological weighting functions have been conceived to calculate the biologically effective UV (UV_{BE}). UVBE encompass UV-A and UV-B, but, given the logarithmic increase in effectiveness with decreasing wavelength, is dominated by UV-B, especially at shorter wavelengths. Therefore, most studies on the effects of UVR have dealt with UV-B. This is especially true since the discovery of the anthropogenic stratospheric ozone reduction, because UV-B (and not UV-A) is absorbed by stratospheric ozone, and thus ozone reduction leads to an increase in surface UV-B level. Nevertheless, the present tendency is to pay also attention to UV-A in the development of biological weighting functions (Flint *et al.*, 2003), considering also that the wavelength limit between UV-B and UV-A is somewhat diffuse.

UVR is a delicate meteorological variable to measure, and for example there exist discrepancies between ground-based measurements and satellite estimates of surface UV irradiance (Seckmeyer *et al.*, 2008). UV irradiance at the ground level depends on a number of factors, such as latitude, season, hour of the day, altitude, presence of clouds or aerosols,

surface reflectivity, and ozone levels (McKenzie *et al.*, 2007). The ozone loss as a result of anthropogenic emissions of halogenated carbon compounds has been most dramatic in the Antarctic continent, although reductions in Arctic and mid-latitudes have also been observed. In mid-latitudes, the resultant increase in solar UV-B has been estimated at 6-12% over the 1980 levels (McKenzie *et al.*, 2003), although this increase may be masked by large seasonal changes and geographic differences (Häder *et al.*, 2007a). Elevated UVR is expected to continue for several decades because of the lack of a full recovery of ozone. Consequently, studies on the effects of ambient and enhanced UV levels on organisms and ecosystems are increasingly important, taking also into account that interactions between ozone depletion and climate change may occur through variations in cloudiness, aerosols and surface reflectivity (McKenzie *et al.*, 2007).

In humans, an excessive UV exposure (mainly to UV-B, but also to UV-A) causes acute and chronic damage to eyes and skin, including sunburn and cancer, and down-regulates immune responses, although some beneficial effects of UV are also clear (Norval *et al.*, 2007). Excessive exposures seem to be caused by irresponsible habits rather than by stratospheric ozone reduction. In photosynthetic organisms, increased UV may cause diverse damage in the photosynthetic apparatus: pigment degradation, photoinhibition, and decreases in quantum yield, photosynthetic rates, and the activity of the Calvin cycle enzymes (Jansen *et al.*, 1998). Also, DNA alterations, oxidative damage, and changes in mineral absorption can occur. This may lead to alterations in growth and development. However, some controversy about the ecological relevance of these effects still persists, because much of the early work concerning the UV effects was conducted indoors using unrealistically high UV doses and UV/PAR proportions. Thus, the extrapolation of these results to field conditions seems difficult (Searles *et al.*, 2001a; Day & Neale, 2002). At the ecosystem level, UV can affect litter decomposition, nutrient cycling, trophic interactions, and the competitive balance between species (Caldwell *et al.*, 2007). Photosynthetic organisms may develop a number of protection and

repair mechanisms against the adverse effects of UV (Jansen *et al.*, 1998): UV-absorbing compounds (flavonoids, phenyl-propanoids, mycosporine-like aminoacids, etc.), antioxidant and photoprotective mechanisms, and repairing or turnover of damaged biomolecules such as DNA and proteins.

The effects of UVR on photosynthetic organisms have been studied mainly in terrestrial plants, especially crops (Caldwell *et al.*, 2007), and in marine phytoplankton and macroalgae (Day & Neale, 2002; Häder *et al.*, 2007b). Effects of UVR on photosynthetic organisms from freshwater ecosystems have been little investigated, in line with their minor contribution to the global biomass and primary production. However, lakes and rivers have outstanding ecological importance as local systems and it would be desirable to know the effects of UVR on the organisms inhabiting them and evaluate their potential vulnerability. In lakes, the penetration of UVR and its effects on phytoplankton have been the most studied topics, both under field and laboratory conditions (Häder *et al.*, 2007b). In flowing waters (rivers and streams), most data come from laboratory studies and not much field work has been done (Rader & Belish, 1997; Kelly *et al.*, 2003), probably due to intrinsic methodological problems derived from the strongly dynamic and changeable environmental conditions (discharge, water velocity, bed morphology, depth, etc.). Another type of freshwater ecosystems, with particular importance in high latitudes and altitudes, are peatlands, in which significant field work has been done in relation to UVR.

1.2. BRYOPHYTES AND ULTRAVIOLET RADIATION

Bryophytes are acquiring increasing importance in the context of UVR research and are usually mentioned in the most recent reviews on this topic (Björn, 2007; Caldwell *et al.*, 2007; Häder *et al.*, 2007b; Newsham & Robinson, 2009). This is the logical consequence of the increasing work on the effects of UVR on bryophytes that has been carried out, mainly in the last decade. To our knowledge, around 80 papers containing original data have been published on this topic (Table 1). The research has focused mainly on terrestrial and semiaquatic bryophytes from Antarctic habitats and circumpolar heathlands and peatlands. The most used species belong to mosses: several *Sphagnum* species, *Hylocomium splendens* (typical from forest soils) and *Polytrichum commune* (typical from a wide range of acid habitats in damp to wet situations). Liverworts have been notably less studied than mosses, except the aquatic *Jungermannia exsertifolia* subsp. *cordifolia* and the terrestrial *Cephaloziella varians*, while no hornwort has been investigated.

Diverse methodological approaches have been applied. Studies under both field and controlled conditions have been conducted, and in the latter case both in the laboratory and the greenhouse. Manipulative experiments have included the two main experimental options in the context of UVR research: UVR supplementation using lamps to address the ozone depletion issue (38 studies), and UVR exclusion using filters to assess the effects of current UVR levels (17 studies). When using lamps, most results have been obtained under unrealistic conditions of UV irradiance, UVR daily pattern, proportions of UV-B, UV-A and PAR, etc. If filters are used, above-ambient UV-B levels associated with ozone reduction cannot be provided, and filters may modify the microenvironmental conditions of the samples. All these methodological problems may limit the ecological relevance of the results obtained. A third way to assess UVR effects exploits natural gradients of UVR, such as temporal variations in the Antarctic during the occurrence of "ozone hole", or spatial variations with water depth or altitude. In

bryophytes, 22 studies of this kind have been carried out. Their advantage is that no environmental circumstance of the plants is modified, something greatly needed in the context of UVR works. However, these experiments cannot reproduce the conditions of potential ozone reductions, require an accurate measurement of the UVR gradient, and should differentiate the influence of interacting factors which could obscure the specific effects of UVR.

The duration of the experiments has been diverse, from a few hours of UV exposure (usually under controlled conditions) to several years (under field conditions), and the bryophyte responses have been assessed using morphological and, especially, physiological variables (Table 1).

The results obtained are diverse, since UVR has been found either to stimulate, depress or have no effect on the bryophyte performance. To a certain extent, this may have been caused by the diversity of species and experimental conditions used in the different studies. Several investigations have found a growth reduction in bryophytes exposed to UVR, but this effect seems to depend on the species considered, the experimental design and other additional factors such as temperature, water availability and CO_2 concentration. Other harmful effects (chlorophyll degradation, reduction in photosynthesis rates and F_v/F_m) are even less clear, since contradictory results have been reported. The increase in UV-absorbing compounds, the most usual response of vascular plants to enhanced UV (Searles *et al.*, 2001a), has been manifested less frequently in bryophytes. Beneficial effects of UVR on bryophyte growth have also been reported (Johanson *et al.*, 1995; Björn *et al.*, 1998; Searles *et al.*, 1999; Phoenix *et al.*, 2001), which further complicates the global interpretation of the results. This controversy contrasts with intuitive thoughts that bryophytes would be strongly sensitive to UVR, because of their structural simplicity and the consequent lack of defenses commonly found in higher plants: hairs, epicuticular waxes, thick cuticles, multilayered epidermis, etc. Given that bryophyte leaves are mostly monostratified and lack air spaces, the molecular targets of UVR

could be reached much more easily.

Globally, the responses of bryophytes to UVR and their protecting systems are still poorly characterized, and thus further study is required both under controlled and field conditions. In particular, long-term field studies under realistic enhancements of UVR (see Lappalainen *et al.*, 2008) are badly needed. However, it is already clear that bryophytes as a group are not as strongly UVR-sensitive as could be anticipated taking into consideration their structural limitations, and that many of the species studied can acclimate well to high levels of UVR. In fact, the first study about genetic responses to UV-B that has been conducted in bryophytes (Wolf *et al.*, 2010) has shown that the model moss (*Physcomitrella patens*) is able to survive UV-B stress more easily than the model cormophyte (*Arabidopsis thaliana*).

Table 1. Original papers and reviews about the effects of UV radiation on bryophytes. Key for "Used Species": L, liverwort; M, moss. Key for "Ambient": T, terrestrial; P, peatlands; A, aquatic; R, rivers or streams; L, lakes. Key for "Type of Experiment": F, Field; G, greenhouse; L, laboratory; E, exclusion of UV-B radiation; S, supplement of UV-B radiation; N, samples exposed to natural levels of solar radiation; VSh, very short duration (less than 1 day); Sh, short duration (1-30 days); M, medium duration (longer than one month and shorter than 6 months); Lo, long duration (6 months - 1 year); VLo, very long duration (longer than 1 year); ?, undetermined duration; H, historical study (comparison of samples over a prolonged period). Key for "Variables used": A, alterations in DNA; C, cover; Fl, chlorophyll fluorescence; FlS, fluorescence spectra; G, growth; Gn, genetic responses; H, hydric relations; M, morphology; Mt1, primary metabolites (glucids, proteins, lipids); Mt2, secondary metabolites, including UV-absorbing compounds; N, mineral nutrients; Ox, variables of oxidative stress (peroxide content, lipid peroxidation, ascorbate, superoxide dismutase, peroxidase, catalase); P, photosynthesis; Ph, phenology; PP, photosynthetic pigments; PS1 and PS2, activity of photosystems I and II, respectively; R, respiration; Rf, reflectance indices; Sc, sclerophylly; U, ultrastructure; Z, other variables.

Reference	Used species	Ambient	Type of experiment	Variables used
Arróniz-Crespo *et al.* (2004)	*Chiloscyphus polyanthos* (L), *Jungermannia exsertifolia* subsp. *cordifolia* (L), *Marsupella sphacelata* (L), *Scapania undulata* (L), *Brachythecium rivulare* (M), *Bryum alpinum* (M), *Bryum pseudotriquetrum* (M), *Fontinalis antipyretica* (M), *Palustriella commutata* (M), *Philonotis seriata* (M), *Polytrichum commune* (M), *Racomitrium aciculare* (M), *Rhynchostegium riparioides* (M), *Sphagnum flexuosum* (M)	A (R)	F, N	Mt2, Sc
Arróniz-Crespo *et al.* (2006)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	F, N	Fl, Mt2, P, PP, R, Sc

Reference	Used species	Ambient	Type of experiment	Variables used
Arróniz-Crespo *et al.* (2008a)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	L, S, M	Fl, Mt2, PP, Sc
Arróniz-Crespo *et al.* (2008b)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	L, S, M	Fl, Mt2, PP, Sc
Ballaré *et al.* (2001)	*Sphagnum magellanicum* (M)	P	F, E, VLo	G, Mt2
Barsig *et al.* (1998)	*Polytrichum commune* (M)	P	G, S, M	Mt1, Mt2, PP, U
Björn *et al.* (1998)	*Aulacomnium turgidum* (M), *Dicranum elongatum* (M), *Hylocomium splendens* (M), *Polytrichum commune* (M), *P. hyperboreum* (M), *Sphagnum fuscum* (M)	T, P	F, S, M-VLo	G, H
Blokker *et al.* (2006)	*Ceratodon purpureus* (M), *Polytrichum commune* (M)	-	?	Mt2
Boelen *et al.* (2006)	*Chorisodontium aciphyllum* (M), *Polytrichum strictum* (M), *Sanionia uncinata* (M), *Warnstorfia sarmentosa* (M)	T, P	F, S, Sh, M	A, Mt2
Clarke & Robinson (2008)	*Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M), *Schistidium antarctici* (M)	T	F, N, M	Mt2
Conde-Álvarez *et al.* (2002)	*Riella helicophylla* (L)	A (L)	L, E, VSh	Fl, Mt2, P, PP, R

Csintalan *et al.* (2001)	*Dicranum scoparium* (M), *Leucobryum glaucum* (M), *Mnium hornum* (M), *Pellia epiphylla* (L), *Plagiomnium undulatum* (M), *Plagiothecium undulatum* (M), *Polytrichum formosum* (M), *Sphagnum capillifolium* (M), *Tortula ruralis* (M)	T	L, S, Sh-M	Fl, FlS, Mt2
Dunn & Robinson (2006)	*Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M), *Schistidium antarctici* (M)	T	F, N, M	Mt2
Gehrke (1998)	*Sphagnum fuscum* (M)	P	F, S, VLo	G, M, Mt2, P, PP, R
Gehrke (1999)	*Hylocomium splendens* (M), *Polytrichum commune* (M)	T, P	F, S, VLo	G, M, Mt2, PP
Gehrke *et al.* (1996)	*Hylocomium splendens* (M), *Sphagnum fuscum* (M)	T, P	F, S, VLo	G, H, Mt2, PP
Green *et al.* (2000)	*Bryum argenteum* (M)	T	F, E, VSh	Fl, P
Green *et al.* (2005)	*Bryum subrotundifolium* (M), *Ceratodon purpureus* (M)	T	F, N, Sh	M, Mt2
Haapala *et al.* (2010)	*Warnstorfia exannulata* (M)	P	F, S, M-VLo	
Harris (2009)	*Plagiomnium spp.* (M)	T	N	Mt2
Hooijmaijers & Gould (2007)	*Isotachis lyallii* (L), *Jamesoniella colorata* (L)	T	F, N, ?	Fl, Mt2, PP

Reference	Used species	Ambient	Type of experiment	Variables used
Hughes *et al.* (2006)	*Drepanocladus sp.* (M)	T	F, N, Sh	Z
Huiskes *et al.* (1999)	*Sanionia uncinata* (M)	T	-	-
Huiskes *et al.* (2001)	*Sanionia uncinata* (M)	T	F, E, Sh	Fl
Huttunen *et al.* (1998)	*Dicranum sp.* (M), *Hylocomium splendens* (M), *Polytrichum commune* (M)	T, P	G, S, ?	M
Huttunen *et al.* (2005a)	*Dicranum scoparium* (M), *Funaria hygrometrica* (M), *Hylocomium splendens* (M), *Pleurozium schreberi* (M), *Polytrichum commune* (M), *Polytrichastrum alpinum* (M), *Sphagnum angustifolium* (M), *S. capillifolium* (M), *S. fuscum* (M), *S. warnstorfi* (M)	T, P	N, H	M, Mt2
Huttunen *et al.* (2005b)	*Hylocomium splendens* (M), *Pleurozium schreberi* (M)	T	N, H	M, Mt2
Ihle (1997)	*Conocephalum conicum* (L)	T	L, S, VSh	Mt1
Ihle & Laasch (1996)	*Conocephalum conicum* (L)	T	L, S, VSh-Sh	Fl, Mt1, Mt2, P
Johanson *et al.* (1995)	*Hylocomium splendens* (M)	T	G, S, ?	G, Ph

Kato-Noguchi & Kobayashi (2009)	*Hypnum plumaeforme* (M)	T	L, S, Sh	Mt2
Lappalainen *et al.* (2008)	*Pleurozium schreberi* (M)	T	F, S, VLo	G, Mt2, PP, Sc
Lappalainen *et al.* (2010)	*Polytrichum juniperinum* (M)	T	F, S, VSh, Sh, M, VLo	G, Mt2, PP, Sc
Lewis Smith (1999)	*Bryum argenteum* (M), *Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M)	T	F, E, M	G
Liu *et al.* (2010)	*Aulacomnium turgidum* (M)	T	L, S, VSh	Gn
Lovelock & Robinson (2002)	*Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M), *Grimmia antarctici* (M)	T	F, N, ?	Mt2, PP, Rf
Lud *et al.* (2002)	*Sanionia uncinata* (M)	T	F, L, E, S, VSh-VLo	A, G, Fl, M, P, Mt2, PP
Lud *et al.* (2003)	*Sanionia uncinata* (M)	T	F, E, S, VSh-Sh	A, Fl, Mt2, P, PP, R
Markham *et al.* (1990)	*Bryum argenteum* (M)	T	N, H	Mt2
Markham *et al.* (1998)	*Marchantia polymorpha* (L)	T	G, S, M	G, M, Mt2, Ph

Reference	Used species	Ambient	Type of experiment	Variables used
Martínez-Abaigar *et al.* (2003)	*Jungermannia exsertifolia* subsp. *cordifolia* (L), *Fontinalis antipyretica* (M)	A (R)	L, S, M	Fl, Mt2, P, PP, R, Sc
Martínez-Abaigar *et al.* (2004)	*Jungermannia exsertifolia* subsp. *cordifolia* (L), *Fontinalis antipyretica* (M)	A (R)	L, S, M	G, M
Martínez-Abaigar *et al.* (2008)	*Jungermannia exsertifolia* subsp. *cordifolia* (L), *Fontinalis antipyretica* (M)	A (R)	L, S, M	Fl, Mt2, P, PP, R, Sc
Martínez-Abaigar *et al.* (2009)	*Jungermannia exsertifolia* subsp. *cordifolia* (L), *Marsupella sphacelata* (L), *Scapania undulada* (L), *Brachythecium rivulare* (M), *Bryum pseudotriquetrum* (M), *Racomitrium acicalare* (M)	A (R)	L, S, Sh	Fl, G, Mt2, PP, Sc
Montiel *et al.* (1999)	*Sanionia uncinata* (M)	T	F, S, ?	Fl
Newsham (2003)	*Andreaea regularis* (M)	T	F, N, M	Mt2, PP
Newsham *et al.* (2002)	*Sanionia uncinata* (M), *Cephaloziella varians* (L)	T	F, N, Sh-M	Fl, Mt2, PP
Newsham *et al.* (2005)	*Cephaloziella varians* (L)	T	F, N, E, M	Mt2, PP

Niemi *et al.* (2002a)	*Sphagnum angustifolium* (M), *S. papillosum* (M), *S. magellanicum* (M)	P	F, S, M	G, Mt2, PP
Niemi *et al.* (2002b)	*Sphagnum balticum* (M), *Sphagnum papillosum* (M)	P	F, S, M	G, Mt2, PP
Núñez-Olivera *et al.* (2004)	*Jungermannia exsertifolia* subsp. *cordifolia* (L), *Fontinalis antipyretica* (M)	A (R)	L, S, M	Fl, G, Mt1, Mt2, P, PP, R, Sc
Núñez-Olivera *et al.* (2005)	*Jungermannia exsertifolia* subsp. *cordifolia* (L), *Fontinalis antipyretica* (M)	A (R)	L, S, Sh	Fl, Mt1, Mt2, P, PP, R, Sc
Núñez-Olivera *et al.* (2009)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	F, N, VLo	A, Fl, Mt2, Sc
Otero *et al.* (2006)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	L, S, Sh	A, Fl, Mt2, P, PP, R, Sc
Otero *et al.* (2008)	*Clasmatocolea vermicularis* (L), *Noteroclada confluens* (L), *Pachyglossa dissitifolia* (L), *Pseudolepicolea quadrilaciniata* (L), *Triandrophyllum subtrifidum* (L), *Breutelia dumosa* (M), *Bryum laevigatum* (M), *Pohlia wahlenbergii* (M), *Racomitrium lamprocarpum* (M), *Scorpidium revolvens* (M), *Scouleria patagonica* (M), *Sphagnum fimbriatum* (M), *Vittia pachyloma* (M), *Warnstorfia exannulata* (M), *Warnstorfia sarmentosa* (M)	A (R)	F, N	Mt2, Sc
Otero *et al.* (2009)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	N, H	M, Mt2
Phoenix *et al.* (2001)	*Hylocomium splendens* (M)	T	F, S, VLo	G, H

Reference	Used species	Ambient	Type of experiment	Variables used
Post & Vesk (1992)	*Cephaloziella exiliflora* (L)	T	F, N, Sh	M, Mt2, P, PP, U
Prasad *et al.* (2004)	*Riccia sp.* (L)	T	L, S, VSh	Ox, PP, PS1, PS2
Rader & Belish (1997)	*Fontinalis neomexicana* (M)	A (R)	F, E-S, M	G
Robinson *et al.* (2005)	*Grimmia antarctici* (M)	T	F, E, VLo	Fl, H, M, Mt2, P, PP, Rf
Robson *et al.* (2003)	*Sphagnum magellanicum* (M)	P	F, E, VLo	G, M
Robson *et al.* (2004)	*Sphagnum magellanicum* (M)	P	F, E, VLo	G, M
Rozema *et al.* (2002)	*Tortula ruralis* (M)	T	F, E, ?	G, Mt2
Rozema *et al.* (2006)	*Polytrichum hyperboreum* (M), *Sanionia uncinata* (M)	T	F, S, VLo	C, G
Ryan *et al.* (2009)	*Bryum argenteum* (M)	T	N, H	Mt2

Schipperges & Gehrke (1996)	*Hylocomium splendens* (M), *Sphagnum fuscum* (M)	T, P	F-L, S, M- VLo	G, H, P
Searles *et al.* (1999)	*Sphagnum magellanicum* (M)	P	F, E, Lo	G, Mt2, PP
Searles *et al.* (2001b)	*Sphagnum magellanicum* (M)	P	F, E, VLo	G, M, Mt2
Searles *et al.* (2002)	*Sphagnum magellanicum* (M)	P	F, E, VLo	G, M, Mt2, PP
Snell *et al.* (2007)	*Cephaloziella varians* (L)	T	F, N, M	Fl, Mt2, PP
Snell *et al.* (2009)	*Cephaloziella varians* (L)	T	F, E, M	Fl, Mt2, P, PP
Sonesson *et al.* (1996)	*Hylocomium splendens* (M)	T	L, S, M	G, P
Sonesson *et al.* (2002)	*Dicranum elongatum* (M), *Sphagnum fuscum* (M)	P	F, S, VLo	G, H
Taipale & Huttunen (2002)	*Hylocomium splendens* (M), *Pleurozium schreberi* (M)	T	F, S, M	Mt2
Takács *et al.* (1999)	*Dicranum scoparium* (M), *Leucobryum glaucum* (M), *Mnium hornum* (M), *Pellia epiphylla* (L), *Plagiothecium undulatum* (M), *Polytrichum formosum* (M), *Tortula ruralis* (M)	T	G, S, Sh-M	Fl
Turnbull & Robinson (2009)	*Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M), *Grimmia antarctici* (M)	T	F, N, M	A

Reference	Used species	Ambient	Type of experiment	Variables used
Turnbull *et al.* (2009)	*Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M), *Grimmia antarctici* (M)	T	L, S, VSh	A
Wolf *et al.* (2010)	*Physcomitrella patens* (M)	T	L, S, VSh, Sh, M	G, Ph, Gn, M, Mt2, PP

1.3. AQUATIC BRYOPHYTES AND ULTRAVIOLET RADIATION

Typically, bryophytes are able to tolerate desiccation and recover their physiological functions considerably rapidly in the subsequent rehydration. This is an important ecological advantage over other photosynthetic organisms (including cormophytes) in the colonization of certain harsh environments, such as bare rocks, protosoils or tree barks. Thus, bryophytes in general are characterized by this peculiar way of life, in which they take an ecophysiological advantage from the continuous dehydration-rehydration cycles that take place under natural conditions as a function of the availability of external water. Precisely, the water status of bryophytes and their desiccation tolerante is one of the factors influencing decisively on their UVR responses (Takács *et al.*, 1999; Sonesson *et al.*, 2002; Lappalainen *et al.*, 2008; Turnbull *et al.*, 2009). Consequently, the study of the effects of UVR on aquatic bryophytes may result especially important because, if adequate populations are selected, they are not exposed to the repetitive dehydration-rehydration cycles, and this prevents the interference of periodic desiccation on the effects due to UVR.

The concept of "aquatic bryophyte"

Aquatic bryophytes are integrated in a frame defined by two different environmental gradients: current-wave action and water level fluctuation (Vitt & Glime, 1984). With respect to the first factor, aquatic bryophytes may be from limnophilous (living in standing waters) to rheophilous (living in running waters). With respect to the second gradient, they range from obligate aquatics to facultative aquatics and semiaquatic emergents. So defined, a great diversity of bryophytes can be considered as aquatics, and they prevail in different environments, such as mountain streams, deep lakes and certain wetlands, where they play a relevant ecological role in primary production, nutrient cycles and food webs.

Depending on the system where they occur, they can support periphyton and provide a refuge, and occasionally direct food, for protozoa, micro- and macroinvertebrates, amphibia and fish. Bryophyte domination in those environments is primarily based on their tolerance to adverse environmental factors.

The key stresses and disturbances in streams are abrasion by turbulent water and suspended solids, substratum movement, cold water, seasonal desiccation, nutrient limitation in soft waters, CO_2 limitation in the stagnant parts of alkaline streams, high photosynthetic and UV irradiances in unshaded high-altitude streams, and diaspore difficulties to attaching to new substrates. In lakes, the adverse factors are waterlogging, cold water, low irradiances in the deep zones, high hydrostatic pressure, and abrasion along the shores. Finally, in mires, waterlogging, fluctuation of the water table level, high irradiances, and particular mineral stresses dependent on the diverse chemical composition of water in the different systems, are typical unfavorable factors.

Effects of UVR on lake bryophytes

In an important study on the effects of UVR on lake bryophytes (Conde-Álvarez *et al.*, 2002), samples of the thalloid liverwort *Riella helicophylla* from a shallow saline lake were collected and cultivated for one day, throughout a natural daily-light cycle, under two radiation treatments: full solar radiation (UVR + PAR) and solar radiation deprived of UVR (PAR treatment). There were significant differences between the two treatments in the maximum quantum yield of PSII (F_v/F_m), the effective quantum yield of photosynthetic energy conversion of PSII (Φ_{PSII}), the electron transport rate (ETR) and the initial slope of ETR *vs.* irradiance curve (all higher in PAR plants than in UV + PAR plants throughout the day), photosynthetic capacity (higher in PAR plants only at noon), chlorophyll *a* (lower in UV+PAR only at 11.00), and phenolic compounds (higher in UV+PAR only at 13.30). No differences between treatments were found in dark respiration,

photochemical quenching and carotenoid concentration, and only slight ones in nonphotochemical quenching (higher in UV+PAR only in the morning). In conclusion, although solar UVR caused some transitory damage to photosynthesis, recovery took place in the afternoon, and thus no irreversible damage occurred in the short term.

Effects of UVR on bog bryophytes

A certain number of studies have been conducted on bryophytes from circumpolar bogs, most of them under long-term field conditions (Table 1), which emphasize the significance of the results obtained. In southern latitudes, the effects of UVR exclusion have been studied in one bryophyte in a bog in Tierra del Fuego, *Sphagnum magellanicum*. In a several-months-long field experiment, height growth of this species was not affected by either near-ambient or reduced solar UV-B (Searles *et al.*, 1999). However, in a more prolonged experiment (three years), Searles *et al.* (2001b) found that height growth was less under near-ambient UV-B than under reduced UV-B, while mass per unit of height growth was greater under near-ambient UV-B. The increased height growth under reduced UV-B was counteracted by an increased volumetric density under near-ambient UV-B, so that biomass production was not influenced by the different treatments during the three years of the study (Searles *et al.*, 2002). Chlorophylls, carotenoids, chlorophyll *a/b* ratio and UV-absorbing compounds were not affected by UV-B manipulation (Searles *et al.*, 1999; Searles *et al.*, 2002). Robson *et al.* (2003) continued the experiment for another three years and corroborated previously reported results. Thus, ambient levels of UVR seemed to affect morphogenic rather than production processes in *Sphagnum magellanicum*, at least in Tierra del Fuego.

In Arctic bogs, UVR supplements have been applied in the field to simulate realistic ozone reductions (15-20%). The height increment of *Sphagnum fuscum* was reduced by 20% in the first year of exposure to enhanced UVR (25% enhancement over the controls: Gehrke *et al.*, 1996). In a

two-year experiment, height increment, spatial shoot density and dark respiration of the same species decreased under enhanced UVR, but dry mass per unit length increased, and thus biomass production did not change from the start to the end of the experiment (Gehrke, 1998). In the same study, the integrity of the photosynthetic apparatus was somewhat affected by enhanced UVR, since the concentration of chlorophyll *a* and carotenoids decreased, but net photosynthesis, chlorophyll *a/b* ratio and the levels of UV-absorbing compounds hardly changed. As it occurred with *Sphagnum magellanicum* under ambient levels of UVR, global production of *Sphagnum fuscum* was not modified by enhanced UVR although height growth was affected. In addition, Gehrke (1998) pointed out that the great variability in productivity among microsites probably masked any effect of enhanced UVR.

Other studies in Arctic bogs used modulated systems which provide UV supplements proportional to ambient UV levels, so that the simulation of UVR enhancement is more realistic. Using this system, two different studies of three-month duration (one growing season) were conducted by Niemi *et al.* (2002a, 2002b). In the first one, three *Sphagnum* species (*S. angustifolium*, *S. papillosum* and *S. magellanicum*) were used and the effects of UV-A and UV-B radiations were differentiated. Membrane damage, chlorophyll and carotenoids concentration, and UV-absorbing compounds, did not show clear changes under UV-A or UV-B enhancements (30% over ambient levels), and no significant differences between the treatments in either capitulum or stem dry mass of *S. angustifolium* (the only species tested in this respect) were observed. In their second experiment, Niemi *et al.* (2002b) found more clear effects on *Sphagnum balticum* and *S. papillosum*, given that both species showed significantly higher membrane permeability under enhanced UVR. However, the rest of the variables measured (capitulum and stem biomass, chlorophyll, carotenoids, and UV-absorbing compounds) did not show significant changes between samples exposed to current and enhanced UVR levels, except a surprising increase in chlorophyll, carotenoids and

chlorophyll *a/b* ratio under enhanced UVR in *S. balticum*.

In a more complex experimental design, Sonesson *et al.* (2002) tested the effect of increased UV-B, temperature and irrigation during two consecutive seasons in *Sphagnum fuscum* and *Dicranum elongatum*. Increased UV-B had no statistically significant overall effect in length growth, chlorophyll and flavonoid contents, neither in the interaction between the species. However, the growth of *S. fuscum* responded negatively to increased UV-B under increased temperature at the peak of the growing season, probably because of water deficit. Different responses of *S. fuscum* in this study and previous ones (Gehrke *et al.*, 1996; Gehrke, 1998) were attributed to different weather conditions during the experimental periods.

Effects of UVR on mountain stream bryophytes

Mountain streams might be particularly exposed to the effects of UVR, since (1) the biologically active UVR increases between 5% and 20% per 1000 m altitudinal increase (Björn *et al.*, 1998); (2) many organisms live emersed and fully exposed to UVR, or immersed at relatively low depths, where UVR can also reach them because it easily penetrates into the typically-occurring oligotrophic shallow waters (Frost *et al.*, 2005), and (3) the low temperatures prevailing during most of the year may limit the development of metabolically-dependent mitigating mechanisms, such as the synthesis of UV-absorbing compounds and the action of antioxidant and DNA-repairing systems.

In a pioneer study on the effects of UVR on stream bryophytes, Rader & Belish (1997) carried out a ten-week field experiment in which samples of the moss *Fontinalis neomexicana* were transplanted from a reference site to both a shaded and an open section of a mountain stream, and were irradiated with enhanced levels of UV-B radiation. The UV-exposed transplants from the open site showed an important reduction in dry

biomass with respect to those under ambient conditions, whereas the transplants from the shaded site were not affected either by ambient or enhanced UV. The moss failed to grow at any site and under any treatment condition, and there was a loss of material in all samples from the beginning to the conclusion of the experiment. These facts show clearly the difficulties in implementing field manipulative experiments (using filters or lamps) in streams, especially in the long-term.

Much more manipulative work has been done under laboratory conditions (Table 1). This is useful to characterize UVR responses while preventing the interference of other environmental factors. However, laboratory results are not directly comparable to those obtained under field conditions and, in particular, should not be used to predict the consequences of a potential UVR increase due to ozone reduction. In the laboratory, aquatic bryophytes have been cultivated under enhanced UVR simulating a realistic ozone reduction (20%), during periods ranging from 3 to 82 days. In two studies, the effects of UV-A and UV-B were measured separately, but contrasting results were obtained because, in one case, samples exposed to UV-A showed a similar behavior to those exposed to only PAR (Martínez-Abaigar *et al.*, 2003), whereas in the other study the responses of UV-A samples were more similar to those of samples exposed to UV-A plus UV-B (Otero *et al.*, 2006). Results of both studies are probably little comparable because the PAR level applied was notably different (100 - 500 μmol m^{-2} s^{-1}), and thus UV-A effects are still little characterized. In *Sphagnum*, UV-A radiation has been demonstrated to have little biological effect (Niemi *et al.*, 2002a).

Some field studies using natural gradients of UVR have been also carried out (Table 1). In the following sections, we will detail the results obtained on mountain stream bryophytes in both laboratory experiments

using enhanced UVR and in field studies using natural UVR gradients.

Laboratory studies on mountain stream bryophytes: responses of the variables used

Bryophyte responses have been analyzed in terms of diverse physiological variables: the photosynthetic pigment composition (chlorophyll and total carotenoid concentration, chlorophyll *a/b* ratio, chlorophylls/phaeopigments and chlorophylls/carotenoids ratios, xanthophyll index and the relationship (antheraxanthin + zeaxanthin)/chlorophyll *a*); the rates of net photosynthesis; some variables of chlorophyll fluorescence (the maximum quantum yield of PSII, F_v/F_m; the effective quantum yield of photosynthetic energy conversion of PSII, Φ_{PSII}; the apparent electron transport rate through PSII, ETR; and the quenching due to non-photochemical dissipation of absorbed light energy, NPQ); the rates of dark respiration; sclerophylly index (the quotient between the dry mass and the shoot area); the level of UV-absorbing compounds (analyzed both globally by spectrophotometry and individually by HPLC); DNA damage (appearance of thymine dimers); protein concentration; length growth, and morphological symptoms (both macro- and microscopic).

Not all the variables used were equally UV-responsive. UV stress may be preferentially indicated by a decrease in F_v/F_m and chlorophylls/phaeopigments ratios, and, to a lesser extent, by a decrease in chlorophyll *a/b* ratio and net photosynthesis rates. The fact that all these variables are directly related to the photosynthetic process is not surprising, because several of its components are recognized molecular targets of UVR (Jansen *et al.*, 1998). In addition, these variables have been frequently used as indices of physiological vitality because they decline under stress conditions caused by diverse harmful factors, such as cold, high light, water deficit or pollutants (Martínez-Abaigar & Núñez-Olivera, 1998; Maxwell & Johnson, 2000; DeEll & Toivonen, 2003).

Dark adapted values of F_v/F_m provide information about the intactness and potential efficiency of PSII, and are used as a sensitive indicator of plant photosynthetic performance (Maxwell & Johnson, 2000). A decrease in F_v/F_m indicates in particular the phenomenon of photoinhibition, that destroys the central core protein D1 of PSII, and D1 is a well-known target of UVR (Jansen *et al.*, 1998). Stream bryophytes are more easily photoinhibited under enhanced UVR than under only PAR (Martínez-Abaigar *et al.*, 2003).

The brown-colored phaeopigments are products of chlorophyll breakdown. Chlorophylls/phaeopigments ratios may be useful to evaluate the physiological state of a plant because they indicate the proportion of intact chlorophylls and decrease under stress conditions (Martínez-Abaigar & Núñez-Olivera, 1998). The first ratio is based on the fact that the blue maximum of chlorophylls at 430-435 nm shifts to 410-415 nm in the respective phaeopigments, and the quotient between the absorbances at 430 and 410 nm, or between the absorbances at 435 and 415, indicate the proportion between chlorophylls and phaeopigments. The second ratio requires the acidification of the pigment extract, that totally transforms chlorophylls into phaeopigments, and then the ratio between the absorbance at 665 nm before and after acidification is calculated. As the red maxima of phaeopigments at 665 nm is lower than those of their respective chlorophylls, this ratio will indicate again the proportion between chlorophylls and phaeopigments.

The chlorophyll *a/b* quotient has a notable ecophysiological relevance and typically decreases in plants that are experiencing senescence or are under stress conditions (Martínez-Abaigar & Núñez-Olivera, 1998), since the degradation of the light-harvesting complexes of the photosystems, relatively enriched in chlorophyll *b*, is slower than that of the core complexes, in which only chlorophyll *a* occurs. The chlorophyll *a/b* quotient also decreases in shade plants.

The alterations of key components of the photosystems (D1 protein,

chlorophylls) may lead to a general decrease of photosynthesis rates under enhanced UV, that can be intensified by UV-driven damage in photosynthetic enzymes (Jansen *et al.*, 1998). However, photosynthesis rates may remain unaffected under field conditions (Allen *et al.*, 1998) and, even in the laboratory, damage in photosynthetic pigments, proteins or enzymes does not necessarily imply a decrease in photosynthesis rates.

Other physiological variables that have been shown to be UV-responsive in bryophytes from mountain streams, although to a lower extent than those described above, are the sclerophylly index, chlorophyll and carotenoid concentrations, and growth. In cormophytes (ferns and seed plants), leaf sclerophylly increases with the development of non-photosynthetic structures such as epidermal cells, hairs, cuticles, epicuticular materials, cell walls, and vascular and supporting tissues, as well as with increasing contents of organic and inorganic solutes. In bryophytes, shoot sclerophylly may depend on the proportion of leaves (phyllidia) and stems (caulidia), the leaf architecture, the ratio of photosynthetic and non-photosynthetic (vascular, supporting-mechanical) tissues, the leaf thickness and papillosity, the proportion between cell walls and protoplasts, and the organic and inorganic contents. The higher values of the sclerophylly index reported under enhanced UV in bryophytes may be due to lower elongation, which could lead to the production of less soft tissues. Hence, this index may indicate morphogenic changes and could be an indirect measurement of growth. In this line, the sclerophylly index could be interpreted in a similar way to the mass per unit length ratio used in other bryophytes (Gehrke, 1998; Searles *et al.*, 2001b).

Chlorophylls and carotenoids are UV targets (Jansen *et al.*, 1998) and thus both pigments would be expected to decrease under enhanced UVR. However, they may increase, decrease or remain unaltered, and thus the effects of UVR on chlorophylls and carotenoids in bryophytes are still obscure, probably due to interspecific and experimental differences. Usually, the concentrations of total chlorophylls and total carotenoids fluctuate in

the same direction in response to enhanced UVR, because chlorophylls and carotenoids are components of photosystems and, when these are constructed or degraded, both types of pigments increase or decrease, respectively. However, their functions are not the same: chlorophylls are specialized in light absorption, whereas carotenoids are involved either in photoprotection or in light absorption. Thus, if carotenoids implied in photoprotection were individually analyzed, their changes would be probably uncoupled to those of chlorophylls. Photoprotecting carotenoids have been analyzed only once in stream bryophytes, and the ratio (antheraxanthin + zeaxanthin)/chlorophyll *a* increased under enhanced UVR, showing a higher photoprotection or a preferential degradation of chlorophyll *a* (Otero *et al.*, 2006).

With respect to growth measurements, only length growth has been tested in response to enhanced UVR in bryophytes from mountain streams. A reduction in length growth under enhanced UVR was found only in *Jungermannia exsertifolia* subsp. *cordifolia* among the six species tested, but otherwise this liverwort was UV-tolerant on the basis of most physiological variables. This suggests that the reduction in length growth would be a morphogenic rather than a damage response. It should be also taken into account that growth measurement in bryophytes is not an easy task to do, and the high variability of data can obscure their interpretation.

Damages caused by enhanced UVR in stream bryophytes, as indicated by the variables described above, are quite similar to those found in other photosynthetic organisms, from phytoplankton to flowering plants. Those variables are indicative of the basic physiological state of the plant, and the responses found are unspecific and may be caused by other adverse factors than enhanced UVR. Thus, the interpretation of results must be cautious. Nevertheless, when a high number of experimental variables are used, multivariate analyses may help demonstrate a "UV general syndrome" suffered by the plants exposed to enhanced UVR, and indicated by a combination of variables (Núñez-Olivera *et al.*, 2004; Otero *et al.*, 2006).

A separate remark should be made for DNA damage. This kind of damage has been rarely measured in bryophytes and, generally, it increased in samples exposed to UVR levels higher than the natural ones, especially under laboratory conditions (Lud *et al.*, 2002; Otero *et al.*, 2006; Turnbull *et al.*, 2009), although also rarely in the field (Lud *et al.*, 2002, 2003). However, no DNA damage has been found under natural UVR levels (Boelen *et al.*, 2006; Núñez-Olivera *et al.*, 2009, 2010), except when the environmental conditions were so adverse that repairing processes were impaired, as occurred in the Antarctic (Turnbull & Robinson, 2009). In the aquatic species studied, the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* showed DNA damage exclusively in the samples exposed to enhanced UVR (mainly consisting of UV-B) in the laboratory (Otero *et al.*, 2006). This is congruent with the target character of DNA with respect to UV-B. However, no damage was demonstrated in either this liverwort or two mosses under field conditions, probably due to the efficient repair mechanisms in nature (Núñez-Olivera *et al.*, 2009, 2010). The specific dependence of DNA damage with respect to enhanced UV-B may be particularly interesting in the evaluation of the consequences of stratospheric ozone depletion on bryophytes.

Effects of UVR on respiration remain obscure because contrasting responses have been found.

Finally, morphological responses to enhanced UVR were studied in two species, between which the moss *Fontinalis antipyretica* showed some damage manifested in brown color, development of the central fibrilar body in the cells, chloroplast disappearance, and presence of protoplasts progressively vesiculose, vacuolized and finally hyaline (Martínez-Abaigar *et al.*, 2004). These symptoms are little specific and have been described in several pleurocarpous mosses as a response to diverse processes of senescence and stress (both natural and anthropogenic: Glime & Keen, 1984; Gimeno & Puche, 1999). The only specific response of the moss to enhanced UVR was a colour change in the cell walls, from yellow to orange-brown.

The morphological damage found in the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* were more subtle, and only when enhanced UVR was combined with chilling, leaves showed greyish brown colours, together with modest cellular damage.

Laboratory studies on mountain stream bryophytes: responses of UV-absorbing compounds

While the variables described above may be differently sensitive markers to detect UVR damage, other variables may rather show acclimation processes. This is the case of the accumulation of UV-absorbing compounds, which is the most common response of vascular plants to enhanced UVR, with an average increase around 10% (Searles *et al.*, 2001a). There is not a simple direct relationship between this accumulation and UVR tolerance, but UV-absorbing compounds could reduce UVR penetration and, consequently, damage to the potential targets. They may offer additional protection through free-radical scavenging activity (Sroka, 2005).

The most used variable to quantify the levels of UV-absorbing compounds, both in bryophytes and other photosynthetic organisms, has been the bulk UV absorbance. It should be noted that this measurement is based on a simple methanol extraction, and thus the UV-absorbing compounds bound to the cell wall may be underextracted or not extracted at all, leading to the underestimation of the total UV absorption capacity. An adequate evaluation of the contribution of methanol-extractable and non-extractable UV-absorbing compounds to the bulk UV-absorption capacity of bryophytes, both mosses and liverworts, remains to be done. UV-absorbing compounds have been demonstrated to be located in cell walls, where they could contribute decisively to UV protection (Clarke & Robinson, 2008), but the fraction more easily extractable in methanol is probably located in vacuoles, as in vascular plants.

In bryophytes, an increase in UV absorbance (soluble fraction) with

increasing UVR has been much more frequently found in liverworts (in ten out of 14 experiments, conducted on six species) than in mosses (in seven out of around 45 experiments, in which about 20 species were used). Similar results have been found in bryophytes from mountain streams. In addition, liverworts show much higher values of UV absorbance (soluble fraction) than mosses under natural conditions, and the absorption spectra in the UV band are notably different in both groups (Arróniz-Crespo *et al.*, 2004; Otero *et al.*, 2008). These facts suggest that (1) the accumulation of methanol-extractable UV-absorbing compounds represents a frequent and constitutive protecting mechanism against UVR in liverworts, but not in mosses; and (2) this mechanism is inducible by UVR much more frequently in liverworts than in mosses. The apparently different behavior of both bryophyte groups with respect to the accumulation of methanol-extractable UV-absorbing compounds could be related to the fact that mosses and liverworts are considered nowadays to be more phylogenetically separated than previously thought (Qiu *et al.*, 2006). Given the important role of liverworts in the water-to-land transition (Zobell *et al.*, 2010), their efficient accumulation of UV-absorbing compounds could have been one factor favoring success in land colonization, supporting higher UV levels than the aquatic habitat.

Another critical point regarding UV-absorbing compounds in bryophytes is that the measurement of the bulk UV absorbance might be insufficient to assess the effects of UVR because each individual compound may respond to UVR in a different manner. Thus, it would be better to use HPLC methods allowing the chemical characterization of the individual compounds. This approach has been progressively more frequently used in bryophytes (Markham *et al.*, 1998; Blokker *et al.*, 2006; Harris, 2009; Kato-Noguchi & Kobayashi, 2009; Snell *et al.*, 2009), especially through the measurement of hydroxycinnamic acid derivatives in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* (Arróniz-Crespo *et al.*, 2006, 2008a, 2008b; Otero *et al.*, 2006, 2009; Martínez-Abaigar *et al.*, 2008; Núñez-Olivera *et al.*, 2009). These studies make clear that, although all the individual compounds contribute to the bulk UV absorbance, their UVR

responses can be different: some of them may increase under enhanced UVR while others may decrease or remain unaltered. These contrasting responses, together with the limited capacity of methanol to extract cell-wall-bound compounds, could be reasons for the relatively frequent failure in demonstrating an increase in the bulk UV absorbance of bryophytes (especially in mosses) under enhanced UVR.

Laboratory studies on mountain stream bryophytes: factors influencing UVR responses

The factors influencing the UVR responses of aquatic bryophytes can be divided into internal and environmental ones. With respect to internal factors, UVR responses may depend primarily on the species, as in the case of vascular plants, and bryophytes should not be grouped as a single functional type regarding UVR effects. The most used aquatic bryophytes in laboratory studies have been the foliose liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, together with the moss *Fontinalis antipyretica*, but five additional species, two liverworts and three mosses, have been used (Table 1). Overall, effects of enhanced UVR are not dramatic for most species under laboratory conditions, except for some particularly sensitive species, such as *Fontinalis antipyretica*. The UVR sensitivity of this moss may be only relative under field conditions, because some populations can be found withstanding high UVR levels above the upper limit of the forest.

Interspecific differences in UVR tolerance may be based on the degree of development of repairing and protecting mechanisms, such as the accumulation of UV-absorbing compounds (Martínez-Abaigar *et al.*, 2003), but the influence of structural characteristics cannot be discarded. Also, a direct relationship between the UV tolerance and the desiccation tolerance of the different species has been suggested in bryophytes (Takács *et al.*,

1999; Csintalan *et al.*, 2001; Turnbull *et al.*, 2009).

Another internal factor is the tissue age. In UV-exposed samples of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, five UV-absorbing compounds (hydroxycinnamic acid derivatives) were differentially distributed between apical and basal segments of the shoots, thus providing presumably different levels of UV protection (Arróniz-Crespo *et al.*, 2008b).

Among environmental factors, a low culture temperature (2ºC) has been shown to enhance the adverse effects of UVR in the UV-sensitive *Fontinalis antipyretica*, but not in the more UV-tolerant *Jungermannia exsertifolia* subsp. *cordifolia* (Núñez-Olivera *et al.*, 2004). Thus, the adverse effects of cold and UVR were apparently additive in the moss, whereas this additiveness was lacking in the liverwort. The combined effect of cold and UVR would probably be due to the limitation of the development of metabolically-related protection and repairing mechanisms against UVR. Other culture conditions apart from temperature, such as the PAR level and the proportions UVR/PAR, may influence the UVR responses.

UVR responses are also influenced by the previous light acclimation of the different samples to sun or shade conditions (Núñez-Olivera *et al.*, 2005). Shade samples were more susceptible to enhanced UVR than sun samples, whose protection against UVR would be more efficient. Again, this effect was found only in a UVR-sensitive species (*Fontinalis antipyretica*), but not in a UVR-tolerant one (*Jungermannia exsertifolia* subsp. *cordifolia*). In the latter, sun and shade samples responded to enhanced UVR in a similar way, and both types of samples would be efficiently protected. Another example of acclimation to high UVR levels was suggested for samples of aquatic bryophytes collected in high-altitude sites and/or in dates near the summer solstice (Martínez-Abaigar *et al.*, 2009). These samples were more tolerant to enhanced UVR than samples of the same species collected from lower altitudes or in periods of the year with lower UVR levels. Thus, the responses to UVR could be influenced by the collection place and collection

date of the samples.

Exposure to heavy metals, such as cadmium, may exacerbate the damaging consequences of enhanced UVR, since both agents are harmful for plant metabolism through some similar mechanisms, such as the production of reactive oxygen species, DNA damage, or alterations in the photosynthetic machinery (Otero *et al.*, 2006). Cadmium and UVR may interact differently depending on the variable considered. In the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, both cadmium and enhanced UVR caused chlorophyll degradation and a strong inhibition of PSII activity, together with an increase in the mechanisms of non photochemical dissipation of energy (increase in the xanthophyll index). Both adverse factors may affect, in a different manner, PSII activity and the photosynthetic machinery in general: UVR radiation inactivates PSII reaction centres, while cadmium acts mainly on the oxygen evolving complex but also on several other photosynthetic sites and processes. Consequently, both factors may have additive effects on those variables. However, cadmium caused a diminution in photosynthesis rates, probably because the activity of Rubisco and other enzymes of Calvin cycle were affected, whereas UVR did not. UVR increased the levels of UV-absorbing compounds, such as *p*-coumaroylmalic acid, and cadmium did the same with, especially, phaselic and feruloylmalic acids. These responses could be related to both the more efficient absorption of harmful UVR and the enhanced protection against oxidative stress. DNA damage was almost specifically provoked by UV-B radiation, whereas cadmium itself had modest effects in this respect. Nevertheless, the strongest DNA damage was recorded under the presence of both UV-B and cadmium, which could act synergistically through two different mechanisms: UV-B would primarily induce the formation of thymine dimers and cadmium would impair the enzymatic repair mechanisms of DNA. In conclusion, UVR and cadmium seemed to operate additively on some physiological processes, while other responses were probably due to either factor alone. The samples exposed to

both UVR and cadmium showed the most intense damage.

The interaction between UVR and mineral availability has been little investigated in bryophytes, in spite of their important peculiarities regarding mineral nutrition. Among mineral nutrients, phosphorus frequently exerts a limiting effect for aquatic phototrophs, because of its importance in ATP production and thus in the overall cell metabolism (in particular, in the repairing of UV-damaged proteins and DNA). Some evidence from microalgae suggests that phosphorus limitation increases UV sensitivity and phosphorus enrichment minimizes UV damage. However, in two bryophytes from mountain streams, an improvement in the availability of phosphorus, with the consequence of a 1.7- to 3.7-fold increase in phosphorus tissue concentration, did not modify the responses of diverse physiological variables to enhanced UVR (Martínez-Abaigar *et al.*, 2008). This was probably due to the fact that the bryophytes studied had low nutritional requirements, and their natural tissue phosphorus concentrations would be enough for their metabolic activity. Thus, increasing phosphorus would result only in luxury consumption, without any improvement either in the photosynthetic performance or the mechanisms of UVR protection and repairing.

Field studies using natural gradients of UVR: stream bryophytes as UVR bioindicators

Three field studies using natural gradients of UVR have been conducted on stream bryophytes. In the first one, Arróniz-Crespo *et al.* (2006) demonstrated a direct association between the increased UVR levels along an altitudinal gradient and both the bulk UV absorbance and the concentrations of several hydroxycinnamic acid derivatives (in particular, two coumarins) in *Jungermannia exsertifolia* subsp. *cordifolia*. These compounds could play a role as UVR protectors. In the second study, Núñez-

Olivera *et al.* (2009) evidenced that, for three consecutive years, the concentration of *p*-coumaroylmalic acid in *Jungermannia exsertifolia* subsp. *cordifolia* was higher in summer-autumn than in winter-spring, being positively correlated with UVR levels. In addition, the best model explaining UV-B radiation took into consideration the concentration of this compound and the ozone level. Ozone maximum was reached in early spring (March-April), UV-B in summer (June-July) and *p*-coumaroylmalic acid in autumn (September-October, although its values were high from June). Thus, ozone and *p*-coumaroylmalic acid would compensate each other in UV-B modelization. Finally, in two mosses monitorized also during three years (*Bryum pseudotriquetrum* and *Fontinalis antipyretica*: Núñez-Olivera *et al.*, 2010), the physiological variables related to photoprotection (the activity of xanthophyll cycle and the bulk UV absorbance) showed more clear seasonal variations than the variables related to physiological activity, such as the sclerophylly index or F_v/F_m.

The relationships found between the concentrations of hydroxycinnamic acid derivatives in *Jungermannia exsertifolia* subsp. *cordifolia* and the spatial and temporal changes in UVR, make progress in the study of using stream bryophytes as UVR bioindicators. This may take advantage of the well-known bioindication ability of aquatic bryophytes in a number of pollution processes and environmental changes (Glime, 1992). In addition, the concentration of *p*-coumaroylmalic acid in herbarium samples of *Jungermannia exsertifolia* subsp. *cordifolia* was useful in the reconstruction of past ozone and UVR levels in northern Europe (Otero *et al.*, 2009). This reconstruction showed that there was no significant temporal trend in UVR in the period 1918-2006, which agrees with previous UVR reconstructions based on both purely climatic models and biological proxies. These studies, together with those of Ryan *et al.* (2009) and Snell *et al.* (2009) in the Antarctic, are the first ones using individual UV-absorbing compounds of bryophytes in UVR bioindication, given that all the previous studies were based on the bulk UV absorbance of methanolic extracts (Newsham *et al.*, 2002, 2005; Newsham, 2003; Huttunen *et al.*, 2005a, 2005b;

Robinson *et al.*, 2005).

For UVR bioindication purposes, an adequate selection of both variables and species must be carried out. The use of hydroxycinnamic acid derivatives in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* seems to be particularly useful for this aim, for several reasons: (1) liverworts are known for their great variety of secondary metabolites, such as many different phenolic constituents; (2) UV-absorbing compounds of liverworts in general, and of this species in particular, are usually more UVR-responsive than those of mosses; (3) the selected species is large enough (2-10 cm long) to allow for easy manipulation, and it forms extensive and frequently unmixed masses that provide plenty of biomass; (4) given the long-lived perennial character of this species, healthy biomass may be available throughout the year; (5) if permanently submerged populations are selected, the interference of the typical transitory desiccation of bryophytes is prevented, and thus the responses may be easier to interpret and model; (6) this species has a wide distribution range over the northern hemisphere, which facilitates studies across wide geographical scales; (7) one of its hydroxycinnamic acid derivatives, *p*-coumaroylmalic acid, is specifically induced by enhanced UVR in laboratory studies under different PAR levels, and correlates well with the temporal changes in UVR under field conditions; and (8) a chemically similar UV-absorbing compound (*p*-coumaric acid) was used in the Antarctic graminoid *Deschampsia antarctica* as a UV-B indicator (Ruhland *et al.*, 2005), and has been repeatedly recommended for the reconstruction of past UV-B (Rozema *et al.*, 2001; Blokker *et al.*, 2006; Lomax *et al.*, 2008).

More studies are needed to better use aquatic bryophytes for UVR bioindication. Some aspects that merit further investigation are: (1) the evaluation of the responses of cell wall-bound UV-absorbing compounds to UVR; (2) the assessment of bryophyte responses to UVR changes in temporal scales little studied until now, such as diel scales; and (3) the comparison of results obtained under laboratory and field conditions.

CHAPTER 2

OBJECTIVES

The general objective of the present Doctoral Thesis is to study in depth the ecophysiological responses of aquatic bryophytes to UV-B radiation, especially considering the physiological damage induced by UV-B and the protection mechanisms developed by these organisms, under both laboratory and field conditions, and taking into account different temporal scales. This may serve to a better understanding of the acclimation of bryophytes to UV-B radiation, in the context of the UV-B enhancement caused by the degradation of stratospheric ozone.

This general objective can be divided into several more specific ones (Table 1):

1. To properly evaluate the role of UV-absorbing compounds as protectants against UV radiation in bryophytes. This requires the measurement of the UV-absorbing compounds located in two different cell compartments: vacuoles (soluble fraction) and cell walls (insoluble fraction). Given that only the traditional alkaline digestion had been applied previously to extract insoluble UV-absorbing compounds in bryophytes, we assayed, for the first time in bryophytes, enzymatic methods to try to improve extraction.

2. To examine, in a one-month duration study under laboratory conditions, the responses of aquatic bryophytes to enhanced UV-B radiation simulating the degradation of stratospheric ozone. We analyzed both a) physiological damage, in terms of the maximum quantum yield of PSII (F_v/F_m), DNA damage, and sclerophylly index (as an indirect indicator of growth); and b) protection mechanisms, particularly attending to the induction of UV-absorbing compounds in two different cell compartments: vacuoles (soluble fraction) and cell walls (insoluble fraction). In addition, UV-absorbing compounds were measured not only as a whole, using the standard procedure (the measurement of the bulk UV absorbance of methanolic extracts by spectrophotometry), but also individually, by measuring by HPLC the concentrations of compounds belonging to the two main types of UV-protective phenolics: hydroxycinnamic acids and

flavonoids. This differentiation between global and individual UV-absorbing compounds is important because each individual compound may respond differently to UV-B. This study was performed in three different species, the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* and the mosses *Bryum pseudotriquetrum* and *Fontinalis antipyretica*, because it has been previously shown that liverworts and mosses may have different mechanisms to cope with high UV-B levels. This is the first time that individual UV-absorbing compounds from both the soluble and the insoluble fractions are measured in bryophytes in response to enhanced UV-B radiation.

3. To study, in a daily scale, the changes produced in the physiology of the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* in response to photosynthetic, UV-A and enhanced UV-B radiations. The study was conducted, firstly, under laboratory conditions, to prevent the interference of other factors than radiation, and then under field conditions. Again, we measured response variables related to both physiological damage (photosynthetic pigments, chlorophyll fluorescence, DNA damage) and protection mechanisms (induction of UV-absorbing compounds, measured both globally and individually, in two cell compartments: vacuoles and cell walls). This is the first time in which daily changes in response to UV radiation are assessed in a liverwort.

4. To relate, in the long-term (three years) and under field conditions, both seasonal and interannual variations in ambient UV-B radiation with changes in the concentration of the flavonol kaempferol 3,7-*O*-diglucoside, one potential UV-protective compound, in the moss *Bryum pseudotriquetrum*.

Table 1. Summary of experiments performed in the context of the objectives of the present Thesis. *Jec*, *Jungermannia exsertifolia* subsp. *cordifolia*. *Bp*, *Bryum pseudotriquetrum*. *Fa*, *Fontinalis antipyretica*. Flu, chlorophyll fluorescence variables. DNA, DNA damage. SI, sclerophylly index. Pig, photosynthetic pigments. UVAC, UV-absorbing compounds.

Chapter	Species	Lab or Field	Duration	Ambient or Enhanced UV-B	Variables related to UV damage	Variables related to UV protection
4	*Jec*	Lab	-	-	-	UVACs: - globally-individually - insoluble
5-6	*Jec* *Bp* *Fa*	Lab	1 month	Enhanced	Flu DNA SI	UVACs: - globally-individually - soluble - insoluble
7-8	*Jec*	Lab	17 days	Enhanced	Pig Flu DNA SI	UVACs: - globally-individually - soluble - insoluble
9	*Jec*	Field	2 days	Ambient	Flu DNA	UVACs: - globally-individually - soluble - insoluble

10	*Bp*	Field	3 years	Ambient	-	UVACs: Individual soluble

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. PLANT MATERIAL AND COLLECTION SITES

Three bryophytes were used in this work: the liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váňa and two mosses, *Bryum pseudotriquetrum* (Hedw.) P.Gaertn. *et al.*, and *Fontinalis antipyretica* Hedw.

Jungermannia exsertifolia subsp. *cordifolia* (Fig. 1) is a large (shoots up to 10 cm long and 5 mm wide) strongly aromatic leafy liverwort typically growing in extense, swollen masses. The leaves are about 2 mm long and wide, characteristically heart-shaped, with a broad base narrowing rapidly to the stem and a narrower, rounded tip. Its polygonal cells have thin walls and 2-3 oil bodies per cell, and lack trigones. It may have red-brown tones, and even black, to its dominantly deep green colour. It is dioicous and has colourless rhizoids. No sign of sexual reproduction has been observed in our samples, but fragments may contribute to vegetative propagation. This is a characteristic species of oligotrophic circumneutral mountain streams.

Bryum pseudotriquetrum (Fig. 2) is a relatively large acrocarpous moss which forms green, reddish or brown tufts composed by several cm tall shoots covered in its basal part with a dense brown mat of rhizoids. Leaves are lanceolate and decurrent, equally spaced along the stem, 2–3.5 mm long, and have a reddish base, a border of narrow cells and a thick and shortly excurrent nerve. Cells are hexagonal. It is dioicous and often produces inclined capsules at the end of a long seta (2–3 cm). It is quite common in marshes, fens, flushes and along stream margins.

Fontinalis antipyretica (Fig. 3) is a large pleurocarpous moss with shoots up to 8 mm wide and often exceeding 15 cm in length. It forms dangling submerged masses ranging in colour from bright green to brownish. Its 4–5 mm long, ovate and strongly keeled leaves, which lack a nerve, give the shoots a characteristic three-sided appearance. Cells are long and narrow. Capsules are very uncommon, and they have never been

observed in our samples. This species is very common in a wide range of slowly or rapidly flowing water bodies, especially in streams and rivers, and can tolerate prolonged periods of exposure above the water.

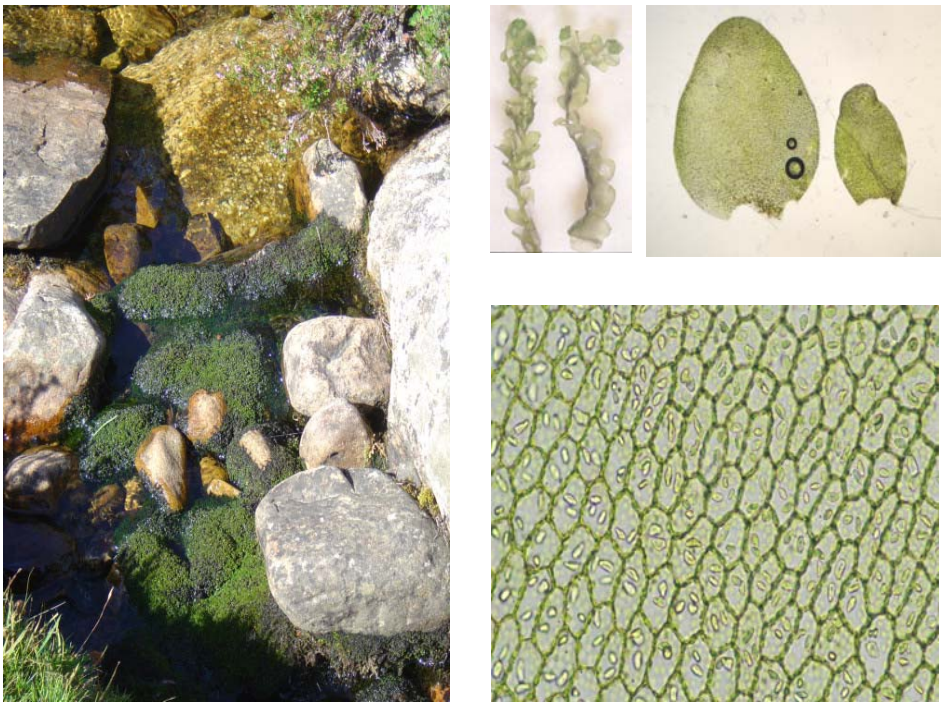

Fig. 1. An extense mass of *Jungermannia exsertifolia* subsp. *cordifolia* in a mountain stream (Lumbreras, La Rioja, Spain). Two leafy shoots, heart-shaped leaves, and polygonal cells with oil bodies and lacking trigones are shown.

Fig. 2. Masses of *Bryum pseudotriquetrum* in a mountain stream (Lumbreras, La Rioja, Spain). Several shoots showing red stems, a typical lanceolate leaf with a strong margin and decurrent leaf base, and hexagonal cells with chloroplasts, are shown.

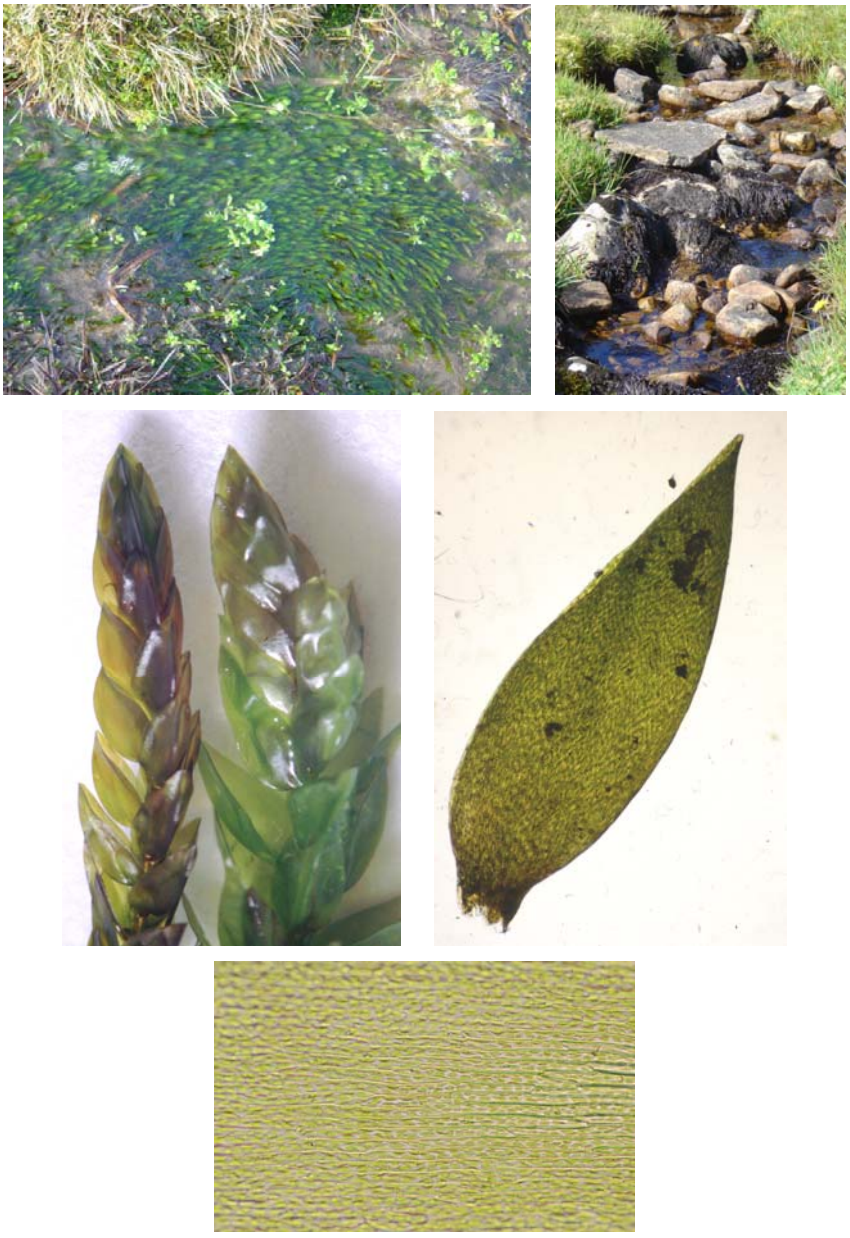

Fig. 3. Masses of submerged (deep green) and emersed (brownish black) *Fontinalis antipyretica* in a mountain stream (Lumbreras, La Rioja, Spain), together with two three-sided shoots, a typical leaf (which is plicated by the midline), and long cells with chloroplasts, are shown.

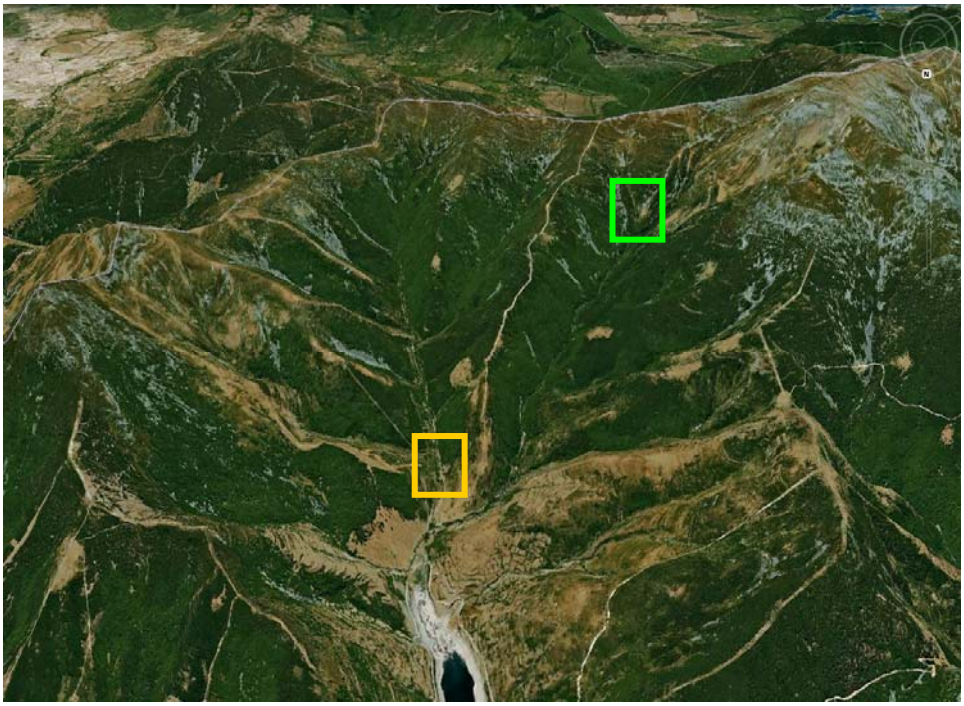

Fig. 4. Bryophyte sampling points in the upper basin of the River Iregua. Yellow quadrat, stream Lavieja. Green quadrat, stream Lumbreras.

Bryophyte samples were collected at the first-order streams Lumbreras (at around 1900 m altitude) and Lavieja (1280 m) (Fig. 4). They are located in the upper basin of the River Iregua, within the limits of the Natural Park of Sierra Cebollera (La Rioja, northern Spain). These oligotrophic and circumneutral streams flow mainly over sandstones and quartzites (Purbeck-Weald facies, Jurassic-Cretaceous). The vegetation of the sampling zone in the river Lumbreras (42°00'30'' N, 02°38'40'' W) is a subalpine shrubland dominated by *Vaccinium myrtillus* L., *Juniperus communis* L. subsp. *alpina* (Suter) Celak and *Calluna vulgaris* (L.) Hull, and intermingled with scattered *Pinus sylvestris* L. In the sampling zone of Lavieja stream (42°03'00'' N, 02°35'30'' W), the vegetation is a clear *Fagus sylvatica* L. woodland.

All the samples were submerged and exposed to full sun when collected. The material was rinsed with stream water and transported to the laboratory in a portable icebox. Once in the laboratory, samples were cultured and/or directly exposed to the specific experimental conditions that were required.

3.2. EXPERIMENTAL DESIGN IN UV-B ENHANCEMENT LABORATORY EXPERIMENTS

For laboratory experiments, green healthy shoot apices were selected. In some experiments, a preculture of the samples was done in a circulating bath system filled with air-bubbled stream water within a growth chamber. Plants were submerged at a depth of 1-2 cm and were maintained at 10ºC. The photosynthetic photon flux density (PFD) was around 100 μmol m^{-2} s^{-1} at the water surface (LI-190SA quantum sensor, LI-COR, Lincoln, NE, USA), and was provided by Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) and True-lite full spectrum (True Sun, Steubenville, OH, USA) fluorescent tubes. After the preculture, apices were distributed into separate plastic tubes (12 cm of diameter) with a basal net which prevented material losses (Fig 5). The tubes were placed in recipients filled with air-bubbled stream water which were permanently circulating and kept at 10ºC. Each recipient was placed on a rotating platform to prevent possible place-dependent differences in the radiation received by the plants, which was provided by a Hönle SOL 1200RF2 lamp (Dr. Hönle AG UV-Technologie, Gräfelfing/Munich, Germany) and Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) and True-lite full spectrum (True Sun, Steubenville, OH, USA) fluorescent tubes. Spectral characteristics have been published elsewhere (Gröniger e*t al*, 1999).

In the typical experiments, the samples were subjected to the following three radiation regimes by covering the tubes with specific UV cut-off foils (PAR is photosynthetically active radiation):

- P (PAR alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which cut off all UV radiation.

- PA (PAR + UV-A), using Folex 320 (Folex GmbH, Dreieich, Germany), which cut off UV-B and UV-C radiation.

- PAB (PAR + UV-A + UV-B), using Ultraphan 295 (Digefra GmbH, Munich, Germany), which cut off UV-C radiation.

The filters were pre-irradiated for one hour and periodically replaced. Table 1 shows the radiation conditions in the different regimes. The biologically effective UV-B irradiance (UV-B_{BE}) was estimated using classic and modern action spectra: the generalized plant damage action spectrum (Caldwell, 1971) normalized at 300 nm, the DNA damage spectrum (Setlow, 1974), and that of Flint & Caldwell (2003). Given that the latter also takes into account UV-A radiation, biologically effective UV irradiance (UV_{BE}) was estimated in this case. The spectral irradiances were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland), and PAR was measured with a quantum sensor (LI-190SA). The UV source (Hönle lamp) was switched on around noon for an adequate period using a square-wave system. The plants under the PAB regime received an appropriate daily biologically effective UV-B dose to mimic a 20% ozone reduction as calculated with a computer model (Björn & Teramura, 1993), taking into account the maximum biologically effective UV-B dose in the collection sites and the radiation amplification factor of ozone for biologically effective UV-B. Water in the culture was replaced periodically.

Table 1. Radiation conditions in the three radiation regimes (P, PA, and PAB) under which the bryophyte samples were usually cultivated. UV-BBE (biologically effective UV-B radiation) and UVBE (biologically effective UV radiation) were calculated on the basis of the action spectra specified. UV radiation and high PAR were provided to the plants during the central hours of the day, and this period was preceded and followed by periods of low PAR.

	P	**PA**	**PAB**
PAR in the low-PAR period (µmol m^{-2} s^{-1})	98	96	101
PAR in the low-PAR period (W m^{-2})	18	18	19
PAR in the high-PAR period (µmol m^{-2} s^{-1})	522	512	543
PAR in the high-PAR period (W m^{-2})	97	98	100
PAR dose (kJ m^{-2} d^{-1})	27.04	27.29	27.94
UV-A (W m^{-2})	2.80	32.8	35.4
UV-A dose (kJ m^{-2} d^{-1})	70.6	826.6	892.1
UV-B (W m^{-2})	0.009	0.015	1.085
UV-B dose (kJ m^{-2} d^{-1})	0.23	0.38	27.3
$UV\text{-}B_{BE}$ (W m^{-2}) (Caldwell, 1971)	0.00	0.00	0.40
UV_{BE} (W m^{-2}) (Flint & Caldwell, 2003)	0.02	0.55	0.96

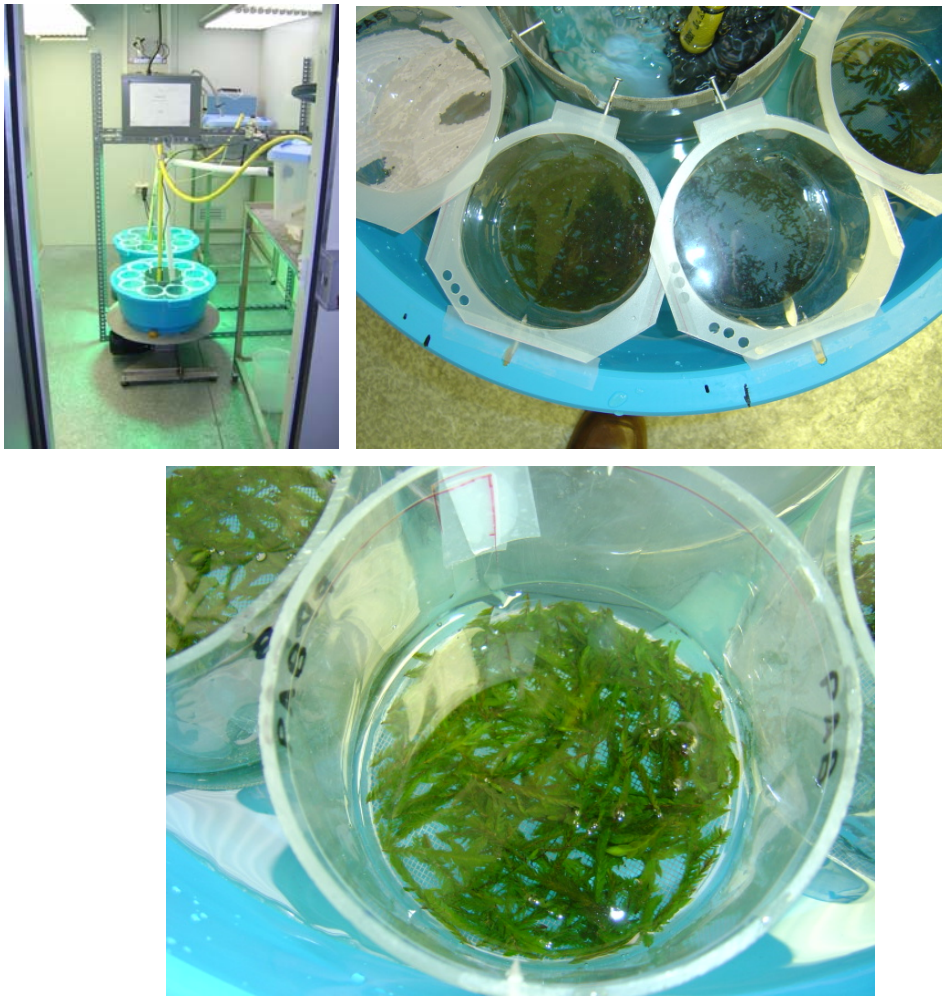

Fig. 5. Experimental design for a typical UV-B enhancement experiment under laboratory conditions. Lamps for providing PAR and UV radiation, recipients with samples, cut-off filters used to impose the different radiation regimes, and bryophyte samples in a plastic tube (once the cut-off filter has been removed) are shown.

3.3. PHYSIOLOGICAL VARIABLES

Chlorophyll fluorescence measurements

Chlorophyll *a* fluorescence was measured using two instruments:

- Mini-PAM (Walz Instruments, Effeltrich, Germany), which was used for both field and laboratory measurements.

- AquaPen-P AP-P 100 (Photon Systems Instruments, Brno, Czech Republic), which was used in the field to obtain OJIP curves.

Measurement of F_v/F_m, Φ_{PSII}, NPQ and ETR

In vivo chlorophyll fluorescence of PSII was measured with a portable pulse amplitude modulation fluorometer (MINI-PAM) using the saturation pulse method (Fig. 6) following Schreiber *et al.* (1995) and Núñez-Olivera *et al.* (2004a). Minimal and maximal fluorescence (F_0 and F_m) were measured in samples dark-adapted for 20 min. The maximum quantum yield of PSII was given by the ratio F_v/F_m, where $F_v = F_m - F_0$, and the effective quantum yield of photosynthetic energy conversion of PSII (Φ_{PSII}) by the ratio (Fm'-Ft)/Fm'. The apparent electron transport rate through PSII (ETR) was calculated as: ETR = Φ_{PSII} x PFD x 0.5 x 0.84, where PFD is photon flux density. Quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined as (Fm - Fm')/Fm'. When obtained, the curve ETR *vs.* PFD was fitted to Jassby and Platt's hyperbolic tangent model equation.

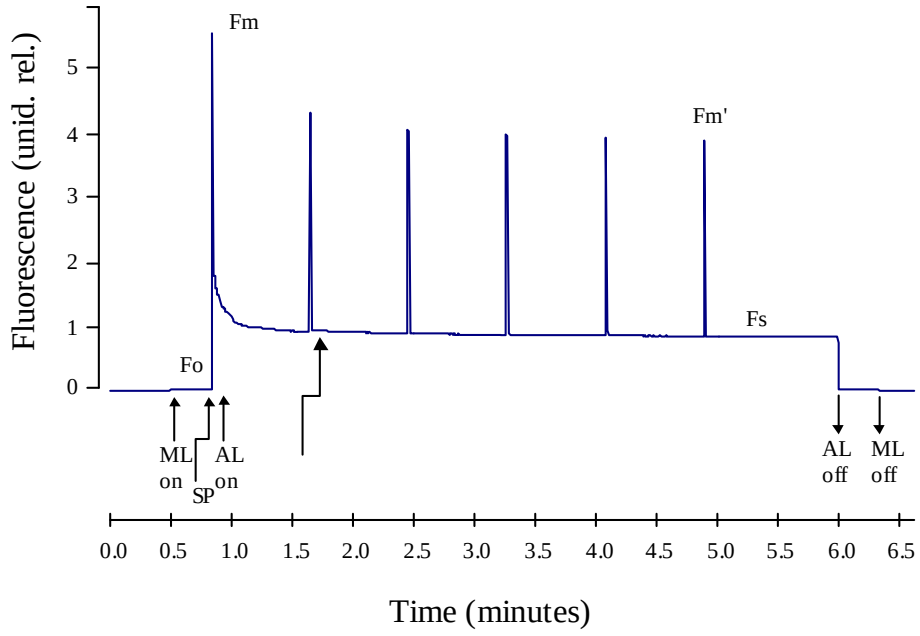

Fig. 6. A typical fluorescence induction signal obtained by the saturating pulse technique. LM, measurement light. PS, saturating pulse. LA, actinic light. RL, far red.

OJIP curves

OJIP curves were obtained in the field with the AquaPen. Chlorophyll fluorescence transients were induced by a saturating PFD of 3000 µmol m^{-2} s^{-1} and fluorescence was recorded at different frequencies between 10 µs to 2 s. Cardinal points of the OJIP curve and derived parameters were calculated with the Fluorpen 2.0 software, based on Strasser *et al.* (2000).

Sclerophylly Index

The sclerophylly index (SI) was calculated as the quotient between the dry mass (DM: 60ºC for 24 h) and the surface area of the prostrate apex of the bryophyte (LI-COR LI-3000 area meter, Lincoln, NE, USA). Previously, fresh mass was measured.

Photosynthetic pigments

Photosynthetic pigments were extracted with 100% acetone buffered with $CaCO_3$ after freezing shoot apices in liquid N_2 and grounding them in a TissueLyser (Qiagen, Hilden, Germany). After 24 h at 4ºC, the extract was filtered with 0.2 µm Nylon filters (Membrane-Solutions). Samples were maintained at a temperature ≤ 4ºC in the dark to avoid pigment alterations.

Pigments separation was performed by HPLC following the methods described in García-Plazaola & Becerril (2001) with the modifications of Otero *et al.* (2006) using an Agilent HP1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The different pigments were separated on a Hypersil ODS-C18 reversed phase column protected by a Hypersil ODS guard column (5 µm, 4 x 4 mm i.d.). Column was maintained at 20ºC. The mobile phase was composed by two solvents, A (acetonitrile: methanol: tris 0.1 M pH=8 84:2:14) and B (methanol: ethyl acetate 68:32). A linear gradient was used from 100% solvent A to solvent B for the first 12 min following by an isocratic elution with 100% B for the next 6 min. After that, 1 min linear gradient from 100% B to 100% A was performed. During 5 min an isocratic elution with 100% A was carried out to allow the column reequilibration. The solvent flow rate was 1.2 ml min^{-1}, with an injection volume of 15 µl. All the process was carried out with samples maintained at 4ºC in the refrigerated autosampler (Agilent 1200) The identification and quantification of photosynthetic pigments was made with a photodiode array detector

(Agilent 1100 DAD).

For quantification, commercial standards of chlorophylls *a* and *b* (Fluka), lutein, zeaxanthin and β-carotene (CaroteNature) were used. Total chlorophyll (Chl *a*+*b*), chlorophyll *a*/*b* ratio (Chl *a*/*b*), β-carotene and xanthophylls (lutein, neoxanthin, violaxanthin, antheraxanthin and zeaxanthin), were determined. The Xanthophyll Index was calculated as (Antheraxanthin + Zeaxanthin) / (Violaxanthin + Antheraxanthin + Zeaxanthin).

UV-absorbing compounds (UVACs)

Methanol-soluble, and methanol-insoluble cell wall-bound UVACs (respectively, SUVAC and WUVAC) were analyzed. In both cases, the bulk level of UVACs and the concentrations of several individual UVACs were measured by, respectively, spectrophotometry and HPLC. The extraction methods were based on Schmitz-Hoerner & Weissenbock (2003), Rozema *et al.* (2001) and Arróniz-Crespo *et al.* (2006).

For these analyses, 50 mg of fresh shoot apices was frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany). Then, 5 ml of methanol: water: 7M HCl (70:29:1 v/v/v) was added for extraction (24 h at 4ºC in the dark). The extract was centrifuged at 6000 *g* for 15 min and the supernatant and pellet were considered the source of SUVAC (presumably mainly located in the vacuoles) and WUVAC, respectively (Clarke & Robinson, 2008).

Bulk level of UVACs

In the supernatant of the methanol extraction described above, the bulk level of SUVAC was measured, in arbitrary units, as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM,

using a Perkin-Elmer λ35 spectrophotometer (Perkin-Elmer, Wilton, CT, USA).

The pellet of the methanol extraction was hydrolysed with NaOH, acidified with HCl and extracted three times with ethyl acetate. After evaporation, the material was dissolved in methanol and the bulk level of WUVAC was spectrophotometrically measured in the same units previously described for the bulk level of SUVAC.

Alternatively to this alkaline hydrolysis, several different enzymatic digestions were performed for comparison on the pellet remaining from the first methanol extraction. For enzymatic digestions, the pellet was washed twice with 2 ml sodium acetate buffer (100 mM NaAc, pH 5.0), centrifuging and discarding the supernatant following each wash. The following enzymes (Sigma-Aldrich) were used for the treatments: cellulase (EC 3.2.1.4), pectinase from *Rhizopus sp.* (polygalacturonase EC 3.2.1.15), pectolyase from *Aspergillus japonicus* (containing two types of pectinases, endopolygalacturonase EC 3.2.1.15, endo-pectin lyase EC 4.2.2.10 and a maceration stimulating factor), hemicellulase from *Aspergillus niger* (a mixture of glycolytic enzymes containing, endo-1,4-beta-xylanase, xylan 1,4-beta-xylosidase, alpha-L-arabinofuranosidase, cellulase, galactomannanase and other activities), and driselase (crude powder containing laminarinase EC 3.2.1.39, xylanase EC 3.2.1.8, and cellulase). The enzymes, in single or in combination, were added to the final pellet in different concentrations, filling up to 1 ml with NaAc buffer. Afterwards, the resuspended samples were incubated while agitating. After that, the same procedure used in the alkaline digestion was applied (acidification, ethyl acetate extraction, etc.).

Individual UVACs

Individual UVACs were measured by high-performance liquid chromatography (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA), following Arróniz-Crespo *et al.* (2006). The different

compounds were separated on a Hypersil ODS-C18 reversed phase column (5 µm, 250 x 4 mm i.d.) protected by a Hypersil ODS guard column (5 µm, 4 x 4 mm i.d.) The mobile phase consisted on two components: solvent A, acetic acid pH 2.4, solvent B acetonitrile:methanol:water (v/v/v). For analysis, 90% A and 10% B were pumped during the first 10 min following by a linear gradient till 100% B during the following 15 min and an isocratic elution with 100% B for a further 5 min period. Starting conditions were restored followed by column equilibration for 8 min. The solvent flow rate was 1 ml min^{-1} with 20ºC column temperature The injection volume was 15 µl. The detection was carried out by an Agilent photodiode array detector at 324 nm (ref. 700 nm) and absortion spectra were recorded in the wavelength range between 200 and 500 nm.

In *Jungermannia exsertifolia* subsp. *cordifolia*, five hydroxycinnamic acid (HCA) derivatives were detected and quantified in the soluble fraction: *p*-coumaroylmalic acid, caffeoylmalic acid (or phaselic acid), feruloylmalic acid, 5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid, and 5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid. These compounds will be hereafter referred to as C1 to C5, respectively. Commercial markers of trans-hydroxycinnamic acid derivatives and coumarin derivatives were used as the most similar external standard (ES) found: caffeic acid (Sigma-Aldrich, St. Louis, MO, USA) for phaselic acid, *p*-coumaric acid (Sigma-Aldrich) for *p*-coumaroylmalic acid, ferulic acid (Fluka) for feruloylmalic acid, and 7,8-dihydroxy-4-phenyl-coumarin (Aldrich) for C4 and C5. Concentrations were usually expressed in nmol per unit of DM. Chemical structures of these compounds are represented in Fig. 7. In the cell wall-bound fraction (WUVAC), and after alkaline or enzymatic digestion, *p*-coumaric and ferulic acids were detected and quantified by HPLC. These two compounds will be hereafter referred to as C6 and C7, respectively. C1 to C7 compounds had been identified using Nuclear Magnetic Resonance and Mass Spectrometry (Arróniz-Crespo *et al.*, 2006). The ratio between *p*-coumaric and ferulic acids has been proved to be useful in the evaluation of long-term changes in UV-B radiation (Lomax *et al.*,

2008), and thus the ratio C6/C7 was sometimes calculated in the cell wall-bound fraction. By analogy, the ratio C1/C3 was obtained in the soluble fraction.

In the soluble fraction of *Bryum pseudotriquetrum*, the flavonols kaempferol-3-*O*-glucoside and kaempferol-3,7-*O*-diglucoside were unequivocally identified, taking into account their retention times and UV spectra, and their quantification was made by calibration curves with the commercial external standard kaempferol (Sigma-Aldrich). Concentration was expressed as nmol per unit of DM.

In the insoluble fraction of *Fontinalis antipyretica*, *p*-coumaric acid was detected and quantified.

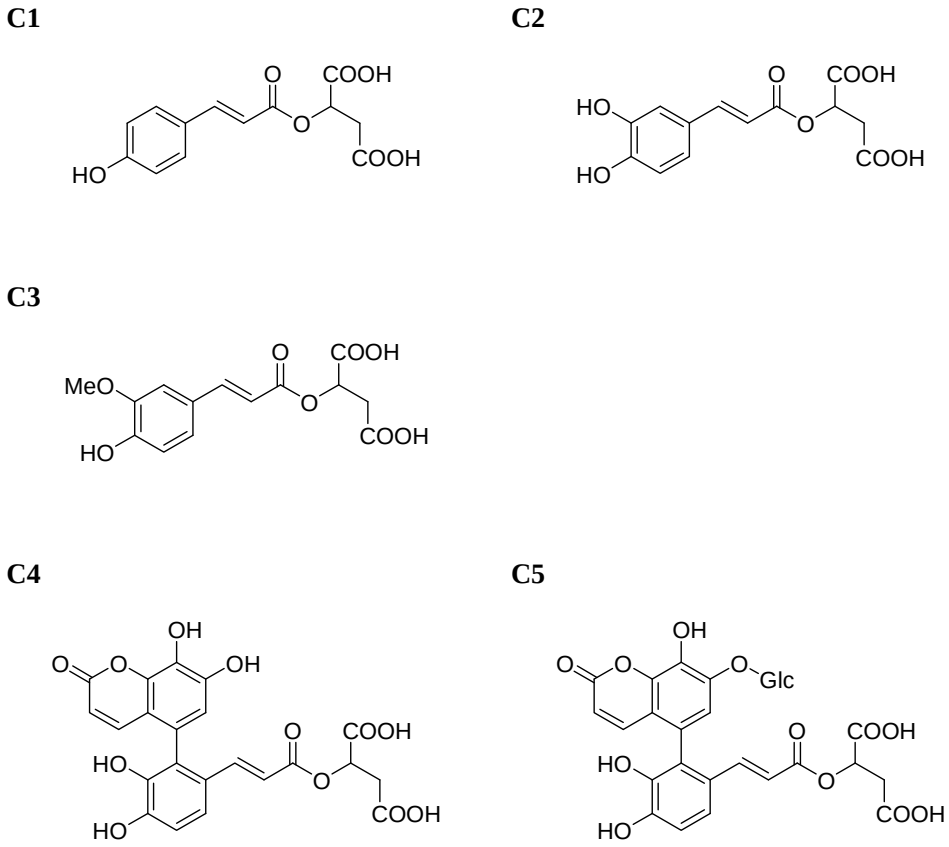

Fig. 7. Chemical structures of the five compounds analyzed in the soluble fraction of *Jungermannia exsertifolia* subsp. *cordifolia*. The compounds are as follows: C1, *p*-coumaroylmalic acid; C2, phaselic acid; C3, feruloylmalic acid; C4, (5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid), and C5, (5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid).

DNA damage (CPDs detection)

DNA damage was evaluated by detection of thymine dimers (Sinha *et al.*, 2001). In order to establish a standard with a known thymine dimer frequency, the plasmid pBSK (obtained from Prof. D.-P. Häder, Erlangen, Germany) was used and its DNA was isolated (QIAprep Spin Kit, Qiagen, Hilden, Germany). After quantification, plasmid DNA was irradiated for 1 h with an UV-C (254 nm) irradiance capable of inducing all possible thymine dimers. Once the DNA of both the bryophyte samples and the irradiated plasmid had been obtained, it was fixed to a Hybond-N^+ membrane (Amersham Biosciences, GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK), which was blocked and incubated with the primary antibody (anti-thymine dimer TDM-2, obtained from Prof. Osamu Nikaido, Kanazawa University, Japan). Afterwards, the membrane was incubated with the secondary antibody (antimouse-IgG) using the ECL western blotting system (Amersham Biosciences). Thymine dimers were detected and quantified by chemiluminiscence using the ChemiGenius Bio Imaging System and associated software (Syngene, Cambridge, UK).

Statistics

The effects of the different main factors (radiation regime, culture period, etc.) on the physiological responses of the bryophytes were usually tested using analysis of variance (ANOVA), once proved that the data met the assumptions of normality (Shapiro–Wilks's test) and homoscedasticity (Levene's test). In the case of significant differences, means were usually compared by Tukey's test. Non-parametric tests (Kruskal-Wallis) were used if the data did not meet the abovementioned assumptions. In this case, and when significant differences occurred, means were compared by Mann-Whitney test. When only two set of data had to be analzed, differences between them were assessed using Student's t tests. All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

CHAPTER 4

ALKALINE *VS.* ENZYMATIC DIGESTION FOR THE EXTRACTION OF UV-ABSORBING COMPOUNDS FROM BRYOPHYTE CELL WALLS

4.1. ABSTRACT

The accumulation of ultraviolet-absorbing compounds (UVACs) in the cell walls represents one important protection mechanism of bryophytes against the adverse effects of ultraviolet-B (UV-B) radiation, given that it provides a spatially uniform UV-B filter. We compared the capacity of several cell wall-degrading enzymes and an alkali (NaOH) to extract cell wall-bound UVACs from the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, in order to select the most appropriate method and to optimally evaluate its protection capacity against UV-B. We analyzed both the bulk level of UVACs and the concentrations of two hydroxycinnamic acids (*p*-coumaric and ferulic). The significantly best extraction of cell wall-bound UVACs, in terms of both their bulk levels and the concentration of individual compouds, was achieved with the alkaline digestion, which was more efficient than any of the enzymatic digestions assayed. This could be due to the capacity of the alkali to simultaneously break both the hydrogen and covalent bonds within the cell wall, including the ester linkages between phenolics and carbohydrates. In addition, the alkaline digestion was more rapid than the enzymatic ones, and was not excessively aggressive because the molecular integrity of the individual compounds analyzed was preserved. Thus, the alkaline digestion can be recommended to extract cell wall-bound UVACs in *J. exsertifolia* subsp. *cordifolia*, both when these compounds are to be evaluated globally and individually.

RESUMEN

La acumulación de compuestos absorbentes de radiación UV (CAUV) en las paredes celulares representa un mecanismo de protección importante de los briófitos contra los efectos adversos de la radiación UV-B, ya que proporciona un filtro UV-B distribuido uniformemente en el espacio. Se ha comparado la capacidad de varios enzimas degradativos de la pared celular, y de un álcali (NaOH), para extraer los CAUV unidos a la pared celular en la hepática acuática *Jungermannia exsertifolia* subsp. *cordifolia*, con el objetivo de seleccionar el método más apropiado y poder evaluar de manera óptima su capacidad de protección frente a la radiación UV-B. Se analizó tanto el nivel global de CAUV como las concentraciones de dos ácidos hidroxicinámicos (*p*-cumárico y ferúlico). La mejor extracción, significativamente, se consiguió con la digestión alcalina, que fue más eficiente que cualquiera de las digestiones enzimáticas ensayadas, en la obtención tanto del nivel global de CAUV como de las concentraciones de los compuestos individuales. Esto pudo deberse a la capacidad del álcali para romper simultáneamente los enlaces covalentes y de hidrógeno dentro de la pared celular, incluyendo los enlaces éster entre los fenoles y los carbohidratos de la pared. Además, la digestión alcalina era más rápida que las enzimáticas, y no era excesivamente agresiva porque se conservó la integridad molecular de los compuestos individuales analizados. Por lo tanto, la digestión alcalina se puede recomendar para extraer los CAUV unidos a la pared celular en la hepática acuática *Jungermannia exsertifolia* subsp. *cordifolia*, tanto para evaluarlos de manera global como individual.

4.2. INTRODUCTION

As a component of the solar spectrum, ultraviolet (UV) radiation, in particular UV-B wavelengths (280-315 nm), may induce diverse adverse effects in photosynthetic organisms, such as DNA damage, chlorophyll degradation, photosynthesis decline, oxidative damage, and alterations in growth and development (Jansen *et al.*, 1998). The study of these effects has notably increased since the discovery of the stratospheric ozone depletion, given that this process leads to an enhancement of UV-B in the biosphere. In bryophytes, UV-B effects are overall similar to those demonstrated in other organisms (Boelen *et al.*, 2006; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011).

Plants have evolved a number of protection mechanisms against the adverse effects of UV-B, such as DNA reparing systems, antioxidants, and the accumulation of UV-absorbing compounds (UVACs), mainly phenolics such as flavonoids, coumarins, and hydroxycinnamic acids (HCAs). In particular, UVACs accumulation is the most consistent response of plants, including bryophytes, to enhanced UV-B (Searles *et al.*, 2001a; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011).

In tracheophyte leaves, the compartmentation of UVACs is considerably complex, because they can be located in the vacuoles and cell walls of epidermal and subepidermal cells, and also in trichomes and waxes (Bornman *et al.*, 1997). UVACs may play different physiological roles depending on their location, and thus methods to extract UVACs from each compartment have been developed. Specifically, UVACs located in vacuoles and cell walls can be differentiated after centrifuging the methanol extract of the plant. At this moment, the supernatant is considered the direct source of vacuolar (soluble) UVACs, whereas the cell debris in the pellet are digested to degrade the cell wall matrix and release the cell wall-bound (insoluble) UVACs (Schnitzler *et al.*, 1996). This digestion is indispensable because many phenolic compounds are covalently bound onto cell wall

carbohydrates. To degrade the cell wall, usually, a hot alkaline digestion with NaOH is applied for several days (Lichtenthaler & Schweiger, 1998; Fischbach *et al.*, 1999; Ruhland *et al.*, 2005), but this procedure might result too aggressive to the integrity of the molecules. Thus, a milder digestion with cell wall-degrading enzymes has been used as an alternative method (Schnitzler *et al.*, 1996; Hoque & Remus, 1999).

In contrast to tracheophytes, bryophyte leaves are composed of only a single cell layer, and thus the compartmentation of UVACs is much more simple: they can be located either in the vacuoles or the cell walls. This differentiation is important because accumulation of UVACs in the cell walls would provide a much more spatially uniform UV-B filter and thus a more efficient structural protection than accumulation in vacuoles (Clarke & Robinson, 2008). In spite of its importance, only a few studies have dealt with this issue (Searles *et al.*, 1999; Clarke & Robinson, 2008; Lappalainen, 2010). One of the main conclusions that can be derived from these studies is that, in mosses, cell wall-bound UVACs are equal or more abundant than vacuole UVACs, whereas in liverworts occurs the contrary. In all these studies, cell wall-bound UVACs were extracted by alkaline digestion, so that enzymatic digestion has never been asssayed in bryophytes.

Another important point regarding UVACs accumulation, both in tracheophytes and bryophytes, is the method used to measure their concentration. The most common method is to spectrophotometrically measure the UV absorbance of methanolic extracts (Searles *et al.*, 2001a; Newsham & Robinson, 2009), either at specific wavelengths in the UV region (*e.g.* 290, 300, or 315 nm) or in a certain range within the UV band (*e.g.* 280-310, 280-315, 280-320, or 280-400 nm). In this last case, the area under the absorbance curve is subsequently integrated. All these variables are highly intercorrelated (Otero *et al.*, 2008), but they may be insufficient to optimally evaluate UV-B protection capacity, because each individual compound contributing to that bulk absorbance may respond differently to UV-B (Kotilainen *et al.*, 2008). Thus, studies analyzing individual UVACs are

especially needed in the context of UV research, in particular if the location of UVACs in the vacuoles or the cell walls is also assessed. This combined approach has been much more commonly carried out in tracheophytes (see for example Schnitzler *et al.*, 1996; Lichtenthaler & Schweiger, 1998; Fischbach *et al.*, 1999; Hoque & Remus, 1999; Ruhland *et al.*, 2005; Piston *et al.*, 2010) than in bryophytes (Taipale & Huttunen, 2002; Snell *et al.*, 2009).

The aim of the present study was to compare the capacity of diverse commercial cell wall-degrading enzymes and an alkali (NaOH) to extract UVACs from the cell walls of a liverwort. This is the first time in which enzymatic digestion was assayed in bryophytes for this aim because, until now, only alkaline digestion had been used (Searles *et al.*, 1999; Clarke & Robinson, 2008; Lappalainen, 2010). We analyzed not only the bulk level of UVACs but also the concentrations of individual UVACs present in the cell wall fraction. All this can be useful to select the most appropriate method to extract cell wall-bound UVACs in bryophytes, which would allow an optimal evaluation of their protection capacity against UV-B radiation.

4.3. MATERIALS AND METHODS

Samples of the leafy aquatic liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váňa (hereafter *J. cordifolia*) were collected on 15 November 2009 at the stream Lavieja (1280 m a.s.l., 42º03'00'' N, 02º35'30'' W), in La Rioja (northern Spain). Samples were submerged and exposed to full sun. The material was rinsed with stream water and transported to the laboratory in a portable icebox. Once in the laboratory, green healthy shoot apices were cut and immediately processed. Groups of apices containing 50 mg fresh mass each were frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany). Then, 5 ml of methanol:water:7 M HCl (70:29:1 v/v/v) was added for extraction of UVACs (24 h at 4ºC in the dark). The extract was centrifuged at 6000 g for 15 min. After this centrifugation, the supernatant and pellet are considered the source of soluble (presumably mainly located in the vacuoles) and insoluble (cell wall-bound) UVACs, respectively (Clarke & Robinson, 2008). The supernatant was discarded and, from the pellet remaining, the cell wall-bound UVACs were extracted by both alkaline and enzymatic digestions.

Alkaline digestion was performed as described in Chapter 3. In brief, the pellet was washed twice with 2 ml methanol, centrifuging and discarding the supernatant following each wash. The washed pellet was then resuspended and digested with 2 ml 1 M NaOH for 3 h at 80ºC.

For the enzymatic digestion, the pellet remaining from the first methanol extraction was washed twice with 2 ml sodium acetate buffer (100 mM NaAc, pH 5.0), centrifuging and discarding the supernatant following each wash. The following enzymes (Sigma-Aldrich, St Louis, MO, USA) were used for the treatments: cellulase (EC 3.2.1.4), pectinase from *Rhizopus sp.* (polygalacturonase EC 3.2.1.15), pectolyase from *Aspergillus japonicus* (containing two types of pectinases, endopolygalacturonase EC 3.2.1.15, endo-pectin lyase EC 4.2.2.10 and a maceration stimulating factor), hemicellulase from *Aspergillus niger* (a mixture of glycolytic enzymes

containing endo-1,4-beta-xylanase EC 3.2.1.8, xylan 1,4-beta-xylosidase EC 3.2.1.37, alpha-L-arabinofuranosidase EC 3.2.1.55, cellulase, galactomannanase and other activities), and driselase (crude powder containing laminarinase EC 3.2.1.39, xylanase EC 3.2.1.8, and cellulase). The enzymes, in single or in combination, were added to the final pellet in different concentrations (Table 1), filling up to 1 ml with NaAc buffer. Afterwards, the resuspended samples were incubated while agitating. Two incubation temperatures (37°C and 27°C) and two incubation periods (2 and 5 days) were tested.

Table 1 Digestion treatments (alkaline or enzymatic) applied to extract UV-absorbing compounds from the cell walls of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*. Enzyme concentrations are specified for each enzymatic treatment

Number	Digestion treatment
1	Without NaOH or enzymes (control)
2	NaOH
3	Cellulase 0.1%
4	Pectinase 0.1%
5	Pectolyase 0.1%
6	Hemicellulase 0.1%
7	Driselase 0.1%
8	Cellulase 0.1% + Hemicellulase 0.1%
9	Cellulase 0.1% + Pectinase 0.1%
10	Cellulase 0.025% + Pectolyase 0.025%
11	Cellulase 0.05% + Pectolyase 0.05%
12	Cellulase 0.1% + Pectolyase 0.1%
13	Cellulase 0.1% + Pectinase 0.1% + Pectolyase 0.1% + Hemicellulase 0.1%
14	Cellulase 0.2%
15	Cellulase 0.2% + Pectolyase 0.2%
16	Cellulase 0.4%
17	Cellulase 0.4% + Pectolyase 0.4%
18	Cellulase 0.8% + Pectolyase 0.2%
19	Cellulase 1%

Additionally to the digestion treatments, a control without NaOH or enzymes was established (Table 1).

The resulting alkaline or enzymatic digestions, together with the control, were acidified to a pH of 1.0 with HCl and extracted three times with ethyl acetate. After evaporation, the material was dissolved in methanol and the bulk level of cell wall-bound UVACs was measured, in arbitrary units, as the area under the absorbance curve in the intervals 280-315 and 280-400 nm ($AUC_{280-315}$ and $AUC_{280-400}$, respectively) per unit of dry mass (DM: 80ºC for 24 h), using a Perkin-Elmer λ35 spectrophotometer (Perkin-Elmer, Wilton, CT, USA). In addition, an only for the alkaline digestion and the enzymatic one which rendered the most efficient extraction (number 18 in Table 1), two HCAs, *p*-coumaric and ferulic, were quantified by HPLC (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA) following Arróniz-Crespo *et al.* (2006). Their concentrations were expressed per unit of DM. All the treatments were assayed at least in triplicate.

The global effect of the digestion treatment on the values of $AUC_{280-315}$ and $AUC_{280-400}$ per DM was tested using a one-way analysis of variance (ANOVA), once proved that the data met the assumptions of normality (Shapiro–Wilks's test) and homoscedasticity (Levene's test). In the case of significant differences, means were then compared by Tukey's test. For each enzymatic treatment, differences between both incubation temperatures and both incubation periods were examined by Student's *t* test. This test was also used to examine the differences in the concentrations of *p*-coumaric and ferulic acids extracted using alkaline digestion and enzymatic digestion number 18. All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

4.4. RESULTS

Fig. 1 shows the bulk level of cell wall-bound UVACs that was extracted using alkaline and enzymatic digestions (Table 1); the control extraction, performed without using NaOH or enzymes, is also shown. Extraction by enzymatic digestions was significantly more efficient (p<0.001) at 37ºC than at 27ºC incubation temperature, whereas no significant differences were found between 2 and 5 days incubation period. This occurred for every enzymatic treatment. Thus, only results obtained at 37ºC and 2 days incubation are shown in Fig. 1.

The significantly best extraction (p<0.001) was achieved with alkaline digestion, both when using $AUC_{280\text{-}315}$ and $AUC_{280\text{-}400}$ as a measure of the bulk level of cell wall-bound UVACs (Fig. 1). With respect to enzymatic digestions, there were only slight differences between most of them when using $AUC_{280\text{-}315}$ (Fig. 1, top). In particular, there was no significant difference between the different enzymes (cellulase, pectinase) or commercial mixtures (pectolyase, hemicellulase and driselase) when used individually (except for the significant difference between cellulase and pectinase). The control did not differ significantly either. Most of our combinations did not achieve much better results, even when a mixture of cellulase, pectinase, pectolyase and hemicellulase was assayed. Only when a combination of cellulase and pectolyase was used in concentrations of 0.2% or higher, the extraction efficiency improved significantly (p<0.001). The ordination produced by the use of $AUC_{280\text{-}400}$ (Fig. 1, bottom) was considerably similar to that depicted by $AUC_{280\text{-}315}$, although some slight differences could be observed. Among the treatments in which one only enzyme or commercial mixture was applied, and for the same enzyme concentration (0.1%), cellulase significantly (p<0.001) produced the most efficient extraction in comparison with hemicellulase, pectinase, driselase and pectolyase. All these four treatments did not differ significantly from the control. The increase in cellulase up to 1% did not improve extraction.

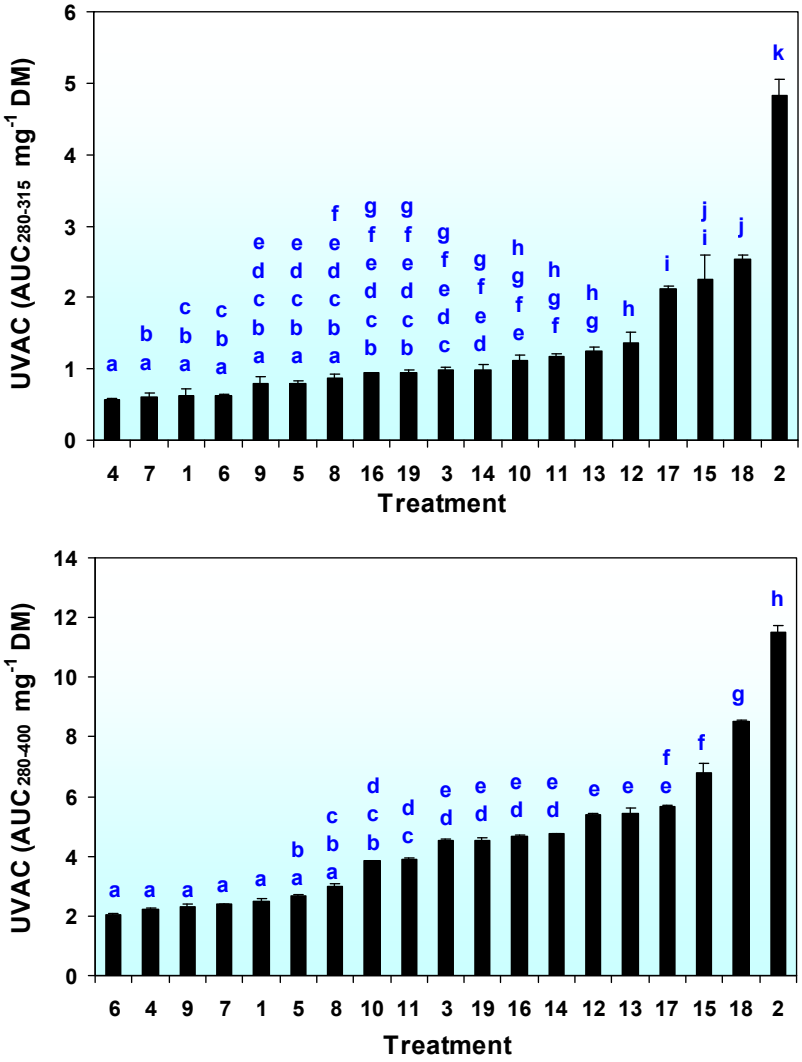

Fig. 1 Values (mean ± SE) of the bulk level of cell wall-bound UV-absorbing compounds (UVAC) in samples of *Jungermannia exsertifolia* subsp. *cordifolia* subjected to alkaline (number 2) or enzymatic (numbers 3-19: see Table 1) digestion. Number 1 represents non-digested control samples. UVAC are quantified in terms of the area under the absorbance curve (AUC) of the liverwort extracts in the intervals 280-315 (top) and 280-400 nm (bottom) per dry mass (DM). Bars marked with different letters are significantly different at least at $p<0.05$.

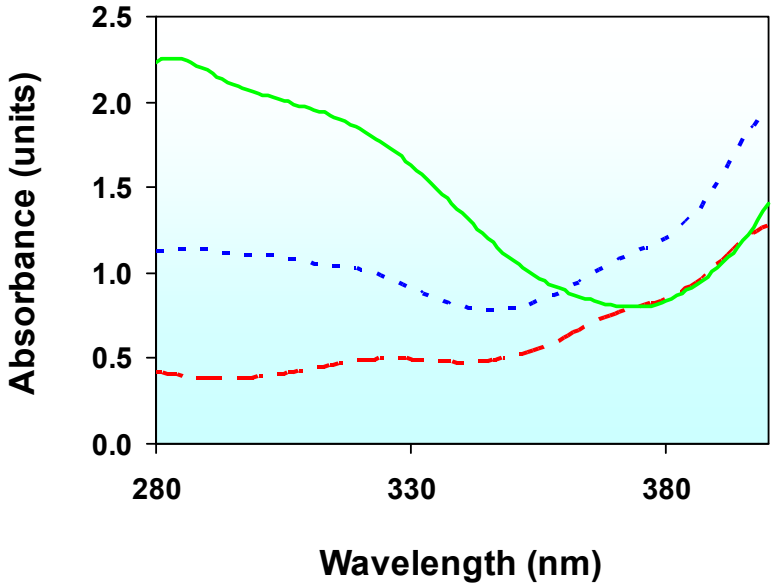

Fig. 2 Representative absorption spectra, in the UV-B and UV-A ranges, of *Jungermannia exsertifolia* subsp. *cordifolia* cell-wall extracts subjected to alkaline digestion (green continuous line) and to the enzymatic digestion rendering the best extraction results (blue dot line). The spectrum of non-digested control samples (red dashed line) is shown for comparison.

Our combinations of 2-4 enzymes or commercial mixtures did not produce significantly higher extractions than that achieved with only cellulase, except when a combination of cellulase and pectolyase was applied in high concentrations (especially, 0.8% cellulase and 0.2% pectolyase). In this last case, the extraction efficiency was significantly higher (p<0.001) than in the rest of enzymatic treatments.

The higher efficiency of the alkaline digestion, in comparison with the best enzymatic one (the combination of 0.8% cellulase and 0.2% pectolyase), was especially due to a better extraction of compounds absorbing between 280 and 360 nm (Fig. 2). In turn, the higher extraction capacity of this enzymatic treatment, when compared to the control, was especially based on a better extraction of compounds absorbing between 280 and 330 nm.

Fig. 3 shows that the two individual cell wall-bound UVACs identified in *J. cordifolia*, *p*-coumaric and ferulic acids, were also significantly ($p<0.001$) better extracted by alkaline digestion than by the enzymatic digestion (number 18 in Fig. 1) which had rendered the best extraction results when the cell wall-bound UVACs had been measured globally as AUC. Specifically, the concentrations of *p*-coumaric and ferulic acids extracted by alkaline digestion were 2.71 ± 0.15 and 11.59 ± 0.57 nmol mg^{-1} DM, respectively, whereas the concentrations extracted by the enzymatic digestion number 18 were 0.28 ± 0.02 and 2.27 ± 0.09, respectively.

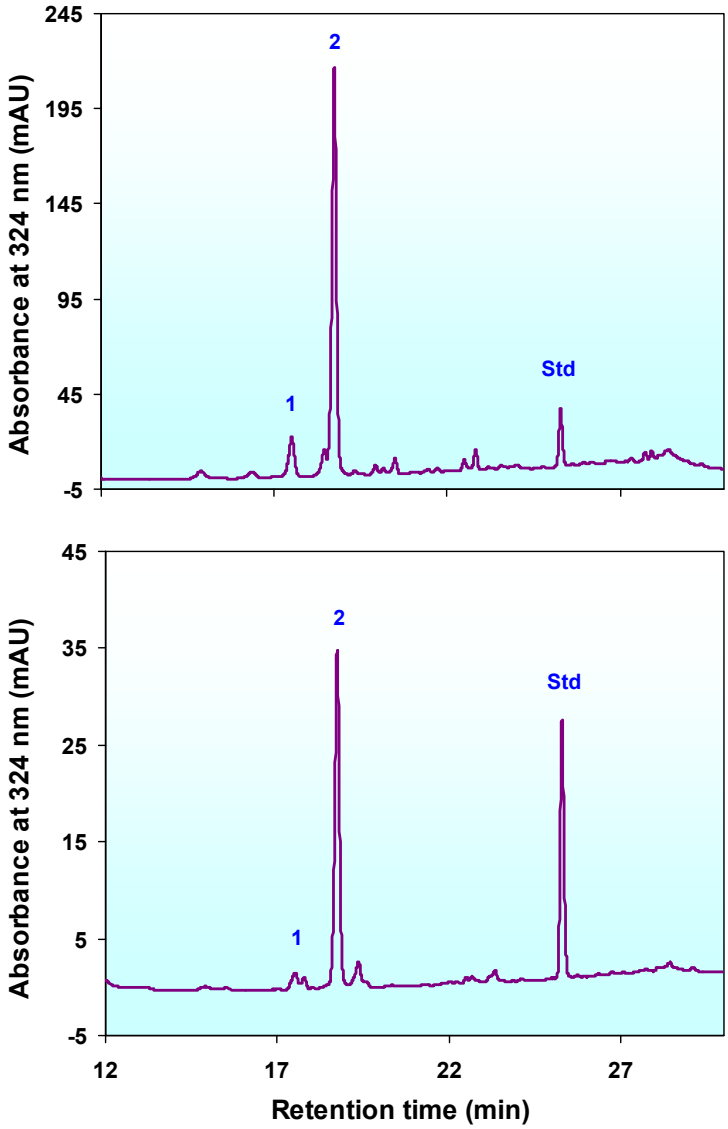

Fig. 3 Chromatograms (unprocessed absorbance at 324 nm in milliabsorbance units, mAU, *vs* retention time) of *Jungermannia exsertifolia* subsp. *cordifolia* cell-wall extracts subjected to alkaline digestion (top) and to the enzymatic digestion rendering the best extraction results (bottom). The peaks corresponding to the two isolated compounds, *p*-coumaric acid (1) and ferulic acid (2), are shown, together with the internal standard (Std: quercetin).

4.5. DISCUSSION

The significantly best extraction of cell wall-bound UVACs, in terms of both $AUC_{280-315}$ and $AUC_{280-400}$, was achieved with the alkaline digestion, which was more efficient than any of the enzymatic digestions assayed (Fig. 1). This could be due to the capacity of the alkali to simultaneously break both the hydrogen bonds binding hemicelluloses to cellulose and the different covalent bonds which occur within the cell wall, including the ester linkages between phenolics and carbohydrates (Ruhland *et al.*, 2005; Santiago *et al.*, 2009; Albersheim *et al.*, 2011). The enzymatic treatments applied may not have some of these capacities, in particular that needed to easily break the ester linkages between phenolics and carbohydrates, thus limiting the biodegradability of cell walls. Furthermore, the treatment temperature was much higher in the alkaline than the enzymatic digestions. This more drastic hydrolysis could lead to a stronger degradation of cell walls and a better UVACs extraction. In addition, alkaline digestion is more rapid than enzymatic ones, so that it can be recommended as the best method to extract cell wall-bound UVACs from *J. cordifolia*.

Among the enzymatic digestions, the best one was a combination of cellulase and pectolyase in relatively high concentrations (0.8% and 0.2%, respectively), especially when using $AUC_{280-400}$ to measure the bulk level of cell wall-bound UVACs. Pectolyase has the advantage over pectinase that combines the pectin-degrading action of polygalacturonase with that of pectin lyase. Given that two important components of pectin, galacturonic and glucuronic acids, are present in liverworts in high concentrations (higher than in tracheophytes: Popper & Fry, 2003), pectin dismantling could be crucial in liverworts for a more complete cell wall degradation, especially taking into account that pectin removal facilitates an easier access of other wall-degrading enzymes (Albersheim *et al.*, 2011), such as cellulase.

It was somewhat surprising that the treatment with hemicellulase, in single or in combination, did not strikingly improve UVACs extraction,

given that many phenolic UVACs are bound to hemicellulose (Albersheim *et al.*, 2011). However, it should be taken into account that xyloglucan, an important component of hemicellulose, appears in low concentration in bryophytes (Popper & Fry, 2003), and thus its degradation might not be especially relevant to release UVACs.

In general, the complete cell wall degradation is a difficult process that requires the combined action of very diverse enzymes (Albersheim *et al.*, 2011). Among them, esterases efficiently releasing certain UVACs, such as HCAs (Kuhnel *et al.*, 2012), are especially needed, because polysaccharide cross-linking by HCAs may protect the cell walls from enzymatic degradation (Carnachan & Harris, 2000; Piston *et al.*, 2010). In the future, new enzymatic combinations more adapted to the peculiar cell wall composition of bryophytes could be assayed to improve the extraction of cell wall-bound UVACs.

The higher efficiency of the alkaline digestion, in comparison with the best enzymatic one, was especially due to the better extraction of compounds absorbing between 280 and 360 nm (Fig. 2). These wavelengths fit well with the absorption maxima of HCAs in general, which lie in the range 285-330 nm (Waterman & Mole, 1994). More specifically, the absorption maxima of the two HCAs identified in the cell walls of *J. cordifolia*, *p*-coumaric and ferulic acids, are respectively 310 and 324 nm (Chapter 5). Thus, these compounds were probably responsible for the increased absorption of the alkaline digestion in the interval 280-360. They could be also responsible for the higher absorption shown by the enzymatic extract with respect to the control, given that the most striking difference between both extracts appeared between 280 and 330 nm (Fig. 2).

The alkaline digestion was the most efficient not only to extract cell wall-bound UVACs in general (in terms of AUC), but also individual UVACs such as *p*-coumaric and ferulic acids (Fig. 3). In tracheophytes, both compounds, especially the latter, are involved in the cross-linking of cell

wall polysaccharides through ester bonds (Albersheim *et al.*, 2011). Alkaline digestion could break the ester bonds much more easily than enzymes, so that both compounds would be more efficiently released. Additionally to their stabilization function in the cell wall, their privileged location would allow them to act as important UV-B screens in *J. cordifolia*, as occurs in tracheophytes (Schnitzler *et al.*, 1996; Agati & Tattini, 2010). Nevertheless, the alkaline digestion used in our study is expected to release only the ester-linked HCAs, but not those linked by other linkages (Du *et al.*, 2009; Piston *et al.*, 2010). Thus, the real UV-B absorption capacity of cell wall-bound HCAs in *J. cordifolia* may be being underestimated.

p-Coumaric and ferulic acids have been detected in the cell walls of many gymnosperms and angiosperms, especially grasses (Lichtenthaler & Schweiger, 1998; Hoque & Remus, 1999; Carnachan & Harris, 2000; Santiago *et al.*, 2009; Piston *et al.*, 2010). Both compounds can be extracted from tracheophyte cell walls by alkaline digestion. They have also been found in the soluble fraction of thracheophytes (Ruhland *et al.*, 2005). In bryophytes, *p*-coumaric acid had been previously reported from the cell walls and soluble fraction of *Sphagnum* species (Verhoeven & Liefveld, 1997), and both *p*-coumaric and ferulic acids from the soluble fraction of the moss *Kindbergia praelonga* (Jockovic *et al.*, 2008). The reported presence of non-esterified HCAs in the soluble fraction of bryophytes and tracheophytes is surprising, because HCAs in the vacuoles may be esterified with organic acids, such as malic, to facilitate their solubility and storage (Chapter 5).

Although with a different efficiency, *p*-coumaric and ferulic acids were extracted from *J. cordifolia* cell walls by both alkaline and enzymatic digestions. This would mean that the apparently aggressive conditions of alkaline digestion did not affect the molecular integrity of these compounds. The stability of HCAs and other penolics under diverse conditions (Wehling *et al.*, 1989; Caasi-Lit *et al.*, 1997; Huttunen *et al.*, 2005) may have contributed to the preservation of this integrity.

In conclusion, the alkaline digestion can be recommended to extract cell wall-bound UVACs in *J. cordifolia*, both when these compounds are to be evaluated globally (as AUCs) and individually (concentrations of *p*-coumaric and ferulic acids). This method was more efficient and rapid than any enzymatic treatment tested, and was not so aggressive as could be thought in advance, because the molecular integrity of the individual compounds analyzed was not altered.

ENHANCED UV-B RADIATION, PHYSIOLOGICAL DAMAGE AND PROTECTION MECHANISMS (SOLUBLE AND INSOLUBLE UV-ABSORBING COMPOUNDS) IN AN AQUATIC LIVERWORT

5.1. ABSTRACT

We examined the responses of UV-absorbing compounds (UVAC) to enhanced UV-B radiation in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* for 31 days under laboratory conditions. Samples were exposed to three radiation regimes: P (only photosynthetic radiation), PA (photosynthetic + UV-A radiation) and PAB (photosynthetic + UV-A + UV-B radiation). We measured both the bulk UV absorbance of the methanolic extracts and the levels of individual UVAC. In both cases, the methanol-soluble and the methanol-insoluble, alkali-extractable cell wall-bound fractions were analyzed. The bulk UV absorbance of the soluble fraction was higher than that of the cell wall-bound fraction. The bulk UV absorbances of both fractions increased under enhanced UV-B (PAB regime). Five different hydroxycinnamic acid (HCA) derivatives were found in the soluble fraction and two additional ones in the cell wall-bound fraction, among which only *p*-coumaroylmalic acid in the soluble fraction and *p*-coumaric acid in the cell wall-bound fraction increased under enhanced UV-B. The maximum quantum yield of PSII (F_v/F_m) decreased and DNA damage (amount of thymine dimers) strongly increased under enhanced UV-B, showing UV-B-induced damage. We conclude that methanol-soluble and cell wall-bound fractions of the liverwort studied have different UVAC, and each individual compound may respond in a different way to UV-B radiation. Thus, the analysis of individual UVAC in both the methanol-soluble and cell wall-bound fractions is advisable to better evaluate the protection mechanisms of liverworts against UV-B radiation. In particular, *p*-coumaric acid and *p*-coumaroylmalic acid seem to be especially UV-B responsive and merit further investigation.

RESUMEN

Se examinaron las respuestas de los compuestos absorbentes de radiación UV (CAUV) a un suplemento de radiación UV-B en la hepática acuática *Jungermannia exsertifolia* subsp. *cordifolia* durante 31 días en condiciones de laboratorio. Las muestras se expusieron a tres regímenes de radiación: P (sólo radiación fotosintética), PA (radiación fotosintética + UV-A) y PAB (radiación fotosintética + UV-A + UV-B). Se midieron tanto la absorbancia UV global de los extractos metanólicos como los niveles de algunos CAUV individuales. En ambos casos, se analizó por una parte la fracción soluble en metanol y, por otra, la fracción insoluble en metanol pero extraíble con un álcali por estar unida a la pared celular. La absorbancia UV global de la fracción soluble era mayor que la de la insoluble, y las absorbancias de ambas fracciones aumentaron cuando se suplementó con UV-B (régimen PAB). Se encontraron cinco derivados diferentes del ácido hidroxicinámico en la fracción soluble y dos más en la insoluble, entre los cuales solamente dos aumentaron con el suplemento de UV-B: el ácido *p*-cumaroilmálico en la fracción soluble y el ácido *p*-cumárico en la insoluble. El rendimiento cuántico máximo del PSII (F_v/F_m) disminuyó con el suplemento de UV-B, mientras que aumentó con fuerza el daño al ADN (cantidad de dímeros de timina), que se mostraba como un daño inducido por UV-B. Se concluye que las fracciones soluble e insoluble en metanol de la hepática estudiada tienen diferentes CAUV, y que cada compuesto individual puede responder de distinta manera a la radiación UV-B. Por lo tanto, resulta aconsejable analizar los CAUV individuales en ambas fracciones para evaluar mejor los mecanismos de protección de las hepáticas frente a la radiación UV-B. En particular, los ácidos *p*-cumárico y *p*-cumaroilmálico parecen ser especialmente sensibles a la radiación UV-B y se recomienda continuar la investigación sobre ellos.

5.2. INTRODUCTION

Ultraviolet radiation is an important natural environmental factor influencing organisms, and it has been involved in the appearance of diverse adaptive changes through the development of life on Earth (Cockell & Knowland, 1999). In particular, the effects of increased solar ultraviolet-B (UV-B: 280–315 nm) irradiance at the ground level, resulting from stratospheric ozone depletion, have stimulated considerable research in recent decades (Paul & Gwynn-Jones, 2003).

In photosynthetic organisms, increased UV-B may cause diverse damage in the photosynthetic apparatus (pigment degradation, photoinhibition, and decreases in quantum yield, photosynthetic rates and enzyme activities), together with DNA alterations and oxidative damage (Jansen *et al.*, 1998). Plants have evolved a variety of counteracting protective mechanisms against the harmful effects of UV-B, including the accumulation of UV-absorbing compounds (UVAC), mainly phenolics.

In the context of UV research, the most used variable to measure UVAC (and thus to partially evaluate the UV protection capacity) has been the bulk UV absorbance of methanolic extracts. Meta-analysis of field experiments have demonstrated that this is the only variable that significantly changes (increases) under enhanced UV-B (Searles *et al.*, 2001a; Newsham & Robinson, 2009). However, the bulk UV absorbance may be insufficient to adequately evaluate the UV protection capacity, because individual compounds contributing to that bulk absorbance may respond to UV in a different manner. Thus, detailed studies of phenolic metabolite composition should be preferred (Kotilainen *et al.*, 2008).

Two classes of phenolics are mainly involved in UV screening: hydroxycinnamic acids (HCA) and flavonoids (Dixon & Paiva, 1995). HCA have received less attention than flavonoids in the context of UV research, probably because HCA have been considered to be a constitutive rather

than inducible shield against UV-B (Bornman *et al.*, 1997; Burchard *et al.*, 2000). However, HCA are particularly effective in screening out UV-B because they absorb effectively in this region, whereas flavonoid absorbance peaks are more displaced to the UV-A range. As a consequence, HCA may provide a greater UV-B attenuation than flavonoids (Sheahan, 1996). The most usual HCA are *p*-coumaric, caffeic, ferulic and sinapic acids.

Both the bulk UV absorbance and individual UVAC can be measured in at least two cell compartments (Schnitzler *et al.*, 1996): 1) the soluble fraction, which would be mainly located in the vacuole, by means of a simple methanolic extraction; and 2) the cell wall-bound fraction, whose measure requires a hydrolysis of the remaining cell debris after the methanolic extraction. This differentiation may be important because the two fractions can be different, and the cell wall-bound UVAC would provide a more spatially uniform UV screen for the cell contents than intracellular UVAC (Clarke & Robinson, 2008). Both flavonoids and HCA can be located in the soluble and cell wall-bound fractions (Dixon & Paiva, 1995; Schmitz-Hoerner & Weissenbock, 2003).

Bryophytes are structurally simple plants which often prevail in harsh environments for tracheophytes, such as bare stones, dry soils and turbulent montane streams (Glime, 2007). Bryophyte responses to UV-B are far from being properly understood, although significant advances have been achieved in the last decade (see recent reviews in Boelen *et al.*, 2006; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). In this context, the most studied bryophyte species belong to mosses, whereas liverworts have been notably less studied, with the exceptions of the aquatic *Jungermannia exsertifolia* subsp. *cordifolia* (Núñez-Olivera *et al.*, 2009) and the terrestrial *Cephaloziella varians* (Snell *et al.*, 2009). Liverworts are considered the earliest diverging land plants (Zobell *et al.*, 2010), and thus their responses to UV-B radiation may be of evolutionary importance in the water-to-land transition.

In tracheophyte leaves, UVAC may be mainly found in the vacuoles and cell walls of epidermal and subepidermal cells, and also in trichomes and waxes (Bornman *et al.*, 1997). In contrast, leaves of foliose liverworts are composed of only a single cell layer and lack hairs, and thus the compartmentation of UVAC is much more simple than that of tracheophytes. In this sense, liverworts, as bryophytes in general, would constitute a highly suitable model system for studying the differentiation of soluble (mainly vacuolar) and insoluble cell wall-bound UVAC fractions. However, this issue has been little studied (Taipale & Huttunen, 2002; Clarke & Robinson, 2008; Snell *et al.*, 2009), in spite that it could contribute to obtain a clearer understanding of the role of the different UVAC in plants. Among the mentioned studies, Clarke and Robinson (2008) measured the bulk UV absorbance of methanolic extracts in the methanol-soluble and alkali-extractable cell wall-bound fractions of three Antarctic mosses, Taipale and Huttunen (2002) assessed the location of flavonoids in two Arctic mosses, both in the cell wall and intracellularly, and Snell *et al.* (2009) identified the anthocyanidin riccionidin A in the cell walls of the liverwort *Cephaloziella varians*. None of these studies, however, differentiated individual UVAC in both the soluble and cell wall-bound fractions of the bryophytes studied.

The aim of the present study was to examine, under laboratory conditions, the response to enhanced UV-B radiation of the HCA derivatives present in the methanol-soluble and the methanol-insoluble, alkali-extractable cell wall-bound fractions of the aquatic leafy liverwort *J. exsertifolia* subsp. *cordifolia*. This is the first time that individual UVACs from both fractions are measured in a bryophyte in response to enhanced UV-B radiation. The use of a liverwort in this research could be interesting because, regarding this specific point, some data on mosses exist (Clarke & Robinson, 2008), but mosses and liverworts may have different mechanisms to cope with high UV-B levels (Otero *et al.*, 2008). We also measured the maximum quantum yield of PSII (F_v/F_m), DNA damage, and sclerophylly

index to assess the possible UV-B-damage suffered by the cultured samples.

Table 1. Radiation conditions in the three radiation regimes (P, PA, and PAB) under which the samples were cultivated. $UV\text{-}B_{BE}$ (biologically effective UV-B radiation) and UV_{BE} (biologically effective UV radiation) were calculated on the basis of the action spectra specified.

	P	PA	PAB
PAR (µmol m^{-2} s^{-1})	522	512	543
PAR (W m^{-2})	97	98	100
UV-A (W m^{-2})	2.61	32.6	35.2
UV-B (W m^{-2})	0.006	0.010	1.21
$UV\text{-}B_{BE}$ (W m^{-2}) (Caldwell, 1971)	0.00	0.00	0.36
$UV\text{-}B_{BE}$ (W m^{-2}) (Setlow, 1974)	0.00	0.00	0.34
UV_{BE} (W m^{-2}) (Flint & Caldwell, 2003)	0.01	0.46	0.83

5.3. MATERIALS AND METHODS

Experimental design

Unshaded and submerged samples of the foliose aquatic liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Vána (hereafter *J. cordifolia*) were collected on 15 November 2007 at the first-order stream Lumbreras (1903 m altitude, 42º00'30'' N, 02º38'40'' W), in La Rioja (northern Spain). The stream flows mainly over sandstones and quartzites, and the vegetation of the sampling zone is a subalpine shrubland dominated by *Vaccinium myrtillus* L., *Juniperus communis* L. subsp. *alpina* (Suter) Celak and *Calluna vulgaris* (L.) Hull, and intermingled with scattered *Pinus sylvestris* L. The material was rinsed with stream water and transported to the laboratory in a portable icebox (temperature always below 5ºC).

The material was then rinsed again with stream water and green healthy apices were selected and distributed into 15 separate plastic tubes (12 cm of diameter) with a basal net which prevented material losses. The 15 tubes were placed in a circulating bath system within a growth chamber. The bath was filled with air-bubbled stream water (pH 6.8, conductivity 21 μS cm^{-1}) which was maintained at a constant temperature of 10ºC using an immersion chiller. The radiation was provided by a Hönle SOL 1200RF2 lamp (Dr. Hönle AG UV-Technologie, Gräfelfing/Munich, Germany) and Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) and True-lite full spectrum (True Sun, Steubenville, OH, USA) fluorescent tubes. Spectral characteristics have been published elsewhere (Gröniger *et al.*, 1999). Three radiation regimes were randomly assigned to the tubes (in five replicates for each regime) by covering the tubes with specific UV cut-off foils:

- P (photosynthetically active radiation, PAR, alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which cut off all UV radiation.

- PA (PAR + UV-A), using Folex 320 (Folex GmbH, Dreieich, Germany), which cut off UV-B and UV-C radiation.

- PAB (PAR + UV-A + UV-B), using Ultraphan 295 (Digefra GmbH, Munich, Germany), which cut off UV-C radiation.

The filters were pre-irradiated for one hour and replaced every week. Table 1 shows the radiation conditions in the three different regimes. The biologically effective UV-B irradiance ($UV\text{-}B_{BE}$) was estimated using classic and modern action spectra: the generalized plant damage action spectrum normalized at 300 nm (Caldwell, 1971), the DNA damage spectrum (Setlow, 1974), and that of Flint & Caldwell (2003). Given that the latter also takes into account UV-A radiation, biologically effective UV irradiance (UV_{BE}) was estimated in this case. Plants were submerged at 1-2 cm depth, which attenuated less than 0.01% the photosynthetic and UV wavelengths. The spectral irradiances were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland), and PAR was measured with a quantum sensor (LI-190SA, LI-COR, Lincoln, NE, USA). Samples were cultivated with a 12:12 photoperiod for 31 days, replacing weekly the water in the culture. The UV source (Hönle lamp) was switched on around noon for 7 h per day (square-wave). The plants under the PAB regime received a daily UV-B dose of 28.2 kJ m^{-2}, which was required to mimic a 20% ozone depletion as calculated with a computer model (Björn & Teramura, 1993).

Physiological measurements

The sclerophylly index (SI) was calculated, at the beginning and end of the experiment, as the quotient between the dry mass (DM: 60ºC for 24 h) and the surface area of the prostrate apex (LI-COR LI-3000 area meter, Lincoln, NE, USA). Previously, fresh mass was measured. The maximum quantum yield of PSII (F_v/F_m) was periodically measured at predawn with a portable pulse amplitude modulation fluorometer (MINI-PAM, Walz, Effeltrich, Germany) following Núñez-Olivera *et al.* (2004). DNA damage was evaluated by detection of thymine dimers, using UV-C irradiated DNA of the plasmid pBSK for calibration (for details, see Otero *et al.*, 2006). Samples for this analysis were taken at the end of the light period on both the beginning and end days of the experiment.

Methanol-soluble and methanol-insoluble, alkali-extractable cell wall-bound UV-absorbing compounds (respectively, SUVAC and WUVAC) were analyzed. In both cases, the bulk level of UVAC and the concentrations of several HCA derivatives were measured by, respectively, spectrophotometry and HPLC. The extraction methods were based on Schmitz-Hoerner & Weissenbock (2003), Rozema *et al.* (2001) and Arróniz-Crespo *et al.* (2006). For these analyses, 50 mg of fresh shoot apices was frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany). Then, 5 ml of methanol : water : 7M HCl (70:29:1 v/v/v) was added for extraction (24 h at 4ºC in the dark). The extract was centrifuged at 6000 *g* for 15 min and the supernatant and pellet were considered the source of SUVAC (presumably mainly located in the vacuoles) and WUVAC, respectively (Clarke & Robinson, 2008). In the supernatant, the bulk level of SUVAC was measured, in arbitrary units, as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM, using a Perkin-Elmer λ35 spectrophotometer (Perkin-Elmer, Wilton, CT, USA). Also in the supernatant, five HCA derivatives were detected and quantified by HPLC (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA) following Arróniz-Crespo *et al.* (2006): *p*-coumaroylmalic acid, caffeoylmalic

acid (or phaselic acid), feruloylmalic acid, 5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid, and 5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid. These will be hereafter referred to as C1 to C5, respectively. Concentrations were expressed per unit of DM. Chemical structures of the compounds are represented, and specific HPLC conditions are detailed, in Arróniz-Crespo *et al.* (2006). The pellet remaining from the methanol extraction was hydrolysed with 2 ml 1 M NaOH for 3 h at 80ºC, acidified to a pH of 1.0 with HCl and extracted three times with ethyl acetate. After evaporation, the material was dissolved in methanol and the bulk level of WUVAC was spectrophotometrically measured as previously described for the bulk level of SUVAC. In the cell wall-bound fraction, *p*-coumaric and ferulic acids were detected and quantified by HPLC (Arróniz-Crespo *et al.*, 2006). These two compounds will be hereafter referred to as C6 and C7, respectively. C1 to C7 compounds were identified using Nuclear Magnetic Resonance and Mass Spectrometry (Arróniz-Crespo *et al.*, 2006). Other minor peaks, especially in the soluble fraction, could not be identified. The ratio between *p*-coumaric and ferulic acids has been proved to be useful in the evaluation of long-term changes in UV-B radiation (Lomax *et al.*, 2008), and thus the ratio C6/C7 was calculated in the cell wall-bound fraction. By analogy, the ratio C1/C3 was obtained in the soluble fraction.

Statistical analysis

The effects of the radiation regime and culture period on the responses of the liverwort were tested using a two-way analysis of variance (ANOVA), with repeated measures for the culture period, once proved that the data met the assumptions of normality and homoscedasticity. In the case of significant differences, means were then compared by Tukey's test. All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

5.4. RESULTS

Representative chromatograms of the soluble and cell wall-bound fractions of the extracts of *J. cordifolia*, together with the absorption spectra of C1 to C7 compounds, are shown in Fig. 1. The two fractions had different compositions: compounds C1 to C5 were found in the soluble fraction, whereas C6 and C7 were present in the cell wall-bound fraction. At the beginning of the experiment, C2 and C5 were the most abundant compounds in the soluble fraction (14.5 and 8.7 nmol mg^{-1}DM, respectively), followed by C1 (7.1), C4 (4.4), and C3 (3.2). In the cell wall-bound fraction, C7 was much more abundant than C6 (4.1 and 0.48 nmol mg^{-1}DM, respectively). The absorption maxima of the seven compounds analyzed were located at 310-330 nm, and secondary maxima occurred in the UV-C region (Fig. 1).

Most of the variables measured were significantly affected by the culture period, but the radiation regime only influenced a few of them: thymine dimers, F_v/F_m, the bulk level of SUVAC and WUVAC, and the concentrations of C1 and C6 (Table 2). SI significantly decreased from 1.74 mg cm^{-2} at the beginning of the experiment to 1.45-1.65 (depending on the radiation regime) at the end, but was not affected by the radiation regime. F_v/F_m significantly changed over the culture period, showing decreases in the PA and PAB regimes but an increase in the P regime (Fig. 2). F_v/F_m was significantly lower in PA and PAB regimes than in P. DNA damage increased in all the samples from the beginning (when no thymine dimer was detected) to the end of the culture period, but while these increases were modest in P and PA samples, PAB samples showed a much stronger increase (Fig. 3).

The bulk levels of SUVAC were about 2.5-fold higher than those of WUVAC at the beginning of the experiment, and remained higher until the end. Both bulk levels were significantly affected by time and regime, with

higher values in the PAB and PA samples than in the P samples (Fig. 2).

C1 was significantly influenced by both culture time and radiation regime. In P samples, C1 remained stable over the experiment, whereas it increased slightly in PA samples and strongly in PAB samples (Fig. 2). Thus, in the final days of culture, C1 values were much higher in PAB than in PA samples, and also higher in these ones than in P samples. The concentrations of the rest of the soluble HCA derivatives (C2 to C5) were influenced by the culture time but not by the radiation regime, and changed over time showing no apparent trend. The ratio C1/C3 was not affected by the radiation regime and showed significant and apparently random changes with time, with values ranging 1.68-3.30 in P samples, 2.02-4.17 in PA samples, and 1.56-5.80 in PAB samples.

Table 2. Overall effects of the culture time and radiation regime on the variables measured in *Jungermannia exsertifolia* subsp. *cordifolia*, tested by a 2-way ANOVA (with repeated measures for the culture time), and interactions between both main factors. ***P<0.001, **P<0.01, *P<0.05, NS non-significant. SUVAC and WUVAC: respectively, soluble and cell wall-bound UV-absorbing compounds. C1 to C7: seven different hydroxycinnamic acid derivatives (see their names in the text).

	Culture time	**Radiation regime**	**Interaction**
Sclerophylly index (SI)	***	NS	*
F_v/F_m	*	*	*
Thymine dimers	***	***	***
Bulk level of SUVAC	**	*	NS
Bulk level of WUVAC	*	***	NS
C1	***	***	**
C2	***	NS	NS
C3	***	NS	NS
C4	***	NS	NS
C5	***	NS	NS
C6	**	**	NS
C7	NS	NS	NS
C1/C3	***	NS	NS
C6/C7	***	NS	NS

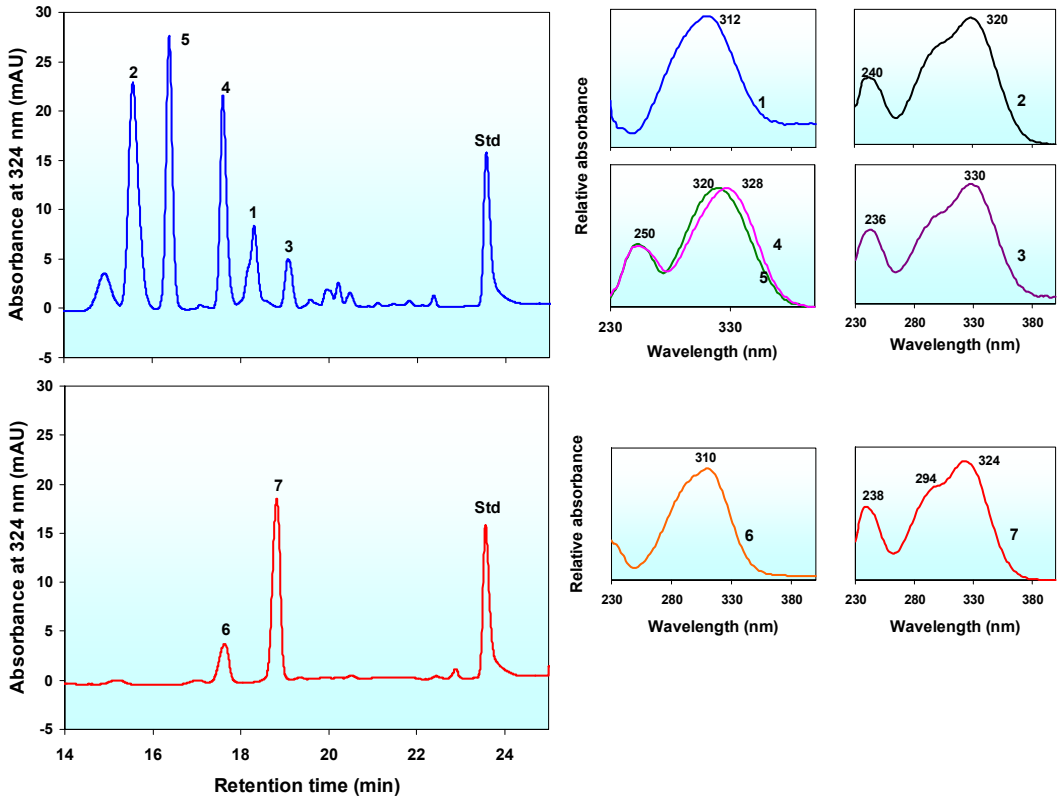

Fig. 1. Representative chromatograms (unprocessed A_{324} in milliabsorbance units, mAU, *vs.* retention time) of the methanol-soluble (top) and methanol-insoluble, alkali-extractable cell wall-bound (bottom) fractions of *Jungermannia exsertifolia* subsp. *cordifolia*. The isolated compounds are shown with the internal standard (Std.: quercetin): 1, *p*-coumaroylmalic acid; 2, phaselic acid; 3, feruloylmalic acid; 4, 5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid; 5, 5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid; 6, *p*-coumaric acid; and 7, ferulic acid. Representative absorption spectra of the respective compounds are also shown.

In the cell wall-bound fraction, C6 was significantly affected by both culture time and radiation regime. It remained stable in all the samples for the first 10 days of culture, but then increased strongly in PAB samples up to reaching a plateau, whereas still remained stable in PA and P samples (Fig. 2). Values of C6 in the cell wall-bound fraction were much lower than those of C1 in the soluble fraction. The other compound present in the cell wall-bound fraction (C7) was not affected either by culture time or radiation regime, and showed no clear trend in its temporal changes. The ratio C6/C7 was significantly influenced by culture time and increased in all the samples in the last days of culture, but was not significantly affected by the radiation regime. Values of C6/C7 ranged 0.12-0.19. A significant interaction between radiation regime and culture time was found only in a few variables: SI, F_v/F_m, DNA damage and C1.

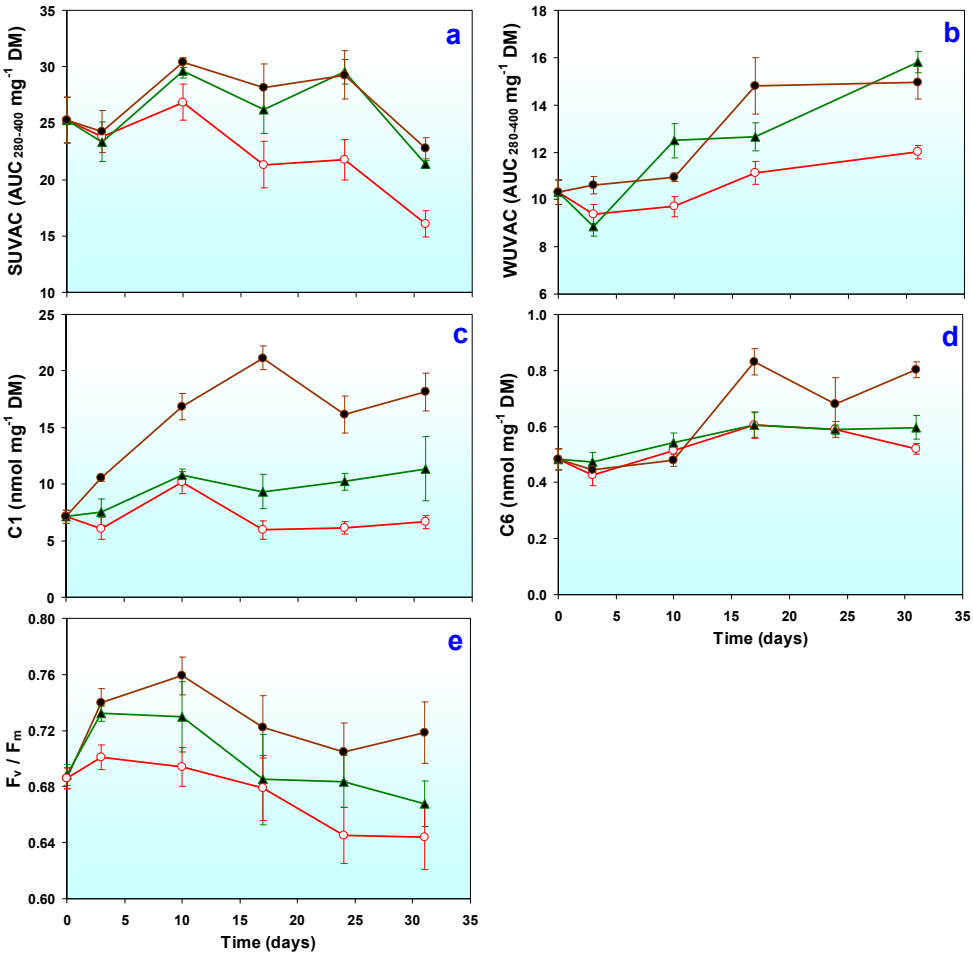

Fig. 2. Temporal changes, over the culture period, of the following characteristics of *Jungermannia exsertifolia* subsp. *cordifolia*: a) the bulk levels of methanol-soluble UV-absorbing compounds (SUVAC), as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM; b) the bulk levels of methanol-insoluble, alkali-extractable cell wall-bound UV-absorbing compounds (WUVAC), in the same units as SUVAC; c) the concentration of C1 (*p*-coumaroylmalic acid) present in the soluble fraction; d) the concentration of C6 (*p*-coumaric acid) present in the cell wall-bound fraction; and e) the maximum quantum yield of PSII (F_v/F_m). Values (mean ± SE) measured in the different

radiation regimes (P, red line; PA, green line; PAB, brown) are shown.

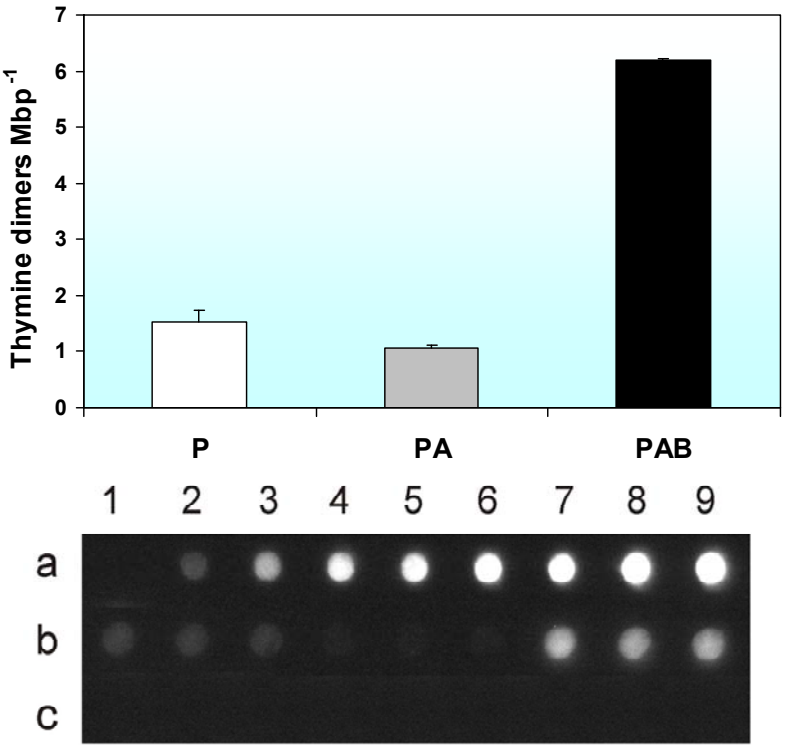

Fig. 3. DNA damage in *Jungermannia exsertifolia* subsp. *cordifolia* at the end of the culture period. Top: number of thymine dimers (means ± SE) under the three radiation regimes (P, PA and PAB). Bottom: dot blot of DNA from plasmid pBSK and from the liverwort showing the presence of thymine dimers. Lane a (columns 1-9) corresponds to increasing concentrations (0, 1, 2, 3, 4, 5, 8, 10 and 15 ng) of plasmid DNA irradiated with UV-C (calibration lane). Lane b represents 1-µg samples of liverwort DNA of the different regimes: P (columns 1-3), PA (columns 4-6) and PAB (columns 7-9). The remaining replicates showed similar results. In the position c1, a 1-µg sample of non-irradiated plasmid DNA is located.

5.5. DISCUSSION

J. cordifolia is the first liverwort in which the bulk UV-screening capacity of both the soluble (SUVAC) and cell wall-bound (WUVAC) fractions has been evaluated, finding that the levels of bulk SUVAC were higher than those of bulk WUVAC from the beginning to the end of the study. This contrasts with the results reported for three Antarctic mosses, given that *Bryum pseudotriquetrum* had almost equal concentrations of soluble and cell wall-bound UV-screening compounds, and both *Ceratodon purpureus* and *Schistidium antarctici* showed a predominant (up to nine times higher) concentration of cell wall-bound screening compounds (Clarke & Robinson, 2008). Thus, although the knowledge of the distribution of the bulk UV absorbance between the soluble and the cell wall-bound fractions in bryophytes is still limited, it may be postulated that liverworts and mosses show, regarding this point, different variants of UV protection. Liverworts would take advantage of their higher bulk SUVAC (Otero *et al.*, 2008), whereas mosses would have higher bulk WUVAC. Further investigation is needed to confirm this hypothesis. In addition, it should be pointed out that our extraction procedure probably cannot extract all the UVAC from the cell wall material.

The bulk SUVAC and bulk WUVAC responded to UV radiation in a similar way and increased under both PA and PAB regimes. Nevertheless, the values of bulk SUVAC in the PA and PAB samples in the final day of the culture period partially distort the clear increase found in the previous days. The positive relationship between the bulk SUVAC and the levels of UV-B radiation was expected in the light of previous laboratory experiments (Martínez-Abaigar *et al.*, 2003; Arróniz-Crespo *et al.*, 2008) and field experiences (Arróniz-Crespo *et al.*, 2006; Núñez-Olivera *et al.*, 2009) using this liverwort. This relationship is also common in tracheophytes (Searles *et al.*, 2001a) and polar bryophytes (in this last case, only in experiments with screens or using unmanipulated natural UV-B: Newsham & Robinson, 2009). In bryophytes in general, this positive relationship between the bulk SUVAC

and UV-B levels has been much more frequently found in liverworts than in mosses (Martínez-Abaigar & Núñez-Olivera, 2011). This finding may additionally support the hypothesis that liverworts and mosses follow different protection mechanisms against enhanced UV-B.

The increase in bulk WUVAC in the PA and PAB samples supports the previous idea that *J. cordifolia* is a UV-responsive species (Martínez-Abaigar & Núñez-Olivera, 2011). There are no comparative data for this variable in bryophytes exposed to artificially enhanced UV-B in the laboratory. Under field conditions, the cell wall-bound UV-screening compounds of three Antarctic mosses did not respond to natural temporal changes in UV-B (Clarke & Robinson, 2008), but epidermal cell wall-bound UV-absorbing compounds increased under enhanced UV-B in the sub-Arctic dwarf shrub *Vaccinium vitis-idaea* (Semerdjieva *et al.*, 2003). Interspecific differences in UV-B responses, together with differences in the experimental and environmental conditions, may justify these contrasting results.

The increase in SUVAC in PA samples which was found in the present study contrasts with the results reported in previous experiments with *J. cordifolia* (Martínez-Abaigar *et al.*, 2003). On the other hand, there is no previous result on the influence of UV-A radiation on the bulk WUVAC in bryophytes. Hence, the precise role of UV-A in the induction of SUVAC and WUVAC in bryophytes requires further study. In a field study using the moss *Pleurozium schreberi*, the bulk SUVAC increased under enhanced UV-B radiation compared with the UV-A control, although it was correlated with both the amount of UV-A and UV-B radiation received by the samples (Lappalainen *et al.*, 2008). In tracheophytes, UV-A may induce a number of phenolics contributing to the bulk SUVAC and WUVAC, and it has recently been suggested the convenience of including UV-A radiation, in addition to UV-B, in the action spectra for the induction of many phenolic metabolites (Kotilainen *et al.*, 2008).

Five UV-absorbing compounds (HCA derivatives) were extracted

from the soluble fraction of *J. cordifolia* (Fig. 1): C1, *p*-coumaroylmalic acid; C2, caffeoylmalic acid; C3, feruloylmalic acid; and C4 and C5, two coumarins. Only two compounds were extracted from the cell wall-bound fraction: C6, *p*-coumaric acid, and C7, ferulic acid. Except for the two coumarins, all these compounds are common in plants (Dixon & Paiva, 1995), although the identity and relative composition of the HCA derivatives may vary depending on the different fractions and species (Wagner *et al.*, 2003). The presence of esterified derivatives in the soluble fraction of *J. cordifolia* may be logical because esterification with organic acids would facilitate the solubility and storage of these compounds in the vacuole (Hause *et al.*, 2002; Meissner *et al.*, 2008). The occurrence of *p*-coumaric and ferulic acids in the cell wall-bound fraction could be expected, because both compounds are usually found in the cell walls, from which are released by alkaline hydrolysis (Wagner *et al.*, 2003; Kaffarnik *et al.*, 2006). Compartmentation of UV-B screening compounds in bryophytes, especially in liverworts with monostratified leaves and lacking a nerve (like *J. cordifolia*), would thus include mainly only two compartments: vacuoles and cell walls. In contrast, the degree of compartmentation in tracheophytes results much more complicated (Fischbach *et al.*, 1999).

Among the seven HCA derivatives analyzed in *J. cordifolia*, only C1 (*p*-coumaroylmalic acid) in the soluble fraction and C6 (*p*-coumaric acid) in the cell wall-bound fraction responded to UV radiation. It may be worth mentioning that the absorption maxima of both compounds are located within the UV-B region (Fig. 1). C1 was higher in PAB samples than in PA samples, and in these latter samples than in P samples, whereas C6 was significantly higher in the PAB samples with respect to PA and P samples. C1 responded to UV-B more clearly and rapidly than C6. The increase in C1 was expected because it is the most UV-B-responsive compound in this liverwort, both under laboratory (Otero *et al.*, 2006; Arróniz-Crespo *et al.*, 2008) and field (Núñez-Olivera *et al.*, 2009) conditions. The increase in C6 is interesting because: 1) it is directly chemically related to C1; and 2) it is one of the few UV-responsive compounds hitherto found in the cell wall-bound

fraction of bryophytes, together with the anthocyanidin riccionidin A of the liverwort *Cephaloziella varians* (Snell *et al.*, 2009). In addition, *p*-coumaric acid has been used in the Antarctic graminoid *Deschampsia antarctica* as a UV-B indicator (Ruhland *et al.*, 2005), has been recommended for the reconstruction of past UV-B using different plant materials and species (Rozema *et al.*, 2001; Blokker *et al.*, 2006; Lomax *et al.*, 2008; Otero *et al.*, 2009), and has been proposed as a chromophore in the photoreception of UV wavelengths (Jenkins, 2009). Because of these facts, the responses of this compound and its malic ester to UV-B merit further investigation.

Apart from C1 and C6, the rest of the compounds analyzed in *J. cordifolia* did not show consistent responses to enhanced UV-B. A diversity of responses of HCA derivatives to UV radiation has also been reported in tracheophytes (Schnitzler *et al.*, 1996; Fischbach *et al.*, 1999; Kolb *et al.*, 2001; Schmitz-Hoerner & Weissenbock, 2003; Ruhland *et al.*, 2005; Luthria *et al.*, 2006; Kotilainen *et al.*, 2008). This diversity was probably due to the differences in the species studied, the compounds considered, the experimental conditions applied, the developmental stage of the leaves, the tissue used and the fraction (soluble or insoluble) from which the compounds were extracted. Additionally, the response of HCA derivatives to enhanced UV-B might be limited because they can be strongly accumulated as constitutive compounds in some species (Schmitz-Hoerner & Weissenbock, 2003). Meissner *et al.* (2008) also hypothesized that, in *Arabidopsis*, HCA esters would be involved in the short-term UV-B response whereas the increased formation of flavonoids would contribute to a sustained adaptation strategy. All these points may make the UV-B responses of HCA still difficult to be interpreted under a global perspective. However, our results show that the role of HCA derivatives as UV-B protectants should be taken into account in liverworts, as it has been done in some tracheophytes (Sheahan, 1996), because these compounds have molar absorptivities in the UV-B range comparable to flavonoids (Liu *et al.*, 1995) and can also act as antioxidants (Rice-Evans *et al.*, 1997).

Regarding the rest of variables, SI decreased over time in all the radiation regimes. SI is an indirect indicator of growth because newly grown shoots are softer (less sclerophyllous) than old shoots (Martínez-Abaigar *et al.*, 2003). Thus, the decrease in SI indicated that all the samples could grow well under the culture conditions imposed. SI (and thus shoot growth) was not affected by the radiation regime, probably because *J. cordifolia* is tolerant to UV-B (Martínez-Abaigar *et al.*, 2003). Also, a longer culture period could have been necessary to observe growth differences between the different treatments, since many bryophytes grow relatively slowly (Glime, 2007).

F_v/F_m was significantly lower in PA and PAB regimes than in P. This was not surprising because F_v/F_m is one of the most UV-B-sensitive variables in *J. cordifolia* (Martínez-Abaigar *et al.*, 2003; Otero *et al.*, 2006). A decrease in F_v/F_m indicates in particular photoinhibition, that destroys the central core protein D1 of PSII, which is a target of UV-B (Jansen *et al.*, 1998). The influence of UV-A on F_v/F_m is far from clear in *J. cordifolia*, because no UV-A effect was observed in a previous study (Martínez-Abaigar *et al.*, 2003), and thus this issue requires further investigation.

In our study, strong DNA damage was caused by UV-B radiation. This is consistent with the target character of DNA with respect to UV-B (Jansen *et al.*, 1998), so that this response seems to be largely UV-B-specific. Our finding is also in agreement with previous studies conducted on bryophytes, since DNA damage increased in samples exposed to enhanced UV-B under laboratory (Lud *et al.*, 2002; Otero *et al.*, 2006; Turnbull *et al.*, 2009) and field (Lud *et al.*, 2002, 2003) conditions. However, DNA damage was not detected when bryophytes were exposed to ambient UV-B levels (Núñez-Olivera *et al.*, 2009), unless the environmental conditions were so harsh that prevented DNA repair, as occurs in the Antarctic (Turnbull &

Robinson, 2009). The specificity of the response of DNA damage to enhanced UV-B makes this variable very suitable to evaluate the consequences of stratospheric ozone depletion in bryophytes.

In conclusion, methanol-soluble and methanol-insoluble cell wall-bound fractions of *J. cordifolia* have different UV-absorbing compounds, and each individual compound may respond in a different way to UV-A and UV-B radiations. Thus, it is interesting to measure not only the bulk SUVAC and WUVAC, but also the specific compounds contributing to these bulk variables. In addition, an adequate extraction of UVAC from both the soluble and cell wall-bound fractions is advisable to better evaluate the protection mechanisms of bryophytes against UV-B radiation, because these two fractions may represent different UV screening mechanisms (Clarke & Robinson, 2008). Among the compounds analyzed, *p*-coumaric acid and its derivative *p*-coumaroylmalic acid merit further investigation, because of their responsiveness to enahnced UV-B and usefulness in UV-B bioindication. Finally, accumulation of both compounds in *J. cordifolia* did not totally prevent the damage induced by enhanced UV-B, that was indicated by the decrease of F_v/F_m and the increase in the amount of thymine dimers. It should be taken into account that our results and conclusions cannot be directy extrapolated to outdoor conditions, because they are directly related to the artificial experimental conditions applied in our study.

CHAPTER 6

ENHANCED UV-B RADIATION, PHYSIOLOGICAL DAMAGE AND PROTECTION MECHANISMS (SOLUBLE AND INSOLUBLE UV-ABSORBING COMPOUNDS) IN TWO AQUATIC MOSSES

6.1. ABSTRACT

The effects of enhanced UV-B radiation on UV-absorbing compounds (UVAC), the maximum quantum yield of photosystem II (F_v/F_m), DNA integrity, and the sclerophylly index, were analyzed in the mosses *Bryum pseudotriquetrum* and *Fontinalis antipyretica* for 31 days under laboratory conditions. Enhanced UV-B increased the bulk level of the vacuolar soluble UVAC (SUVAC) in both mosses and the concentration of two different soluble kaempferols in *B. pseudotriquetrum*, but had no effect on the bulk level of cell wall-bound insoluble UVAC (WUVAC) in both mosses and the concentration of insoluble *p*-coumaric acid in *F. antipyretica*. Thus, the insoluble fraction would be less UV-B-responsive than the soluble one, probably because the constitutively high bulk level of WUVAC (and noticeably higher than that of SUVAC) would already provide a notably effective protection. Also, WUVAC would be less responsive due to their relative immobilization in the cell wall. The protective mechanisms developed by both mosses could not totally prevent UV-B damage, indicated by the modest decrease of F_v/F_m and the increase in DNA damage. Ecological and phylogenetic implications of the differences in UVAC compartmentation between liverworts and mosses are discussed.

RESUMEN

Se analizaron los efectos de un suplemento de radiación UV-B sobre los CAUV, el rendimiento cuántico máximo del PSII (F_v/F_m), los daños al ADN y el índice de esclerofilia de los musgos *Bryum pseudotriquetrum* y *Fontinalis antipyretica* durante 31 días en condiciones de laboratorio. El suplemento de UV-B aumentó el nivel global de los CAUV solubles (CAUVS) en ambos musgos y la concentración de dos kaempferoles solubles en *B. pseudotriquetrum*, pero no tuvo ningún efecto ni en el nivel global de los CAUV insolubles (CAUVI) en ambos musgos ni en la concentración del ácido *p*-cumárico insoluble en *F. antipyretica*. Por lo tanto, la fracción insoluble sería menos sensible a la radiación UV-B que la soluble. Esto podría haber ocurrido, probablemente, porque el alto nivel constitutivo de CAUVI (considerablemente mayor que el de CAUVS) ya ejercería una protección notablemente efectiva. Otra razón adicional sería que los CAUVI responderían con menor intensidad que los CAUVS por su relativa inmovilización en las paredes celulares. Los mecanismos de protección desarrollados por los dos musgos no pudieron evitar totalmente los daños producidos por la radiación UV-B, que se hacían patentes en un moderado descenso de F_v/F_m y un aumento en el daño al ADN. Se discuten las implicaciones ecológicas y filogenéticas de las diferencias en la compartimentación de los CAUV en las hepáticas y los musgos.

6.2. INTRODUCTION

Ultraviolet (UV) radiation is a minority part of solar radiation, but it has important biological effects on the morphology and physiology of photosynthetic organisms, including bryophytes (Boelen *et al.*, 2006; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). The amount of solar UV-B (280-315 nm) radiation reaching the ground has increased due to the anthropogenic ozone reduction. At northern mid-latitudes, ozone loss has been estimated around 6% in the last 30 years, which might have resulted in a UV-B increase of up to 12% (McKenzie *et al.*, 2003). The future trend for UV-B radiation is notably uncertain, in particular due to the influence of climate change factors (McKenzie *et al.*, 2007; Hegglin & Shepherd, 2009). In addition, ozone mini-holes may cause up to 33% decreases in ozone and consequent transitory UV-B increases between 43 and 75% (Antón *et al.*, 2007). Thus, the study of UV-B effects on organisms will be an important issue in the coming several decades.

In photosynthetic organisms, increased UV-B may trigger diverse damage, repair and acclimation processes, so that the plant can cope with the new situation (Jansen *et al.*, 1998). Thus, damage to DNA and the photosynthetic apparatus (pigment degradation, photoinhibition, and decreases in photosynthetic rates and enzyme activities) may be counteracted by protective mechanisms, such as DNA repariring, antioxidant systems and the accumulation of UV-absorbing compounds (UVAC), mainly phenolics.

The bulk UV absorbance of methanolic plant extracts has been the most used variable to globally measure UVAC and the associated UV protection capacity. This variable has been notably valuable in the context of UV research, being the one most consistently responding to enhanced UV-B in tracheophytes and bryophytes (Searles *et al.*, 2001a; Newsham & Robinson, 2009). Nevertheless, this variable can be usefully complemented by the measurement of the specific individual UVAC contributing to that

bulk UV absorbance, since each compound may respond to UV-B differently (Kotilainen *et al.*, 2008). Furthermore, Schnitzler *et al.* (1996) measured both the bulk UV absorbance and specific UVAC in two different cell compartments: the soluble fraction (mainly located in the vacuole), and the insoluble cell wall-bound fraction. This differentiation is relevant, especially in the monostratified leaves of bryophytes, because cell wall-bound UVAC would provide a more efficient UV screen than intracellular UVAC, and thus could enhance UV-B tolerance (Clarke & Robinson, 2008).

The effects of UV-B on bryophytes are not properly understood yet, in spite of the important work carried out mainly in the last decade (see recent reviews in Boelen *et al.*, 2006; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). In particular, UVAC compartmentation between vacuoles and cell walls has been little studied (Taipale & Huttunen, 2002; Clarke & Robinson, 2008; Snell *et al.*, 2009; Lappalainen, 2010). This issue may be important in such structurally simple plants as bryophytes, since their UVAC compartmentation is much simpler than that of tracheophytes and thus easier to model. In addition, it has been hypothesized that liverworts and mosses may show different compartmentations: liverworts would have a higher bulk level of SUVAC than of WUVAC (Chapter 5), whereas the inverse pattern would be found in mosses (Clarke & Robinson, 2008; Lappalainen, 2010). More studies on the compartmentation of UVAC in bryophytes and its responses to enhanced UV-B are needed to test this hypothesis.

The aim of the present study was to examine, under laboratory conditions, the response of the UVAC compartmentation of two mosses (*Bryum pseudotriquetrum* and *Fontinalis antipyretica*) to enhanced UV-B radiation, given that previous studies with these mosses (Martínez-Abaigar *et al.*, 2003; Núñez-Olivera *et al.*, 2004; Martínez-Abaigar *et al.*, 2009) did not take into account this important issue. We measured the bulk levels of SUVAC and WUVAC, together with specific UVAC belonging to the two main types of UV-protective phenolics, hydroxycinnamic acids and

flavonoids, that can be located in both the soluble and cell wall fractions (Dixon & Paiva, 1995; Schmitz-Hoerner & Weissenbock, 2003). We also measured the maximum quantum yield of photosystem II (F_v/F_m), DNA integrity, and the sclerophylly index, which could indicate the damage effects caused by enhanced UV-B on the photosynthetic apparatus, DNA, and growth. The simultaneous evaluation of damage and protection mechanisms would serve to a better understanding of the tolerance and acclimation to enhanced UV-B in the mosses used, and also to accumulate information on the different strategies developed by bryophytes regarding UVAC compartmentation.

6.3. MATERIALS AND METHODS

Experimental design

Unshaded and submerged samples of two aquatic mosses, *Bryum pseudotriquetrum* (Hedw.) P.Gaertn. *et al.* and *Fontinalis antipyretica* Hedw., were collected on 15 November 2007 at the first-order stream Lumbreras (1903 m altitude, 42º00'30'' N, 02º38'40'' W), in La Rioja (northern Spain). The material was rinsed with stream water and transported to the laboratory in a portable icebox. For each species, green healthy apices were selected and distributed into 15 plastic tubes with a basal net which prevented material losses. The 30 tubes were placed in a circulating bath system within a growth chamber. The bath was filled with air-bubbled stream water (pH 6.8, conductivity 21 μS cm^{-1}) which was maintained at a constant temperature of 10ºC using an immersion chiller. The radiation was provided by a Hönle SOL 1200RF2 lamp (Dr. Hönle AG UV-Technologie, Gräfelfing/Munich, Germany) and Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) and True-lite full spectrum (True Sun, Steubenville, OH, USA) fluorescent tubes. Spectral characteristics have been published elsewhere (Gröniger *et al.*, 1999). By covering the tubes with specific UV cut-off foils, three radiation regimes were randomly assigned to the tubes (in five replicates for each regime):

- P (photosynthetically active radiation, PAR, alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which cut off all UV radiation.

- PA (PAR + UV-A), using Folex 320 (Folex GmbH, Dreieich, Germany), which cut off UV-B and UV-C radiation.

- PAB (PAR + UV-A + UV-B), using Ultraphan 295 (Digefra GmbH, Munich, Germany), which cut off UV-C radiation.

Table 1. Radiation conditions in the three radiation regimes (P, PA, and PAB) under which the samples were cultivated. UV_{BE} (biologically effective UV radiation) were calculated on the basis of the action spectra specified.

	P	**PA**	**PAB**
PAR (μmol m^{-2} s^{-1})	522	512	543
PAR (W m^{-2})	97	98	100
UV-A (W m^{-2})	2.61	32.6	35.2
UV-B (W m^{-2})	0.006	0.010	1.21
UV_{BE} (W m^{-2}) (Caldwell, 1971)	0.00	0.00	0.36
UV_{BE} (W m^{-2}) (Setlow, 1974)	0.00	0.00	0.34
UV_{BE} (W m^{-2}) (Flint & Caldwell, 2003)	0.01	0.46	0.83

The filters were pre-irradiated for one hour and replaced every week. Samples were cultivated with a 12:12 photoperiod for 31 days, replacing weekly the water in the culture. The UV source (Hönle lamp) was switched on around noon for 7 h per day (square-wave). Table 1 shows the radiation conditions in the three regimes. The plants under the PAB regime received a daily UV-B dose of 28.2 kJ m^{-2}, which was required to mimic a 20% ozone depletion as calculated with a computer model (Björn & Teramura, 1993).

The biologically effective UV irradiance (UV_{BE}) was estimated using classic and modern action spectra: the generalized plant damage action spectrum (Caldwell, 1971), the DNA damage spectrum (Setlow, 1974), and that of Flint & Caldwell (2003). Plants were submerged at 1-2 cm depth, which attenuated less than 0.01% the photosynthetic and UV wavelengths. The spectral irradiances were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland), and PAR was measured with a quantum sensor (LI-190SA, LI-COR, Lincoln, NE, USA).

Physiological measurements

The sclerophylly index (SI) was calculated at the end of the experiment, as the quotient between the dry mass (DM: 60ºC for 24 h) and the surface area of the prostrate apex (LI-COR LI-3000 area meter, Lincoln, NE, USA). Previously, fresh mass was measured. The maximum quantum yield of PSII (F_v/F_m) was periodically measured at predawn with a portable pulse amplitude modulation fluorometer (MINI-PAM, Walz, Effeltrich, Germany) following Núñez-Olivera *et al.* (2004). DNA damage was evaluated by detection of thymine dimers, using UV-C irradiated DNA of the plasmid pBSK for calibration (for details, see Otero *et al.*, 2006). Samples for this analysis were taken at the end of the light period on the final day of the experiment.

In both species, the bulk level of UV-absorbing compounds (UVAC) was analyzed by spectrophotometry (Perkin-Elmer λ35 spectrophotometer, Perkin-Elmer, Wilton, CT, USA) in the soluble fraction (SUVAC, presumably mainly located in the vacuoles) and the insoluble cell wall-bound fraction (WUVAC). For these analyses, fresh shoot apices were frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany). Then, 5 ml of methanol : water : 7M HCl (70:29:1 v/v/v) was added for extraction (24 h at 4ºC in the dark). The extract was centrifuged and the supernatant and pellet were considered the source of SUVAC and WUVAC, respectively (Clarke & Robinson, 2008). In the supernatant, the bulk level of SUVAC was measured, in arbitrary units, as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. The pellet was hydrolysed with NaOH, acidified with HCl and extracted three times with ethyl acetate. After evaporation, the material was dissolved in methanol and the bulk level of WUVAC was spectrophotometrically measured as previously described for the bulk level of SUVAC.

Individual UVACs were measured in both species by high-performance liquid chromatography (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA), following Arróniz-Crespo *et al.* (2006). In the soluble fraction of *B. pseudotriquetrum*, we measured the concentration of the flavonols kaempferol-3-*O*-glucoside and kaempferol-3,7-*O*-diglucoside, two of its major flavonoids (Webby *et al.*, 1996). Quantification was made by calibration curves with the commercial external standard kaempferol (Sigma-Aldrich, St. Louis, MO, USA). In the insoluble fraction of *F. antipyretica*, *p*-coumaric acid was detected and quantified.

Statistical analysis

In each moss species, the effects of the radiation regime and culture period on the responses of F_v/F_m and UVAC-related variables were tested using a two-way analysis of variance (ANOVA), with repeated measures for the culture period, once proved that the data met the assumptions of normality and homoscedasticity. In the case of significant differences, means were then compared by Tukey's test. The effect of the radiation regime on SI and the amount of thymine dimers of each species at the end of the experiment was tested using a one-way ANOVA. All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

6.4. RESULTS

Representative absorption spectra of the insoluble and soluble fractions of the extracts of *B. pseudotriquetrum* and *F. antipyretica* are shown in Fig. 1. Insoluble fractions of both species showed several absorption peaks in the 268-316 nm region (mainly in the UV-B band), whereas the soluble fractions showed well-defined unique peaks in the UV-C band (at 267 nm in *B. pseudotriquetrum* and 269 nm in *F. antipyretica*). In the soluble fraction of *B. pseudotriquetrum*, a shoulder around 330 nm could also be noted.

At the beginning of the experiment, the bulk level of WUVAC was higher than that of SUVAC in both species, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per mg DM: 28.5±2.4 *vs.* 14.9±0.5 in *B. pseudotriquetrum*, and 41.1±3.0 *vs.* 4.5±0.1 in *F. antipyretica*. Something similar occurred along the course of the experiment, where the ranges of the bulk level of WUVAC and SUVAC in *B. pseudotriquetrum* were, respectively, 27.5-45.3 and 9.9-19.2, while in *F. antipyretica* they were 29.3-42.1 and 2.9-5.9 (Figs. 2-3).

All the variables measured several times along the experiment were significantly affected by the culture period, except the concentration of *p*-coumaric acid in *F. antipyretica*, and most of these variables (except the bulk level of WUVAC in both species and *p*-coumaric acid in *F. antipyretica*) were also affected by the radiation regime (Table 2).

In *B. pseudotriquetrum* (Fig. 2), SUVAC increased around 25% in PAB samples and then maintained relatively stable levels, showing significantly higher values in PAB than in P samples. Kaempferol-3-*O*-glucoside slightly increased in P samples in the first days of the experiment and then progressively decreased until the final days, whereas in PAB samples it increased in the first days and then remained stable. Kaempferol-3,7-*O*-diglucoside progressively decreased in P samples, whereas in PAB samples it

slightly decreased in the first days and then remained fairly stable. Both flavonols showed significantly higher values in PAB than in P samples. F_v/F_m increased in all the radiation regimes in the first days of culture (more steeply in P and PA than in PAB samples), and then decreased until the end of the experiment (more smoothly in P than in PAB samples). F_v/F_m values were significantly higher in P than in PAB samples.

In *F. antipyretica* (Fig. 3), the bulk level of SUVAC showed unclear temporal trends in the three radiation regimes, but its values were significantly higher in PA and PAB than in P samples. Temporal trends were also ill-defined in the bulk level of WUVAC and *p*-coumaric acid, whose values were not significantly different between the three radiation regimes. With respect to F_v/F_m, it remained fairly stable in P and PA samples along the culture period (except for a final decrease in PA samples), whereas it progressively decreased in PAB samples. PAB samples showed significantly lower F_v/F_m values than P and PA samples.

In both species, PA samples frequently showed temporal trends and values of the variables intermediate between P and PAB samples (Figs. 2-3).

Significant interactions between culture time and radiation regime were found only in a few variables: the bulk level of SUVAC in both species and the concentration of kaempferol-3,7-*O*-diglucoside in *B. pseudotriquetrum* (Table 2).

At the end of the culture period, SI was affected ($P<0.05$) by the radiation regime in *B. pseudotriquetrum* (Fig. 4), showing significantly higher values in P than in PA samples (respectively, 3.44±0.18 and 2.88±0.11 mg cm^{-2}), while PAB samples showed intermediate values (3.11±0.13 mg cm^{-2}). In *F. antipyretica*, SI was not significantly affected by the radiation regime (Fig. 4). In both species, the amount of thymine dimers at the end of the experiment was significantly affected by the radiation regime ($P<0.001$), showing higher values in PAB than in P and PA samples (Fig. 4).

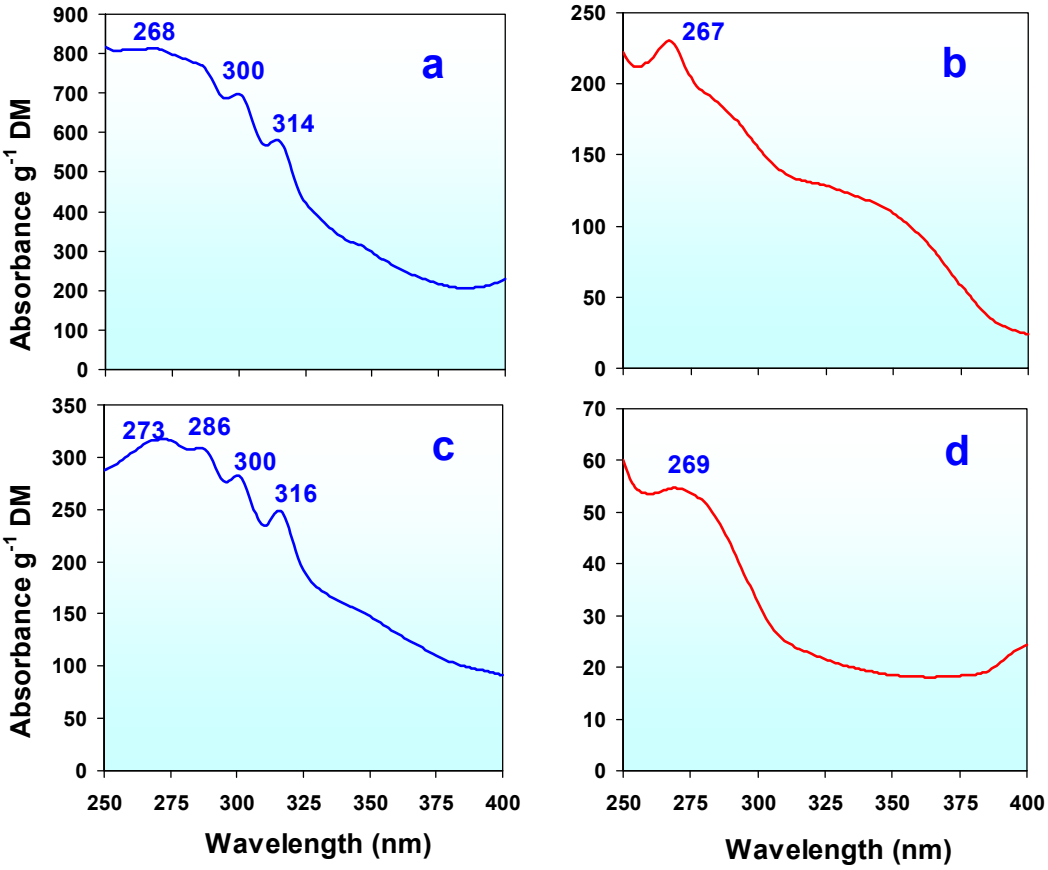

Fig. 1. Representative absorption spectra of the insoluble (a, c) and soluble (b, d) fractions of the extracts of *Bryum pseudotriquetrum* (a, b) and *Fontinalis antipyretica* (c, d).

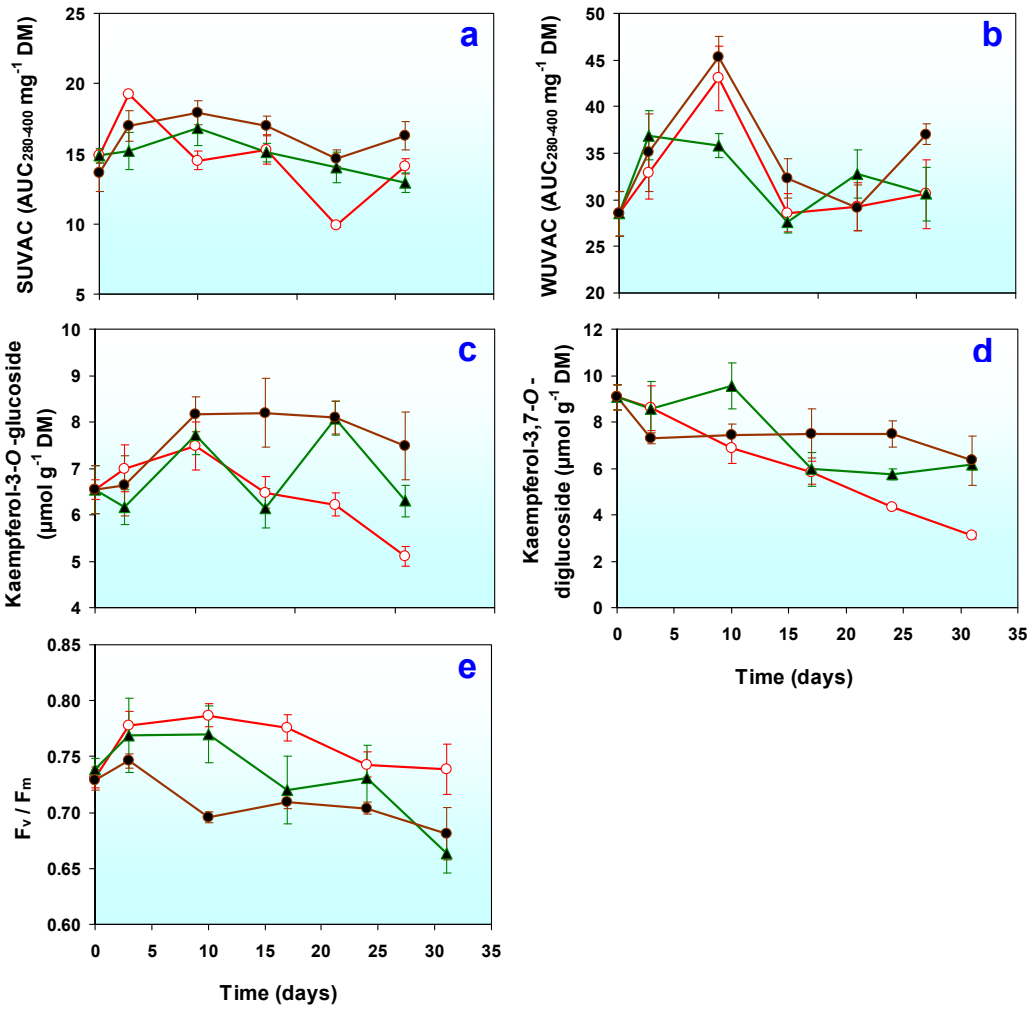

Fig. 2. Temporal changes, over the culture period, of the following variables of *Bryum pseudotriquetrum*: a) the bulk levels of soluble UV-absorbing compounds (SUVAC), as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM; b) the bulk levels of cell wall-bound UV-absorbing compounds (WUVAC), in the same units as SUVAC; c) the concentration of kaempferol-3-*O*-glucoside present in the soluble fraction; d) the concentration of kaempferol-3,7-*O*-diglucoside present in the soluble fraction; and e) the maximum quantum yield of PSII (F_v/F_m). Values (mean ± SE) measured in the different radiation regimes (P, red line; PA, green line; PAB, brown) are shown.

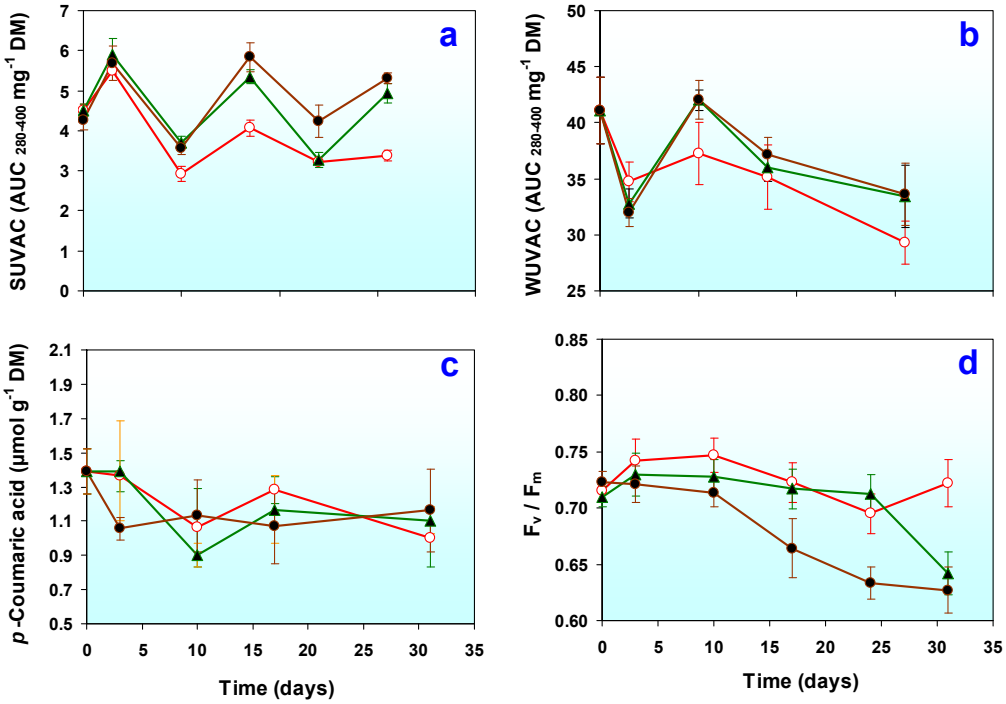

Fig. 3. Temporal changes, over the culture period, of the following variables of *Fontinalis antipyretica*: a) the bulk levels of soluble UV-absorbing compounds (SUVAC), as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM; b) the bulk levels of cell wall-bound UV-absorbing compounds (WUVAC), in the same units as SUVAC; c) the concentration of *p*-coumaric acid present in the insoluble fraction; d) the maximum quantum yield of PSII (F_v/F_m). Values (mean ± SE) measured in the different radiation regimes (P, red line; PA, green line; PAB, brown) are shown.

Table 2. Overall effects of the culture time and radiation regime on the variables measured in *Bryum pseudotriquetrum* and *Fontinalis antipyretica* along the course of the experiment, tested by a 2-way ANOVA (with repeated measures for the culture time), and interactions between both main factors. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, NS non-significant. SUVAC and WUVAC: respectively, soluble and cell wall-bound UV-absorbing compounds.

	Culture time	Radiation regime	Interaction
Bryum pseudotriquetrum			
Bulk level of SUVAC	***	*	**
Bulk level of WUVAC	***	NS	NS
Kaempferol-3-O-glucoside	*	**	NS
Kaempferol-3,7-O-diglucoside	**	***	*
F_v/F_m	*	*	NS
Fontinalis antipyretica			
Bulk level of SUVAC	***	***	**
Bulk level of WUVAC	***	NS	NS
p-Coumaric acid	NS	NS	NS
F_v/F_m	**	**	NS

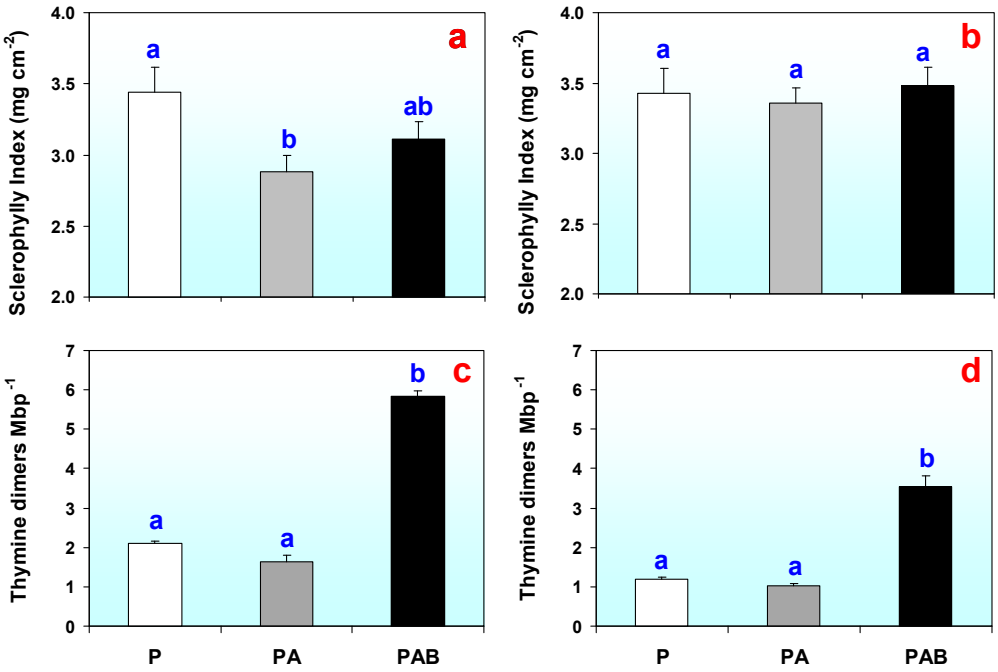

Fig. 4. Sclerophylly Index (SI) and number of thymine dimers (means ± SE) in *Bryum pseudotriquetrum* (a, c) and *Fontinalis antipyretica* (b, d) under the three radiation regimes (P, PA and PAB) at the end of the culture period. For each species and variable, different letters mean significant differences (at least $P<0.05$) between the radiation regimes (one-way ANOVA).

6.5. DISCUSSION

On the basis of the known absorption maxima of common types of phenolic compounds (Waterman & Mole, 1994), the absorption spectra of the insoluble fraction of *B. pseudotriquetrum* and *F. antipyretica* (Fig. 1) might mainly be due to the presence of hydroxycinnamic acids, one of which has already been identified in the latter species (*p*-coumaric acid). In the soluble fraction of *B. pseudotriquetrum*, the shoulder around 330 nm suggested the presence of flavonoids, among which two flavonols (kaempferol-3-*O*-glucoside and kaempferol-3,7-*O*-diglucoside) were identified. The absorbance in the soluble fraction of *F. antipyretica* was very low and no compound could be identified.

In the two species studied, the bulk level of SUVAC was comparable to the values found in these species in a 3-year study under field conditions (8.9-21.1 $AUC_{280-400}$ mg^{-1} DM in *B. pseudotriquetrum* and 2.1-5.7 in *F. antipyretica*: Núñez-Olivera *et al.*, 2010). In comparison with other mosses, the bulk level of SUVAC was relatively high in *B. pseudotriquetrum* and average in *F. antipyretica*, but in both cases lower than that found in most of the liverworts studied (Arróniz-Crespo *et al.*, 2004; Otero *et al.*, 2008). This suggests that the bulk level of SUVAC strongly depends on the species and the type of bryophyte considered. In this last sense, mosses and liverworts seem to show different protection mechanisms against UV radiation, given that liverworts accumulate a higher amount of SUVAC than mosses. Comparative data regarding the bulk level of WUVAC (as $AUC_{280-400}$ mg^{-1} DM) in bryophytes are much more scarce, but it is interesting to note that the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* showed lower values (9-16 $AUC_{280-400}$ mg^{-1} DM: Chapter 5) than the two mosses studied here (27-45). In addition, the bulk level of WUVAC was 2 to 3-fold higher than that of SUVAC in *B. pseudotriquetrum* along the experiment, while in *F. antipyretica* it was 6 to 13-fold. In Antarctic samples, *B. pseudotriquetrum* had rather similar values of both types of compounds, whereas *Ceratodon purpureus* and *Schistidium antarctici* showed a predominant (up to 9-fold

higher) concentration of WUVAC (Clarke & Robinson, 2008). In line with this, in sub-Arctic *Pleurozium schreberi* and *Polytrichum juniperinum*, the bulk level of WUVAC was 10-12 times higher than that of SUVAC (Lappalainen, 2010). These findings in mosses contrast with the fact that, in the only liverwort analyzed to date in this regard (*Jungermannia exsertifolia* subsp. *cordifolia*), the bulk level of SUVAC was about 2.5-fold higher than that of WUVAC (Chapter 5). This would support the hypothesis that liverworts and mosses show different variants of UV protection, with higher bulk SUVAC in liverworts and higher bulk WUVAC in mosses. Given that WUVAC may provide a more spatially uniform and effective UV screen than SUVAC (Clarke & Robinson, 2008), it could be speculated that mosses as a group would be better adapted to colonize UV-rich sun-exposed environments than liverworts. Further investigation in more species is needed to confirm these hypotheses and their ecological relevance.

In our study, the response of the bulk level of both SUVAC and WUVAC to enhanced UV-B was evaluated in mosses for the first time, because previous studies (1) only took into account the bulk level of SUVAC (Martínez-Abaigar & Núñez-Olivera, 2011), (2) analyzed the responses to ambient (not enhanced) UV-B (Clarke & Robinson, 2008), or (3) were dedicated to liverworts (Chapter 5).

The bulk level of WUVAC in both species, together with the concentration of the cell wall-located compound *p*-coumaric acid in *F. antipyretica*, showed unclear temporal variations and did not respond to enhanced UV-B. This could be due to the fact that levels of WUVAC were constitutively high in these mosses, as occurred in three Antarctic mosses including *B. pseudotriquetrum* (Clarke & Robinson, 2008). These constitutive levels of WUVAC would already provide a notably effective protection against UV-B, and there would be no need to increase them when the mosses were exposed to artificially enhanced (this study) or naturally increasing (Clarke & Robinson, 2008) UV-B levels. However, in the liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, both the bulk level of WUVAC and *p*-coumaric acid from the cell wall were induced by enhanced UV-B (Chapter 5). This would additionally support the different behaviour of

mosses and liverworts regarding their protective mechanisms against UV-B.

The bulk level of SUVAC in both species, together with the concentrations of the two flavonols measured in the soluble fraction of *B. pseudotriquetrum*, were higher in PAB than in P samples. Thus, the compounds in the soluble fraction may be more UV-B-responsive than those in the insoluble one, probably because the former would be more directly connected with the cell metabolism than the latter, that would be more or less immobilized in the cell wall. Nevertheless, only the bulk level of SUVAC and the concentration of kaempferol-3-*O*-glucoside in *B. pseudotriquetrum* increased along the experiment with respect to the initial values, and thus only these variables seemed to be clearly inducible by enhanced UV-B. The bulk level of SUVAC had not responded to enhanced UV-B in previous laboratory studies using both *B. pseudotriquetrum* (Martínez-Abaigar *et al.*, 2009) and *F. antipyretica* (Martínez-Abaigar *et al.*, 2003; Núñez-Olivera *et al.*, 2004). These negative results could have been caused, at least in *B. pseudotriquetrum*, because the samples had been collected near the summer solstice, when their natural protection against UV-B would be sufficient and would not have been increased in the laboratory culture. However, in the present study, the samples were collected in November, a period of the year in which the natural protection of the samples would be lower and could have been increased when exposed to the UV-B levels applied in the experiment. The collection date near the summer solstice could also have been the reason underlying the negative results obtained in one of the previous laboratory studies with *F. antipyretica* (Núñez-Olivera *et al.*, 2004), but not in the other one (Martínez-Abaigar *et al.*, 2003), in which samples were collected in December. The bulk level of SUVAC in *F. antipyretica* did not either respond to naturally fluctuating UV-B under field conditions (Núñez-Olivera *et al.*, 2010), whereas it responded in *B. pseudotriquetrum* (Dunn & Robinson, 2006; Núñez-Olivera *et al.*, 2010), although not always (Clarke & Robinson, 2008). Thus, the bulk level of SUVAC is more UV-B responsive in *B. pseudotriquetrum* than in *F. antipyretica*, both in the laboratory and the field. The lack of response reported in some studies using

these species might be due to an inadequate collection date or other unkown factors whose elucidation needs further investigation. In other mosses tested, the bulk level of SUVAC was UV-B-responsive in *Ceratodon purpureus* (Clarke & Robinson, 2008) but not in *Schistidium antarctici*, *Brachythecium rivulare* or *Racomitrium aciculare* (Clarke & Robinson, 2008; Martínez-Abaigar *et al.*, 2009), while the same variable in liverworts responded to UV-B in most cases (Newsham *et al.*, 2005; Núñez-Olivera *et al.*, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). Therefore, species-specific characteristics and the fact of being a moss or a liverwort notably influence the responsiveness of the bulk level of SUVAC to UV-B in bryophytes.

The two flavonols measured in the soluble fraction of *B. pseudotriquetrum* decreased in P samples from the beginning to the end of the experiment, whereas in PAB samples increased (kaempferol-3-*O*-glucoside) or remained stable (kaempferol-3,7-*O*-diglucoside). The decrease in P samples could be due to the experimental elimination of any UV radiation incident on those plants, which resulted in a relaxation of the UV-induced stress they would have been suffering in the field and a consequent diminution of the concentration of UV-protective compounds. In contrast, the intensification of UV-stress in PAB samples led to the maintenance or increase of the levels of those compounds. It should be noted that kaempferols may act not only as UV absorbers (Dixon & Paiva, 1995), but also as potent antioxidants (Sroka, 2005).

In both species, F_v/F_m was significantly lower in PAB than in P samples. In *F. antipyretica*, this was expected in the light of previous results (Martínez-Abaigar *et al.*, 2003; Núñez-Olivera *et al.*, 2004). However, in a prior study with *B. pseudotriquetrum*, F_v/F_m did not change when the moss was exposed to enhanced UV-B (Martínez-Abaigar *et al.*, 2009). As discussed above, this could happen because the samples for this previous study had been collected near the summer solstice and would be better protected than the samples used in the present study, that were collected in November.

Under a general perspective, it is not surprising that F_v/F_m decreases under enhanced UV-B, because this decrease indicates a stress situation and, in particular, the phenomenon of photoinhibition (Maxwell & Johnson, 2000). Photoinhibition is caused through damage of a well-known target of UV-B radiation: the protein D1 in photosystem II (Jansen *et al.*, 1998). In our study, the decrease in F_v/F_m in PAB samples was modest (7% in *B. pseudotriquetrum* and 13% in *F. antipyretica*). This suggests a dynamic and reversible photoinhibition, and consequently a moderate photosynthetic damage.

DNA damage was much stronger in PAB than in P and PA samples. This is congruent with previous results obtained after exposing *B. pseudotriquetrum* and other mosses and liverworts to enhanced UV-B in both the laboratory (Lud *et al.*, 2002; Otero *et al.*, 2006; Turnbull *et al.*, 2009;) and the field (Lud *et al.*, 2002, 2003). All these results are consistent with the fact that DNA is a specific target of UV-B (Jansen *et al.*, 1998). However, no DNA damage was found when *B. pseudotriquetrum*, *F. antipyretica* or the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* were exposed to ambient UV-B levels under field conditions (Núñez-Olivera *et al.*, 2009, 2010). This suggests that efficient DNA repairing mechanisms occur in nature, and that DNA damage takes place only when bryophytes are exposed to higher than ambient UV-B. To date, only Turnbull & Robinson (2009) have demonstrated DNA damage in bryophytes (the mosses *B. pseudotriquetrum*, *Ceratodon purpureus* and *Schistidium antarctici*) exposed to naturally increased ambient UV-B levels in the field, but it should be taken into account that this study was conducted in the Antarctic, where the extremely harsh environmental conditions might prevent DNA repair.

SI was not affected by enhanced UV-B in any of the species studied. Given that SI may be considered as an indirect indicator of growth, because newly grown shoots are softer (less sclerophyllous) than old shoots (Martínez-Abaigar *et al.*, 2003), no difference in growth between UV-B exposed and non-exposed samples could be established at the end of the

culture period. This contrasts with the increase in SI found in *B. pseudotriquetrum* and *F. antipyretica* exposed to enhanced UV-B in previous laboratory studies, which would mean a lower growth in UV-B exposed samples (Martínez-Abaigar *et al.*, 2003, 2009). No convincing explanation can be offered for this discrepancy, apart from the fact that the specific samples used in this study were tolerant, in terms of growth, to the experimental radiation conditions (UV-B dose, proportions of PAR, UV-A and UV-B, etc.) that were applied.

UV-A radiation did not show specially defined effects in any of the species studied, and the values of the variables (and their temporal trends) in PA samples were frequently intermediate between P and PAB samples (Figs. 2-3). This is in line with the diversity of results previously obtained in bryophytes, since UV-A radiation may have effects similar to PAR (Niemi *et al.*, 2002a; Martínez-Abaigar *et al.*, 2003) or to UV-B (Chapter 5). The lack of specific effects caused by UV-A may be logical taking into account the UV-A doses used in the experiments, that in our case were relatively moderate within the annual range measured in the collection site (Núñez-Olivera *et al.*, 2010). In tracheophytes and algae, UV-A may have significant effects on the induction of phenolics and photoinhibition (Kotilainen *et al.*, 2008; Figueroa *et al.*, 2009; Hakala-Yatkin *et al.*, 2010), and it has been included in the modern action spectra in the context of UV research (Flint & Caldwell, 2003). In bryophytes, the precise effects of UV-A are still poorly known and require further study.

In conclusion, the two mosses studied had noticeably higher bulk levels of WUVAC than SUVAC, which confirms previous results in other mosses (Clarke & Robinson, 2008; Lappalainen, 2010). In addition, enhanced UV-B increased the bulk level of SUVAC in the two species and the concentration of two kaempferols in the soluble fraction of *B. pseudotriquetrum*, but had no effect on the bulk level of WUVAC in the two species and the concentration of *p*-coumaric acid in the insoluble fraction of *F. antipyretica*. Thus, the insoluble fraction would be less UV-B-responsive

than the soluble one, probably because the constitutively high bulk level of WUVAC would already provide a notably effective protection. Also, WUVAC would be less responsive due to their relative immobilization in the cell wall with respect to SUVAC. Taking into accout that soluble and insoluble fractions may provide different UV screening mechanisms (Clarke & Robinson, 2008), it is convenient to analyze separately both the bulk levels and the specific compounds of the two fractions to better evaluate the protection of bryophytes against UV-B radiation. The protective mechanisms developed by the two mosses studied could not totally prevent UV-B damage, that was evident in a modest decrease of F_v/F_m and an increase in the amount of thymine dimers.

Our data suggest that the protection strategies of mosses and liverworts may be different, since liverworts show a higher accumulation of SUVAC than WUVAC, and equally UV-B-responsive SUVAC and WUVAC (Chapter 5). It may be speculated that these differerences have ecological, evolutionary and phylogenetic implications: (1) if WUVAC were more efficient UV screens than SUVAC (Clarke & Robinson, 2008), mosses as a group would be more competitive than liverworts in UV-rich environments; (2) these two different plant evolutionary lineages would have acquired different strategies to cope with higher UV-B levels since the water-to-land transition; and (3) this difference could be an additional evidence of the phylogenetic distance between mosses and liverworts, that nowadays is considered to be deeper than previously thought (Qiu *et al.*, 2007).

CHAPTER 7

DIEL CHANGES IN PHYSIOLOGICAL DAMAGE AND PROTECTION MECHANISMS OF AN AQUATIC LIVERWORT EXPOSED TO ENHANCED UV-B UNDER LABORATORY CONDITIONS

7.1. ABSTRACT

We studied the diel responses of the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* to radiation changes under laboratory conditions. The samples were exposed to three radiation regimes: P (only PAR), PA (PAR + UV-A), and PAB (PAR + UV-A + UV-B). The day was divided in four periods: darkness, a first low-PAR period, the high-PAR plus UV period, and a second low-PAR period. After 15 days of culture, we measured photosynthetic pigments, chlorophyll fluorescence and UV-absorbing compounds in the four periods of the day on two consecutive days. With respect to UV-absorbing compounds, we analyzed their global amount (as the bulk UV absorbance of methanolic extracts) and the concentration of seven hydroxycinnamic acid derivatives, both in the soluble (mainly vacuolar) and insoluble (cell wall-bound) fractions of the plant extracts. PAB samples increased the bulk UV absorbance of the soluble and insoluble fractions, and the concentrations of *p*-coumaroylmalic acid in the soluble fraction and *p*-coumaric acid in the cell wall. Most of these variables showed significant diel changes and responded within a few hours to radiation changes (more strongly to UV-B), increasing at the end of the period of high-PAR plus UV. F_v/F_m, Φ_{PSII}, NPQ and the components of the xanthophyll cycle showed significant and quick diel changes in response to high PAR, UV-A and UV-B radiation, indicating dynamic photoinhibition and protection of PSII from excess radiation through the xanthophyll cycle. Thus, the liverwort showed a dynamic protection and acclimation capacity to the irradiance level and spectral characteristics of the radiation received.

RESUMEN

Se estudiaron las respuestas de la hepática *Jungermannia exsertifolia* subsp. *cordifolia* a los cambios en la radiación a lo largo de ciclos diarios en condiciones de laboratorio. Las muestras se expusieron a tres regímenes de radiación: P (con sólo radiación fotosintéticamente activa, PAR), PA (PAR + UV-A), y PAB (PAR + UV-A + UV-B). El día se dividió en cuatro periodos: oscuridad, un primer periodo de baja PAR, un periodo de alta PAR más radiación UV, y un segundo periodo de baja PAR. Después de 15 días de cultivo, se midieron los pigmentos fotosintéticos, la fluorescencia de clorofilas y los CAUV en los cuatro periodos del día en dos días consecutivos. Con respecto a los CAUV, se analizaron su cantidad total (absorbancia UV global de los extractos metanólicos) y la concentración de siete derivados del ácido hidroxicinámico, y se hicieron ambos análisis en las fracciones soluble (principalmente localizada en las vacuolas) e insoluble (unida a las paredes celulares) de los extractos de la planta. En las muestras PAB aumentó la absorbancia UV global de las fracciones soluble e insoluble, y las concentraciones de los ácidos *p*-cumaroilmálico en la fracción soluble y *p*-cumárico en la insoluble. La mayoría de estas variables mostraron cambios diarios significativos y respondieron en unas pocas horas a los cambios en la radiación, especialmente a la radiación UV-B, aumentando al final del periodo de alta PAR más radiación UV. F_v/F_m, Φ_{PSII}, NPQ y los componentes del ciclo de las xantofilas mostraron cambios diarios rápidos y significativos en respuesta al nivel alto de PAR y a las radiaciones UV-A y UV-B. Esto indicaba una fotoinhibición dinámica, y una protección del PSII frente al exceso de radiación, mediante la activación del ciclo de las xantofilas. Por lo tanto, la hepática mostró una gran dinamicidad en la fotoprotección y una notable capacidad de aclimatación a la radiación recibida, tanto en lo referido a los niveles de esta radiación como a sus características espectrales.

7.2. INTRODUCTION

Ultraviolet (UV) radiation is a significant abiotic factor that elicits diverse acclimation responses in plants, influencing their morphology and physiology. In the last decades, the responses to increased solar UV-B (280–315 nm) radiation in biosphere has received a special interest, due to its direct relationship with the stratospheric ozone reduction (Paul & Gwynn-Jones, 2003). Stratospheric ozone seems to be recovering after the Montreal protocol, but its return to the 1970s values is not expected for decades, and the influence of climate change factors may even cause a further increase of UV-B (McKenzie *et al.*, 2007). Therefore, there remains considerable interest in the study of UV-B effects on photosynthetic organisms. These effects have mainly been studied in terrestrial crops and marine phytoplankton and macroalgae (Caldwell *et al.*, 2007; Häder *et al.*, 2007b). Bryophytes have received much less attention, in spite of being important components of many ecosystems. Comparing the two main evolutionary lineages of bryophytes, liverworts have been notably less studied than mosses in the UV context (Martínez-Abaigar & Núñez-Olivera, 2011). However, liverworts are considered the earliest land plants (Zobell *et al.*, 2010), and thus their responses to UV-B may have evolutionary importance in the water-to-land transition, especially when compared with those found in other differentially evolved phototrophs, such as algae and cormophytes (Jansen *et al.*, 1998; Bischof *et al.*, 2006). In addition, leafy liverworts are structurally simple, since their leaves have only a single layer of cells and lack a nerve. Hence, leafy liverworts lack the structural defences against UV typical of cormophytes, such as hairs, thick cuticles and epidermis. These aspects add interest to the study of UV-B effects on liverworts.

The most frequent responses of cormophytes to UV-B (Jansen *et al.*, 1998) have also been demonstrated in bryophytes in general and in liverworts in particular: chlorophyll degradation, photoinhibition, decrease in photosynthetic rates, DNA and oxidative damage, and accumulation of protective screening compounds (Kato-Noguchi & Kobayashi, 2009; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). Nevertheless, bryophyte responses to UV-B are far from being properly understood, mainly because of the diverse methodologies used.

The responses of bryophytes to UV-B show long-term seasonal changes (Núñez-Olivera *et al.*, 2009; Lappalainen *et al.*, 2010), as well as short-term day-to-day variations (Newsham *et al.*, 2002; Newsham, 2003; Newsham *et al.*, 2005) and diel changes (Green *et al.*, 2000; Conde-Álvarez *et al.*, 2002; Lud *et al.*, 2002, 2003; Green *et al.*, 2005; Lappalainen *et al.*, 2010). All these studies were conducted under field conditions, where the interference of water availability and temperature can mask the responses to UV (Lappalainen *et al.*, 2010). In the specific studies on diel changes, a number of response variables were measured, comprising photosynthetic pigments and performance, DNA damage and UV-absorbing compounds. However, the measurement of UV-absorbing compounds was exclusively based on global variables such as the bulk UV absorbance of the soluble fraction of plant extracts, without considering either the insoluble fraction or the responses of individual compounds. The differentiation between the soluble (mainly vacuolar) and the insoluble (cell wall-bound) fractions may be important because the cell wall-bound compounds would provide a more spatially uniform UV screen than the intracellular compounds (Clarke & Robinson, 2008). In addition, the analysis of individual UV-absorbing compounds represents a useful complement to the global estimation of UV protection provided by the bulk UV absorbance, since each compound may respond to UV in a different manner (Morales *et al.*, 2010). It is also remarkable that there is only one study on the diel changes in the responses to UV radiation in liverworts (Conde-Álvarez *et al.*, 2002).

In this context, we studied the diel changes in the physiological and protective responses to photosynthetic, UV-A and UV-B radiations in the leafy aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*. In particular, higher-than ambient levels of biologically effective UV-B were applied to the samples to simulate the effect of stratospheric ozone reduction. The study was conducted under laboratory conditions to prevent the interference of other factors than radiation. We measured photosynthetic pigments, chlorophyll fluorescence, and UV-absorbing compounds. With respect to the analysis of UV-absorbing compounds, we differentiated the soluble and insoluble fractions of the plant extracts, and, in both fractions, we measured the global amount of UV-absorbing compounds (as the bulk UV absorbance of the extracts) as well as the concentration of seven specific individual compounds (hydroxycinnamic acid derivatives). We hypothesized that the variables measured would respond differently to diel radiation changes, depending on their dynamicity and response specificity to the wavelengths used.

7.3. MATERIALS AND METHODS

Experimental design

Samples of the foliose aquatic liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Vána (hereafter *J. cordifolia*) were collected on 26 January 2009 at the stream Lavieja (1280 m a.s.l., 42º03'00'' N, 02º35'30'' W), in La Rioja (northern Spain). Samples were submerged and exposed to full sun. The material was rinsed with stream water and transported to the laboratory in a portable icebox. Green healthy shoot apices were selected and precultured in a circulating bath system filled with air-bubbled stream water (pH 6.8, conductivity 21 μS cm^{-1}) within a growth chamber. Plants were submerged at a depth of 1-2 cm and were maintained at 10ºC with a 11:13 photoperiod (light:dark) for 5 days. The photosynthetic photon flux density (PFD) was around 100 μmol m^{-2} s^{-1} at the water surface (LI-190SA quantum sensor, LI-COR, Lincoln, NE, USA), and was provided by Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) and True-lite full spectrum (True Sun, Steubenville, OH, USA) fluorescent tubes.

After the preculture, apices were distributed into 15 separate plastic tubes (12 cm of diameter) with a basal net which prevented material losses. Water in the culture was replaced every week. The radiation was additionally supplied by a Hönle SOL 1200RF2 lamp (Dr. Hönle AG UV-Technologie, Gräfelfing/Munich, Germany), which provided both photosynthetically active radiation (PAR) and UV radiation (for spectral characteristics of the lamp, see Gröniger *et al.*, 1999). The day was divided in four different periods: the darkness period, the first two-hour-long low-PAR period (in which only fluorescent tubes were switched on, providing around 100 μmol m^{-2} s^{-1}), the seven-hour-long high-PAR plus UV period (in which both fluorescent tubes and Hönle lamp were switched on, providing around 500 μmol m^{-2} s^{-1} PAR and the UV radiation specified in Table 1), and a second two-hour-long low-PAR period (after switching off the Hönle lamp).

After this last period, the fluorescent tubes were switched off and the darkness period started again. Other culture conditions were similar to those applied in the preculture, and were described in detail in chapter 3. Covering the tubes with specific UV cut-off filters, three radiation regimes were established (in five replicates for each regime):

- P (PAR alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which cut off all UV radiation.

- PA (PAR + UV-A), using Folex 320 (Folex GmbH, Dreieich, Germany), which cut off UV-B and UV-C radiation.

- PAB (PAR + UV-A + UV-B), using Ultraphan 295 (Digefra GmbH, Munich, Germany), which cut off UV-C radiation.

The filters were pre-irradiated and replaced every five days. Table 1 shows the radiation conditions in the three regimes, including the biologically effective UV or UV-B irradiances, which were estimated using classic and modern action spectra. The spectral irradiances were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland), and PAR was measured with a quantum sensor. The plants under the PAB regime received a daily biologically effective UV-B dose of 10.1 kJ m^{-2} (according to the action spectrum of Caldwell, 1971: Table 1). This dose was required to mimic a 20% ozone reduction as calculated with a computer model (Björn & Teramura, 1993), taking into account the maximum biologically effective UV-B dose in the collection site (7.4 kJ m^{-2} d^{-1} following Caldwell, 1971) and the radiation amplification factor of ozone for biologically effective UV-B.

Table 1. Radiation conditions in the three radiation regimes (P, PA, and PAB) under which the bryophyte samples were usually cultivated. UV-B_{BE} (biologically effective UV-B radiation) and UV_{BE} (biologically effective UV radiation) were calculated on the basis of the action spectra specified. UV radiation and high PAR were provided to the plants during the central hours of the day, and this period was preceded and followed by periods of low PAR.

	P	**PA**	**PAB**
PAR in the low-PAR period (μmol m^{-2} s^{-1})	98	96	101
PAR in the low-PAR period (W m^{-2})	18	18	19
PAR in the high-PAR period (μmol m^{-2} s^{-1})	522	512	543
PAR in the high-PAR period (W m^{-2})	97	98	100
PAR dose (kJ m^{-2} d^{-1})	27.04	27.29	27.94
UV-A (W m^{-2})	2.80	32.8	35.4
UV-A dose (kJ m^{-2} d^{-1})	70.6	826.6	892.1
UV-B (W m^{-2})	0.009	0.015	1.085
UV-B dose (kJ m^{-2} d^{-1})	0.23	0.38	27.3
$UV\text{-}B_{BE}$ (W m^{-2}) (Caldwell, 1971)	0.00	0.00	0.40
UV_{BE} (W m^{-2}) (Flint & Caldwell, 2003)	0.02	0.55	0.96

The annual range of unweighted UV-B, UV-A and PAR doses (kJ m^{-2} d^{-1}) in the collection site in the year of measurement (2009), estimated by the measurements taken at Valdezcaray (35 km away from the sampling site: Núñez-Olivera *et al.*, 2009), were respectively 0.68-42.0, 50.6-1512 and 265-13659. The unweighted UV-B, UV-A and PAR doses applied in the PAB regime (Table 1) were within the ambient range, and those of biologically effective UV-B were higher-than-ambient to simulate ozone reduction.

Physiological measurements

After 15 days of treatment, diel variations in different physiological variables were measured on two consecutive days at five different moments of the day: in the darkness, just before dawn; at the end of the first low-PAR period; in the middle and at the end of the high-PAR plus UV period; and at the end of the second low-PAR period (Fig. 1-2).

UV-absorbing compounds were analyzed as described in Chapter 5. In brief, fresh shoot apices were frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany), and then 5 ml of methanol : water : 7M HCl (70:29:1 v/v/v) was added for extraction (24 h at 4ºC in the dark). The extract was centrifuged to differentiate two fractions of UV-absorbing compounds. In the supernatant of the centrifugation, we measured the soluble compounds (SUVAC), presumably mainly located in the vacuole. Subsequently, the pellet was subjected to alkaline digestion to extract the insoluble cell wall-bound compounds (WUVAC). Then, using spectrophotometry (Perkin-Elmer λ35 spectrophotometer, Perkin-Elmer, Wilton, CT, USA), we measured the bulk UV absorbance of the two fractions as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of dry mass (DM: 80ºC for 24 h). Using HPLC (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA), seven hydroxycinnamic acid derivatives were analyzed, five in the soluble fraction and two additional ones in the insoluble fraction. The soluble compounds were: *p*-coumaroylmalic acid, caffeoylmalic acid (or phaselic acid), feruloylmalic acid, 5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid, and 5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid. These will be hereafter referred to as C1 to C5, respectively. The insoluble compounds were *p*-coumaric and ferulic acids, which will be referred to as C6 and C7, respectively.

The maximum quantum yield of PSII, F_v/F_m (after dark adaptation), the effective quantum yield of PSII (Φ_{PSII}: $(F_m' - F_t)/F_m'$), and the non-

photochemical quenching (NPQ: $(F_m-F_m')/F_m'$), were measured with a portable pulse amplitude modulation fluorometer (MINI-PAM, Walz, Effeltrich, Germany), as described in Chapter 5.

Photosynthetic pigments were extracted with 100% acetone after freezing shoot apices in liquid N_2 and grounding them in a TissueLyser. Pigments were analyzed by HPLC (Otero *et al.*, 2006) using an Agilent HP1100 HPLC system and an Agilent photodiode array detector. Total chlorophyll (Chl *a*+*b*), chlorophyll *a*/*b* ratio (Chl *a*/*b*), β-carotene and xanthophylls (lutein, neoxanthin, violaxanthin, antheraxanthin and zeaxanthin), were determined. The Xanthophyll Index was calculated as (Antheraxanthin + Zeaxanthin) / (Violaxanthin + Antheraxanthin + Zeaxanthin).

Statistical analysis

The global effects of the radiation regime and the period of the day on the responses of each physiological variable were tested using a two-way analysis of variance (ANOVA), once proved that the data met the assumptions of normality (Shapiro-Wilks test) and homoscedasticity (Levene test). In the case of significant differences, means were then compared by Tukey's test. If the data did not meet those assumptions, Kruskal-Wallis tests were applied. All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

7.4. RESULTS

The radiation regime significantly affected, on both measurement days, the bulk UV absorbance of the soluble and insoluble fractions, and the concentrations of C1, C6 and C7 (Table 2). Except C7, which showed a less clear trend, all these variables showed significantly higher values in PAB samples than in PA and P samples (Fig. 1). Other compounds (C2 and C3) were significantly affected by the radiation regime only in one of the two measurement days, and their levels were not clearly higher in PAB samples than in PA and P samples (data not shown). Among the rest of the variables, only F_v/F_m was significantly affected by the radiation regime, showing higher values in P samples than in PA and PAB samples (Table 2 and Fig. 2). This occurred in both measurement days.

The period of the day significantly influenced, on both measurement days, the bulk UV absorbance of the soluble and insoluble fractions, C2 concentration, the three chlorophyll fluorescence variables (F_v/F_m, Φ_{PSII} and NPQ), the three components of the xanthophyll cycle (violaxanthin, antheraxanthin and zeaxanthin) and the Xanthophyll Index (Table 2). In the case of the bulk UV absorbances, the highest values were found at the end of the high-PAR plus UV period, and this occurred more generally and strongly in PAB samples than in PA and P samples (Fig. 1). C2 showed very different changes in the two measurement days, with a somewhat erratic behaviour (changes not shown). F_v/F_m showed a relatively modest decrease (2-15%, depending on the radiation regime and the measurement day) during the course of the day, and a final increase in the second low-PAR period (Fig. 2). This pattern was most clear in the second measurement day. Φ_{PSII} showed a comparable pattern to F_v/F_m, but with much steeper initial and final changes (Fig. 2). NPQ, together with zeaxanthin, antheraxanthin and the xanthophyll index, showed higher values in the high-PAR plus UV period than in the low-PAR periods, whereas violaxanthin showed an inverse pattern (Fig. 2). In other variables (concentrations of C3, C6 and C7, Chl *a*+*b* and Chl *a*/*b* ratio), the global effect of the period of the day took

place in only one of the two measurement days, but their diel changes showed no clear trend except in C6 and C7, which changed similarly to the bulk UV absorbances (Fig. 1). In C7, this occurred only in the first measurement day. C1 also showed this pattern, especially in the PAB samples, but its diel changes were not significant (Fig. 1). β-Carotene, lutein and neoxanthin were unaffected by the period of the day. There were no significant interactions between the radiation regime and the period of the day in the two-way ANOVA conducted.

Table 2. Global effects of the radiation regime and period of the day on the variables measured in *Jungermannia exsertifolia* subsp. *cordifolia*, tested either by a 2-way ANOVA or by a Kruskal-Wallis test. Effects are shown for each of the two consecutive measurement days. ***P<0.001, **P<0.01, *P<0.05, NS non-significant.

	Measurement day 1		Measurement day 2	
	Rad. Reg.	**Period**	**Rad. Reg.**	**Period**
Bulk level of SUVAC	***	**	***	***
Bulk level of WUVAC	***	***	***	*
C1	***	NS	***	NS
C2	**	**	NS	*
C3	NS	NS	***	*
C4	NS	NS	NS	NS
C5	NS	NS	NS	NS
C6	***	**	***	NS
C7	*	***	**	NS
F_v/F_m	***	**	***	***
Φ_{PSII}	NS	***	NS	***
NPQ	NS	***	NS	***
Chl *a*+*b*	NS	*	NS	NS
Chl *a*/*b* ratio	NS	*	NS	NS
β-carotene	NS	NS	NS	NS
Lutein	NS	NS	NS	NS
Neoxanthin	NS	NS	NS	NS
Violaxanthin	NS	***	NS	***
Antheraxanthin	NS	**	NS	*
Zeaxanthin	NS	**	NS	***
(A+Z)/(V+A+Z)	NS	***	NS	***

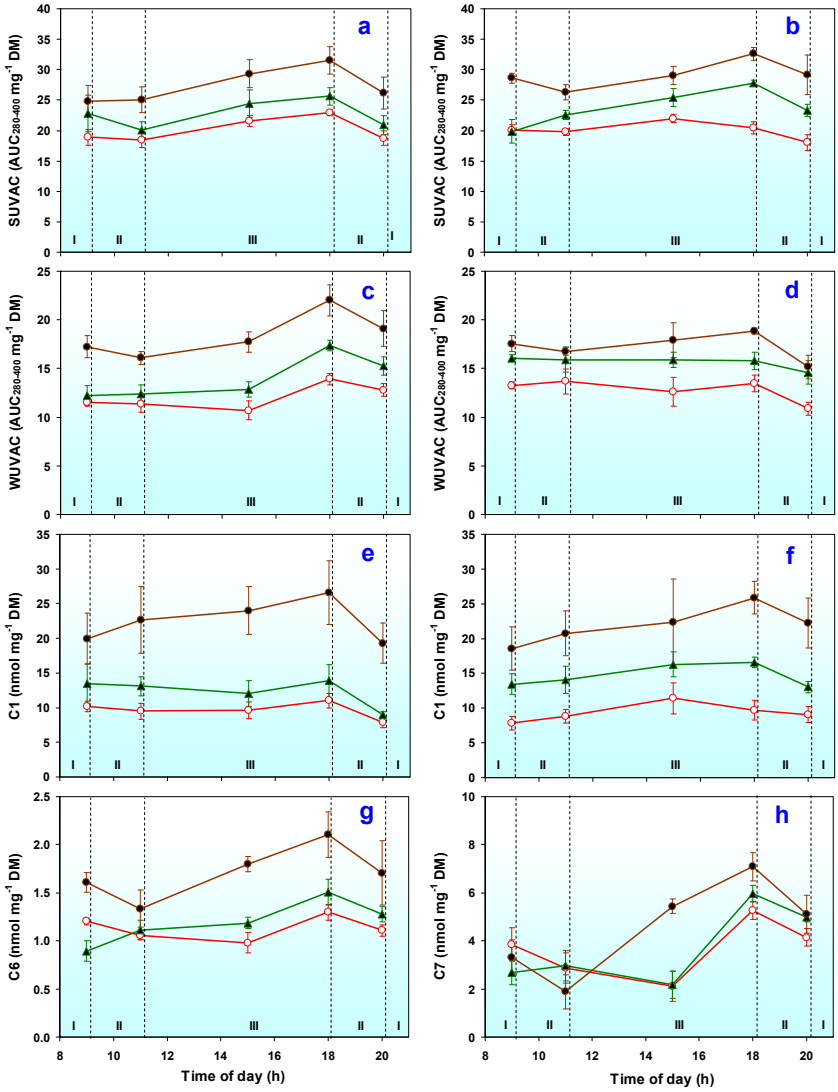

Fig. 1. Diel changes in different variables of *Jungermannia exsertifolia* subsp. *cordifolia*, measured on two consecutive days after 15 days of exposure to three different radiation regimes: P (only PAR, red line), PA (PAR + UV-A, green line), and PAB (PAR + UV-A + UV-B, brown line). The variables shown are: the bulk levels of methanol-soluble UV-absorbing compounds (SUVAC), measured on the first (a) and second (b) day of measurement; the bulk levels of methanol-insoluble UV-absorbing compounds (WUVAC), measured on the first (c) and second (d) day of measurement; the concentrations of C1 measured on the first (e) and second (f) day of measurement; and the concentrations of C6 (g) and C7 (h), measured on the first day of measurement. Means ± SE are shown. I to III, periods in which the day was divided: I, darkness; II, low PAR; III, high-PAR plus UV.

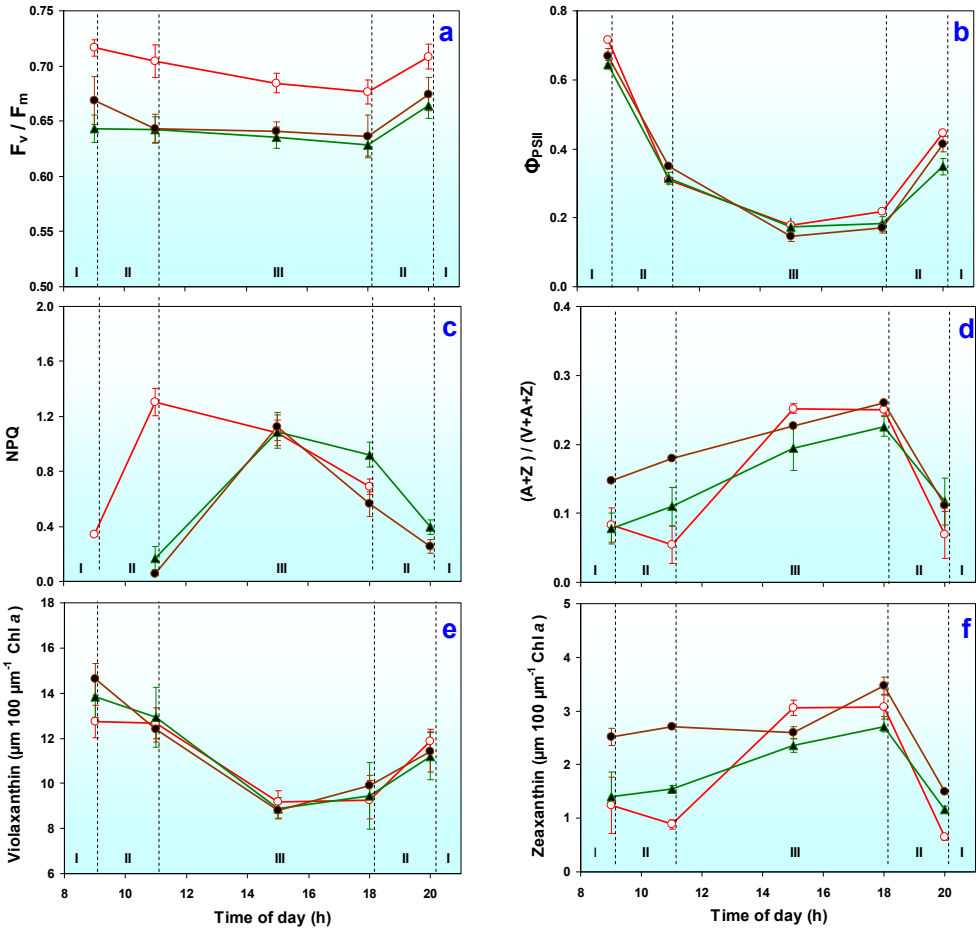

Fig. 2. Diel changes in F_v/F_m (a), Φ_{PSII} (b), NPQ (c), the Xanthophyll Index (A+Z)/(V+A+Z) (d), and the concentrations of violaxanthin (e) and zeaxanthin (f), analyzed in *Jungermannia exsertifolia* subsp. *cordifolia* on the first day of measurement after 15 days of exposure to three different radiation regimes: P (only PAR, red line), PA (PAR + UV-A, green line), and PAB (PAR + UV-A + UV-B, brown line). Means ± SE are shown. I to III, periods in which the day was divided: I, darkness; II, low PAR; III, high-PAR plus UV.

7.5. DISCUSSION

The clear increase in the bulk UV absorbance of both soluble and insoluble fractions that took place in the PAB samples, together with the increase in the concentrations of C1 and C6, corroborates the fidelity of these variables in the response of *J. cordifolia* to enhanced UV-B under laboratory conditions (Chapter 5). C1 and/or C6 have also been used in the reconstruction of past UV-B (Blokker *et al.*, 2006; Lomax *et al.*, 2008; Otero *et al.*, 2009) and as field biomarkers of present UV-B (Ruhland *et al.*, 2005; Núñez-Olivera *et al.*, 2009) in different plant species including *J. cordifolia*. Thus, these compounds could be useful in the biomonitorization of the UV-B enhancement derived from stratospheric ozone reduction.

Most of the variables that clearly increased under enhanced UV-B showed significant diel changes. In particular, the bulk UV absorbance of the soluble and insoluble fractions, together with C6 and C7 concentrations, increased at the end of the high-PAR plus UV period (Fig. 1). This occurred, especially, in the samples exposed to enhanced UV-B. Thus, the bulk UV absorbances and certain specific UV-absorbing compounds can react quickly, within a few hours, to UV-B changes. Apart from UV-B, high PAR and UV-A (P and PA regimes) also occasionally caused an increase in the bulk UV absorbance of the soluble and insoluble fractions, and the concentration of certain compounds (C6 and C7), in the course of the day. Nevertheless, the responses to UV-B were always stronger than the responses to high PAR or UV-A. Thus, although not completely specific to UV-B, the diel changes found in our study were mainly triggered by UV-B.

Quick changes similar to those found in our study have previously been reported in the bulk UV absorbance of the soluble fraction of bryophytes. The time taken for this variable to increase in response to episodes of ozone depletion and concomitant UV-B increase was less than 24 h (even 5.5 h) in the liverwort *Cephaloziella varians* and the mosses *Sanionia uncinata* and *Andreaea regularis* growing in Antarctica (Newsham *et al.*,

2002; Newsham, 2003; Newsham *et al.*, 2005). In addition, the UV absorbance of the moss *Polytrichum juniperinum* in the Arctic responded within hours to changes in either artificially enhanced UV-B or ambient radiation (Lappalainen *et al.*, 2010). However, no clear daily pattern in variables related to UV-absorbing compounds was shown by these species or the Mediterranean thalloid liverwort *Riella helicophylla* exposed to full solar spectrum (Conde-Álvarez *et al.*, 2002). All these studies were conducted under field conditions, where diel variations in UV-absorbing compounds in response to radiation changes may not be easy to detect, probably because other environmental factors (temperature, water availability) obscure the radiation effects. Therefore, it would be interesting to confirm that the diel changes detected in *J. cordifolia* under laboratory conditions occur also in the field.

C1 did not show significant diel changes but an increasing trend in the course of the day, until the end of the high-PAR plus UV period. We expected that the diel changes in C1 would be more clear, because this is the compound that best responded to enhanced UV-B in previous studies with *J. cordifolia* (Otero *et al.*, 2006; Núñez-Olivera *et al.*, 2009). However, it should be taken into account that C1 reached its highest values precisely after 15 days of culture under enhanced UV-B (15-20 nmol mg^{-1} DM: Chapter 5). Thus, in the present experiment, C1 would be around its maximum concentration in the days of measurement, and this fact could have limited its diel variations. The diel changes of the compounds that did not respond clearly to UV-B radiation, such as C2, C3, C4 and C5, were mostly subtle or, in certain cases, somewhat erratic. This was not surprising because these compounds did not respond to enhanced UV-B in previous studies (Otero *et al.*, 2006), so that their function in *J. cordifolia* may not be related to UV protection.

Only F_v/F_m was significantly affected by the radiation regime, and was lower in PA and PAB samples than in P samples. These lower values could be due to a stronger photoinhibition not counteracted by the intensification of the energy dissipation mechanisms, given that the xanthophyll cycle and NPQ were not affected by the radiation regime (Table

2, Fig. 2). In *J. cordifolia*, the decrease in F_v/F_m is a usual response to enhanced UV-B (Otero *et al.*, 2006; Martínez-Abaigar & Núñez-Olivera, 2011), having also been found in the thalloid aquatic liverwort *Riella helicophylla* and the terrestrial moss *Sanionia uncinata* (Montiel *et al.*, 1999; Conde-Álvarez *et al.*, 2002). In contrast, UV radiation had no effect on F_v/F_m in other terrestrial bryophytes, such as the leafy liverwort *Cephaloziella varians* (Newsham *et al.*, 2002) and the mosses *Bryum argenteum* (Green *et al.*, 2000) and, again, *Sanionia uncinata* (Lud *et al.*, 2002; Newsham *et al.*, 2002). Differences in the species and experimental conditions used (laboratory or field experiments, UV-B doses, presence of UV-A, etc.) may help explain these different results.

Φ_{PSII}, NPQ, and photosynthetic pigments were not influenced by UV-A or enhanced UV-B (PA and PAB regimes) in *J. cordifolia*, which is congruent with some results obtained in bryophytes (Lud *et al.*, 2002; Newsham *et al.*, 2002; Newsham, 2003; Otero *et al.*, 2006), but not with other findings. For example, enhanced UV-B caused a decrease in Φ_{PSII}, lutein and zeaxanthin, and increases in total carotenoids, violaxanthin and neoxanthin, in several bryophytes (Newsham *et al.*, 2002; Newsham, 2003; Lud *et al.*, 2003). Again, the use of different species and experimental conditions may justify the discrepancies in the results.

While the radiation regime only affected F_v/F_m, the diel radiation variations resulted in significant and quick changes in a number of variables of *J. cordifolia* (Table 2, Fig. 2). The decrease of F_v/F_m and Φ_{PSII} in the central hours of the day (under high-PAR plus UV), and their recovery in the subsequent low-PAR period, would be indicative of dynamic photoinhibition and loss of photosynthetic efficiency of PSII. These patterns were apparently mainly due to the variations in PAR, and were related to an increased dissipation of excessively absorbed energy by thermal radiation, evidenced by the midday increase in antheraxanthin, zeaxanthin and the Xanthophyll Index, with a concomitant decrease in violaxanthin. The midday increase in NPQ would be also indicative of energy dissipation,

either by the xanthophyll cycle or alternative mechanisms (Maxwell & Johnson, 2000). All these processes would protect PSII from excess radiation. These diel changes were mostly congruent with those found in other bryophytes (Green *et al.*, 2000; Conde-Álvarez *et al.*, 2002; Lud *et al.*, 2002, 2003) and cormophytes (Veit *et al.*, 1996).

In conclusion, *J. cordifolia* was able to react quickly to changes in radiation under laboratory conditions. Protective responses against enhanced UV-B included the increase in the bulk UV absorbance of the soluble and insoluble fractions, and in the concentrations of C1 (*p*-coumaroylmalic acid) in the soluble fraction and C6 (*p*-coumaric acid) in the cell wall. Most of these variables showed significant diel changes and responded within a few hours to radiation variations (especially in UV-B), increasing at the end of the high-PAR plus UV period. In this sense, our study is the first in which clear diel changes in UV-absorbing compounds are found in bryophytes, both in the bulk UV absorbances and in specific compounds, and the first in which diel variations in hydroxycinnamic acid derivatives are studied in plants in relation to changes in UV radiation. F_v/F_m, Φ_{PSII}, NPQ and the components of the xanthophyll cycle showed significant diel changes in response, mainly, to high PAR. These changes were quick and complementary, indicating dynamic photoinhibition and protection of PSII from excess radiation through the xanthophyll cycle. Thus, *J. cordifolia* showed a dynamic protection and acclimation capacity to the levels and spectral characteristics of the radiation used in the present study. It may be worth mentioning that the mechanisms responsible for this capacity have also been found in both more primitive and evolved photosynthetic organisms: algae (Bischof *et al.*, 2006) and cormophytes (Jansen *et al.*, 1998).

CHAPTER 8

DIEL CHANGES IN DNA DAMAGE OF AN AQUATIC LIVERWORT EXPOSED TO ENHANCED UV-B UNDER LABORATORY CONDITIONS

8.1. ABSTRACT

We studied, under laboratory conditions, the diel variations of DNA damage in response to changes in ultraviolet (UV) radiation in the liverwort *Jungermannia exsertifolia* subsp. *cordifolia*. The samples were exposed to three radiation regimes: P (only photosynthetically active radiation, PAR), PA (PAR + UV-A), and PAB (PAR + UV-A + UV-B). The day was divided in four periods: darkness, a first low-PAR period, a high-PAR plus UV period, and a second low-PAR period. After 15 days of culture, we measured the amount of thymine dimers in the four periods of the day on two consecutive days. DNA damage was significantly stronger in PAB than in P and PA samples, which corroborates the fact that it is a good and considerably specific biomarker of artificially enhanced UV-B. The diel changes in DNA damage consisted of a significant increase in thymine dimers at the end of the period of high-PAR plus UV, followed by a quick and complete repair after UV cessation and the instauration of low-PAR. These diel changes occurred much more clearly in PAB samples than in PA and P samples, and thus the differences in DNA damage were mainly due to UV-B. The low amount of thymine dimers produced and the rapid repair indicated that the UV-B levels applied caused little DNA damage in this liverwort, which is well adapted to those UV-B levels. This is the first time in which diel variations in DNA damage in response to artificial changes in UV-B are detected in a liverwort.

RESUMEN

Se han estudiado, en condiciones de laboratorio, los daños producidos en el ADN de la hepática *Jungermannia exsertifolia* subsp. *cordifolia* por exposición a la radiación ultravioleta (UV) a lo largo de ciclos diarios artificiales de 24 horas. Las muestras se expusieron a tres regímenes de radiación: P (con sólo radiación fotosintéticamente activa, PAR), PA (PAR + UV-A), y PAB (PAR + UV-A + UV-B). El día se dividió en cuatro periodos: oscuridad, un primer periodo de baja PAR, un periodo de alta PAR más radiación UV, y un segundo periodo de baja PAR. Después de 15 días de cultivo, se midió la cantidad de dímeros de timina en los cuatro periodos del día en dos días consecutivos. Los daños en el ADN fueron significativamente mayores en las muestras PAB que en las P y PA, lo que confirma que es un biomarcador bueno y considerablemente específico del aumento artificial de la radiación UV-B. Con respecto a los cambios en el ciclo diario, el número de dímeros de timina aumentó significativamente al final del periodo de alta PAR más radiación UV, y posteriormente se produjo una reparación rápida y completa de los daños tras el apagado de la radiación UV y la instauración del segundo periodo de baja PAR. Estos cambios fueron mucho más claros en las muestras PAB que en las P y PA, por lo que las diferencias en los daños al ADN se debieron principalmente a la radiación UV-B. La baja cantidad de dímeros de timina producidos y la rapidez de la reparación indican que los niveles de radiación UV-B aplicados provocaban pocos daños en el ADN de esta especie, que por lo tanto está bien adaptada a dichos niveles. Ésta es la primera vez que se detectan variaciones en los daños al ADN de una hepática en respuesta a cambios artificiales de UV-B a lo largo del ciclo diario.

8.2. INTRODUCTION

Ultraviolet (UV) radiation is a minority part of solar radiation, but it has important biological effects on the morphology and physiology of photosynthetic organisms, including bryophytes (Jansen *et al.*, 1998; Boelen *et al.*, 2006; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). UV consists of three wavelength bands: the most damaging UV-C (<280 nm), which is completely absorbed by stratospheric oxygen and ozone; UV-B (280-315 nm), which is partially absorbed by stratospheric ozone; and UV-A (315-400 nm), which is not absorbed by ozone. The amount of solar UV-B radiation reaching the ground has increased due to the anthropogenic ozone reduction. At northern mid-latitudes, ozone loss has been estimated around 6% in the last 30 years, which might have resulted in a UV-B increase of up to 12% (McKenzie *et al.*, 2003). The future trend is uncertain, in particular due to the influence of climate change (McKenzie *et al.*, 2007). In addition, ozone mini-holes may cause up to 33% decreases in ozone and transitory UV-B increases (43-75%: Antón *et al.*, 2007). Thus, the study of UV-B effects on organisms will be an important issue in the coming several decades. In photosynthetic organisms, UV-B can damage DNA and the photosynthetic apparatus. This damage may be counteracted by protective mechanisms, such as DNA repariring, antioxidant systems and the accumulation of UV-absorbing compounds (Jansen et *al*, 1998).

DNA is one of the key molecular targets of UV-B radiation, given that DNA bases absorb UV-B. This may lead to the formation of different photoproducts, mainly affecting the pyrimidine constituents: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (Ravanat *et al.*, 2001). The ratio between CPDs and (6-4) photoproducts is around 3, and thymine dimer is the major photoproduct, with a yield of formation 10-fold higher than that of the corresponding (6-4) adduct. DNA alterations are mutagenic and cytotoxic, and disrupt cellular metabolism since transcription and replication are blocked. This may affect growth.

Table 1. Original experiments on the effects of UV radiation on DNA damage in bryophytes. The species used and their geographic provenance are indicated, together with the characteristics of the experiments performed and the results obtained.

Reference	Species used and geographic provenance[1]	Type of experiment[2]	Manipulation of radiation[3]	UV-B level[4]	Duration	Results obtained[5]
Boelen *et al.* (2006)	*Chorisodontium aciphyllum* (M An), *Polytrichum strictum* (M An), *Sanionia uncinata* (M An), *Warnstorfia sarmentosa* (M An)	F	S	0.076 W m^{-2} ([a])	3 weeks	=
This Thesis (Chapter 5)	*Jungermannia exsertifolia* subsp. *cordifolia* (L Ml)	L	S	8.6 kJ m^{-2} d^{-1} ([a])	31 days	+
This Thesis (Chapter 6)	*Bryum pseudotriquetrum* (M Ml), *Fontinalis antipyretica* (M Ml)	L	S	8.6 kJ m^{-2} d^{-1} ([a])	31 days	+
This Thesis (Chapter 8)	*Jungermannia exsertifolia* subsp. *cordifolia* (L Ml)	L	S	8.6 kJ m^{-2} d^{-1} ([a])	17 days	+
Lud *et al.* (2002)	*Sanionia uncinata* (M An Ar)	F	S	8.4 kJ m^{-2} d^{-1} ([a])	1-2 days	+
Lud *et al.* (2002)	*Sanionia uncinata* (M An Ar)	F	E	0	1-2 days	-
Lud *et al.* (2002)	*Sanionia uncinata* (M An Ar)	L	S	5.3 kJ m^{-2} d^{-1} ([a])	1 hour	+
Lud *et al.* (2003)	*Sanionia uncinata* (M An)	F	S	8.7 kJ m^{-2} d^{-1} ([a])	10 hours	+
Lud *et al.* (2003)	*Sanionia uncinata* (M An)	F	N	0.07-0.12 W m^{-2} ([b])	16-21 days	-
Núñez-Olivera *et al.* (2009)	*Jungermannia exsertifolia* subsp. *cordifolia* (L Ml)	F	N	3.3-34.3 kJ m^{-2} d^{-1} ([c])	3 years	-
Núñez-Olivera *et al.* (2010)	*Bryum pseudotriquetrum* (M Ml), *Fontinalis antipyretica* (M Ml)	F	N	3.3-34.3 kJ m^{-2} d^{-1} ([c])	3 years	-

Reference	Species used and geographic provenance[1]	Type of experiment[2]	Manipulation of radiation[3]	UV-B level[4]	Duration	Results obtained[5]
Otero *et al.* (2006)	*Jungermannia exsertifolia* subsp. *cordifolia* (L Ml)	L	S	8.6 kJ m^{-2} d^{-1} ([a])	15 days	+
Turnbull & Robinson (2009)	*Bryum pseudotriquetrum* (M An), *Ceratodon purpureus* (M An), *Schistidium antarctici* (M An)	F	N	0-30 kJ m^{-2} d^{-1} ([c])	3 months	(+)
Turnbull *et al.* (2009)	*Bryum pseudotriquetrum* (M An), *Ceratodon purpureus* (M An), *Schistidium antarctici* (M An)	L	S	12 kJ m^{-2} d^{-1} ([b])	4 hours	+

[1] L, liverwort; M, moss; An, antarctic; Ar, Arctic; Ml, mid-latitudes.

[2] F, field experiment; L, laboratory experiment.

[3] E, exclusion of UV-B radiation; N, samples exposed to natural UV-B levels; S, supplement of UV-B radiation.

[4] The highest UV-B doses to which samples were exposed or, in the case of exposures to natural UV-B levels, the UV-B range (doses or irradiances) experienced by the samples through the experimental period.

[5] Results: +, increased DNA damage in UV-B-exposed with respect to control samples; =, no difference between UV-B-exposed and control samples; -, DNA damage not detected; (+), positive association between CPDs accumulation and natural UV-B levels (in two out of the three species studied).

[a] Biologically effective UV-B ($UV\text{-}B_{BE}$), weighted using the DNA damage spectrum (Setlow, 1974).

[b] Biologically effective UV-B ($UV\text{-}B_{BE}$), weighted using the generalized plant damage action spectrum (Caldwell, 1971).

[c] Unweighted UV-B.

UV-A wavelengths are not absorbed by native DNA and thus are less efficient than UV-B in inducing DNA damage, although they can produce a certain damage through indirect photosensitizing reactions (Sinha & Häder, 2002). UV-A photooxidation damage mostly involves the guanine residues of cellular DNA and, when affects pyrimidine bases, it does not lead to the formation of dimers (Ravanat *et al.*, 2001). Even visible light photons up to 700 nm are able to generate singlet oxygen and thus trigger photosensitization reactions that may damage DNA (Häder & Sinha, 2005).

Cells have developed a number of repair or tolerance mechanisms to counteract DNA damage, mainly photoreactivation with photolyase and excision repair with glycosylases and polymerases (Sinha & Häder, 2002; Britt, 2004; Häder & Sinha, 2005). Photolyase specifically binds to CPDs or 6-4 photoproducts and reverses the damage using very efficiently the energy of UV-A (around 380 nm) or blue (around 440 nm) radiation, and splitting approximately one dimer for every photon absorbed. Thus, UV-A and blue radiations can ameliorate UV-B-induced DNA damage, although photoreactivation is apparently saturated at relatively low photon levels (Krizek, 2004). Photolyase may also act in the dark, binding to the pyrimidine dimer and stimulating the nucleotide excision repair system. This system is a part of the other main repair modality, the excision repair (dark repair pathway), that does not directly reverse DNA damage (as photolyases do) but instead replaces the damaged DNA with new nucleotides, with the aid of glycosylases and polymerases (Sinha & Häder, 2002).

The effects of UV-B on bryophytes have been recently reviewed (Boelen *et al.*, 2006; Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). Among the (around) 80 papers published on this topic, DNA damage has been measured only in 10 of them previously to the present study (Table 1). Nine species have been studied, eight mosses and only one liverwort (*Jungermannia exsertifolia* subsp. *cordifolia*). Both laboratory and field studies were conducted, almost in the same proportions. Most

experiments (eight) used artificially enhanced UV radiation, whereas four used natural ambient levels and only one solar radiation deprived of UV-B (exclusion-type experiment). The duration of the experiments was diverse, between 1 hour and 3 years of UV exposure; field studies were longer than laboratory ones. Five papers dealt with Antarctic mosses, one of which included also Arctic mosses (Lud *et al.*, 2002), whereas the remaining five papers used bryophytes from mountain streams at mid-latitudes. In all the experiments, DNA damage was measured in terms of the amount of CPDs, and the amount of (6-4) photoproducts was also measured in one experiment (Turnbull *et al.*, 2009).

In general, DNA damage has been demonstrated in bryophytes exposed to artificially enhanced UV-B radiation under laboratory and field conditions (Table 1), in line with the target character of DNA with respect to UV-B (Jansen *et al.*, 1998). In contrast with this, exposure to natural ambient UV-B levels did not result in DNA damage, probably because of efficient protection and repairing mechanisms in bryophytes acclimated to their specific environmental conditions. The only study in which DNA damage was found in samples exposed to ambient UV-B was performed in Antarctica (Turnbull & Robinson, 2009), where repair mechanisms may be severely limited by low temperature. In another field Antarctic study, Boelen *et al.* (2006) did not find any DNA damage in four mosses exposed to artificially enhanced UV-B, in spite of the harsh environmental conditions for repair. This makes an exception to the general rule of finding damage in mosses exposed to enhanced UV-B. In the only exclusion experiment, no CPDs could be detected in samples of the moss *Sanionia uncinata* when exposed to solar radiation deprived of UV-B (Lud *et al.*, 2002).

Other remarkable results regarding DNA damage in bryophytes are that desiccation may protect mosses from this damage, at least in some species (Turnbull *et al.*, 2009), and that the presence of cadmium intensified the damage specifically caused by UV-B radiation, probably because it would impair the enzymatic repair mechanisms of DNA (Otero *et al.*, 2006).

Diverse responses of bryophytes to UV-B may show changes along the day (Chapter 7). To our knowledge, only Lud *et al.* (2002, 2003) have studied changes in DNA damage along the day in bryophytes, while Lud *et al.* (2001) have considered this issue in algae. This kind of studies may serve to evaluate the quickness of the responses of photosynthetic organisms to UV-B. In bryophytes, daily variations in DNA damage have been assessed only in the moss *Sanionia uncinata*, whereas no liverwort has been studied to date in this respect. This point can be important because liverworts and mosses, the two main evolutionary groups of bryophytes, seem to have different protection mechanisms against UV-B radiation (Chapters 5 and 7). In addition, the responses of DNA damage to UV-B radiation in liverworts may be evolutionary important to better understand the acclimation of plants to a UV-B increase in the water-to-land transition, given that liverworts are now considered the earliest diverging land plants (Zobell *et al.*, 2010).

In this context, we studied the diel changes in DNA damage (in terms of CPDs production) in response to photosynthetic, UV-A and UV-B radiations in the leafy aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*. This is the first time in which this issue is studied in a liverwort. The diel changes in our study were evaluated, for the first time in bryophytes, under controlled conditions, because previous studies on diel changes in this type of plants (Lud *et al.*, 2002, 2003) were conducted under field conditions, where other factors than radiation, such as temperature or desiccation, may mask the plant responses to radiation.

8.3. MATERIALS AND METHODS

Samples of the foliose aquatic liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Vána (hereafter *J. cordifolia*) were collected on 26 January 2009 at the stream Lavieja (1280 m a.s.l., 42º03'00'' N, 02º35'30'' W), in La Rioja (northern Spain). Samples were submerged and exposed to full sun. The material was rinsed with stream water and transported to the laboratory in a portable icebox. Green healthy shoot apices were selected and precultured in a circulating bath system filled with air-bubbled stream water (pH 6.8, conductivity 21 μS cm^{-1}) within a growth chamber. Plants were submerged at a depth of 1-2 cm and were maintained at 10ºC with a 11:13 photoperiod (light:dark) for 5 days. The photosynthetic photon flux density (PFD) was around 100 μmol m^{-2} s^{-1} at the water surface (LI-190SA quantum sensor, LI-COR, Lincoln, NE, USA), and was provided by Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) and True-lite full spectrum (True Sun, Steubenville, OH, USA) fluorescent tubes.

After the preculture, apices were distributed into 15 separate plastic tubes (12 cm of diameter) with a basal net which prevented material losses. The radiation was additionally supplied by a Hönle SOL 1200RF2 lamp (Dr. Hönle AG UV-Technologie, Gräfelfing/Munich, Germany), which provided both photosynthetically active radiation (PAR) and UV radiation (for spectral characteristics of the lamp, see Gröniger *et al.*, 1999). The day was divided in four different periods: a darkness period, a first 2-hour-long low-PAR period (in which only fluorescent tubes were switched on, providing around 100 μmol m^{-2} s^{-1}), a 7-hour-long high-PAR plus UV period (in which both fluorescent tubes and Hönle lamp were switched on, providing around 500 μmol m^{-2} s^{-1} PAR and the UV radiation specified in Table 2), and a second 2-hour-long low-PAR period (after switching off the Hönle lamp). After this last period, the fluorescent tubes were switched off and the darkness period started again. Other culture conditions were as those in the preculture (see Chapter 3). Covering the tubes with specific UV cut-off filters, 3 radiation regimes were established (in 5 replicates for each regime):

- P (PAR alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which cut off all UV radiation.

- PA (PAR + UV-A), using Folex 320 (Folex GmbH, Dreieich, Germany), which cut off UV-B and UV-C radiation.

- PAB (PAR + UV-A + UV-B), using Ultraphan 295 (Digefra GmbH, Munich, Germany), which cut off UV-C radiation.

The filters were pre-irradiated and replaced every 5 days. Table 2 shows the radiation conditions in the three regimes, including the biologically effective UV or UV-B irradiances, which were estimated using classic and modern action spectra: the DNA damage spectrum (Setlow, 1974), which would be the most representative spectrum in the context of the present study, the generalized plant damage action spectrum normalized at 300 nm (Caldwell, 1971), and that of Flint & Caldwell (2003). The spectral irradiances were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland), and PAR was measured with a quantum sensor. The plants under the PAB regime received a daily UV-B dose of 27.4 kJ m^{-2}, which was required to mimic a 20% ozone reduction as calculated with a computer model (Björn & Teramura, 1993). Water in the culture was replaced every week.

Table 2. Radiation conditions in the three radiation regimes (P, PA, and PAB) under which the samples were cultivated. UV-B_{BE} (biologically effective UV-B radiation) and UV_{BE} (biologically effective UV radiation) were calculated on the basis of the action spectra specified. UV radiation and high PAR were provided to the plants during the 7 central hours of the day, and this period was preceded and followed by 2-hour-long periods of low PAR.

	P	**PA**	**PAB**
PAR in the low-PAR period (μmol m^{-2} s^{-1})	98	96	101
PAR in the low-PAR period (W m^{-2})	18	18	19
PAR in the high-PAR period (μmol m^{-2} s^{-1})	522	512	543
PAR in the high-PAR period (W m^{-2})	97	98	100
PAR dose (kJ m^{-2} d^{-1})	270	272	279
UV-A (W m^{-2})	2.80	32.8	892.1
UV-A dose (kJ m^{-2} d^{-1})	70.6	826.6	892.1
UV-B (W m^{-2})	0.009	0.015	1.085
UV-B dose (kJ m^{-2} d^{-1})	0.23	0.38	27.3
UV-B_{BE} (W m^{-2}) (Setlow, 1974)	0.003	0.002	0.341
UV-B_{BE} (W m^{-2}) (Caldwell, 1971)	0.003	0.001	0.402
UV_{BE} (W m^{-2}) (Flint & Caldwell, 2003)	0.016	0.553	0.965

After 15 days of treatment, diel variations in DNA damage were measured on two consecutive days at five different moments of the day: in the darkness, just before dawn; at the end of the first low-PAR period; in the middle and at the end of the high-PAR plus UV period; and at the end of the second low-PAR period. DNA damage was evaluated by detection of thymine dimers (Otero *et al.*, 2006; Sinha *et al.*, 2001). In order to establish a standard with known thymine dimer frequency, the plasmid pBSK (obtained from Prof. D.-P. Häder, Erlangen, Germany) was used and its DNA was isolated (QIAprep Spin Kit, Qiagen, Hilden, Germany). After quantification, plasmid DNA was irradiated for 1 h with UV-C radiation (254 nm) capable of inducing all possible thymine dimers. Liverwort samples were collected from the laboratory culture and were immediately frozen in liquid nitrogen and stored in a freezer at -80°C until analysis. Samples were ground using Tissue Lyser (Qiagen, Hilden, Germany) and DNA was isolated using a standard phenol:chloroform:isoamyl extraction routine. Once the DNA of both the liverwort samples and the irradiated plasmid had been obtained, it was fixed to a Hybond-N^+ membrane (Amersham Biosciences, GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK), which was blocked and incubated with a primary antibody (anti-thymine dimer TDM-2, obtained from Prof. Osamu Nikaido, Kanazawa University, Japan). Afterwards, the membrane was incubated with the secondary antibody (antimouse-IgG) using the ECL western blotting system (Amersham Biosciences). Thymine dimers were detected and quantified by chemiluminiscence using the ChemiGenius Bio Imaging System and associated software (Syngene, Cambridge, UK).

The global effects of the radiation regime and the period of the day on the amount of thymine dimers were tested using Kruskal-Wallis tests since the data did not meet the assumption of homoscedasticity (Levene test). In the case of significant differences, means were then compared by Mann-Whitney test. All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

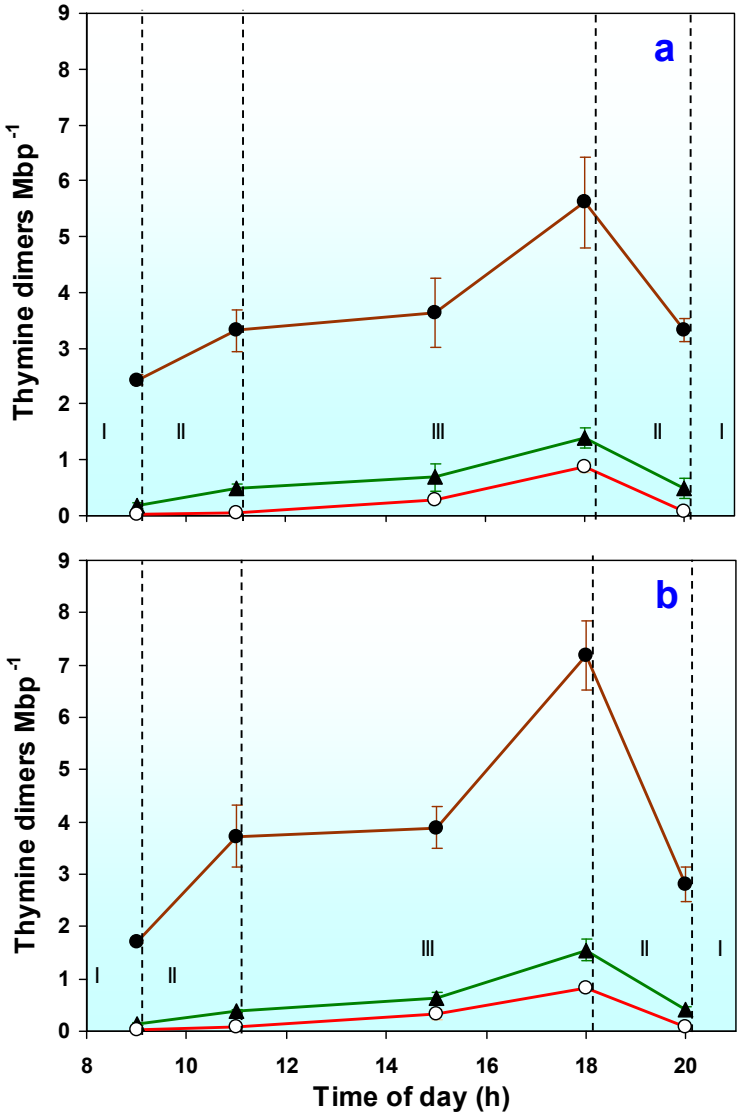

Fig. 1. Diel changes in DNA damage of *Jungermannia exsertifolia* subsp. *cordifolia*, in terms of the amount of thymine dimers, measured on two consecutive days (a and b, first and second days of measurement, respectively) after 15 days of exposure to three different radiation regimes: P (only PAR, red line), PA (PAR + UV-A, green line), and PAB (PAR + UV-A + UV-B, brown line). Means ± SE are shown. I to III, periods in which the day was divided: I, darkness; II, low PAR; III, high-PAR plus UV.

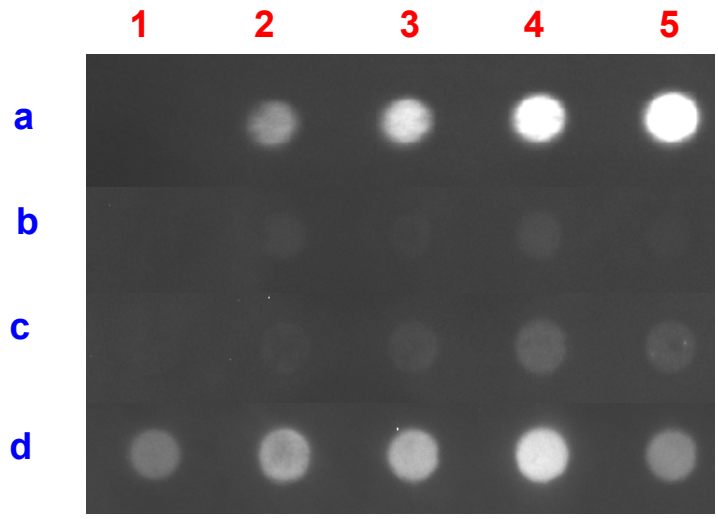

Fig. 2. Dot blot of DNA from plasmid pBSK and from the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* showing DNA damage (presence of thymine dimers) on the second day of measurement after 15 days of exposure to three different radiation regimes: P (only PAR), PA (PAR + UV-A), and PAB (PAR + UV-A + UV-B). Lane a (columns 1-5) corresponds to increasing concentrations (0, 0.25, 0.5, 1 and 2 ng) of plasmid DNA irradiated with UV-C (calibration lane). Lanes b, c and d represent samples of 1-µg of liverwort DNA per dot corresponding to, respectively, the regimes P, PA and PAB. In these lanes, columns show DNA damage at the different moments of the day in which samples were taken: in the darkness, just before dawn (column 1); at the end of the first low-PAR period (column 2); in the middle and at the end of the high-PAR plus UV period (columns 3 and 4, respectively); and at the end of the second low-PAR period (column 5). The results of one replicate are shown.

8.4. RESULTS AND DISCUSSION

Effect of UV-B on DNA damage

The radiation regime significantly affected the amount of thymine dimers on both measurement days (global effect: $P<0.001$). Samples exposed to the three regimes applied were significantly different between them, although with diverse probability levels: P and PAB regimes were the most different regimes ($P<0.001$ on both measurement days), whereas PA and PAB ($P<0.01$ on both measurement days), and P and PA ($P<0.05$ on the first measurement days and $P<0.01$ on the second one) were less significantly different. Thus, clearly, DNA damage was much stronger in PAB than in P and PA samples (Figs. 1-2). This result corroborates the fidelity of DNA damage in the response of *J. cordifolia* to artificially enhanced UV-B under laboratory conditions (Otero *et al.*, 2006). Our finding is also congruent with the DNA damage detected after exposing several mosses (*Bryum pseudotriquetrum*, *Ceratodon purpureus*, *Fontinalis antipyretica*, *Schistidium antarctici* and *Sanionia uncinata*) to an artificial UV-B supplement in both the laboratory (Lud *et al.*, 2002; Turnbull *et al.*, 2009) and the field (Lud *et al.*, 2002, 2003). All these results are consistent with the fact that DNA is a specific target of UV-B (Jansen *et al.*, 1998).

However, no DNA damage was found when three mosses and the liverwort *J. cordifolia* itself were exposed to ambient UV-B levels under field conditions (Lud *et al.*, 2003; Núñez-Olivera *et al.*, 2009, 2010). The two types of results obtained (DNA damage under enhanced UV-B in the laboratory or the field, and lack of damage under ambient UV-B in the field) could be explained because bryophytes in nature would be acclimated to a certain UV-B level, under which their protection mechanisms and repairing systems would be able to prevent DNA damage. Artificially enhanced UV-B would alter this natural state and, consequently, the balance between damage and repair would be inclined towards damage. This might especially happen in experiments using UV-B supplements during relatively short periods (in

bryophytes, this kind of studies have lasted from 1 hour to 31 days: Table 1). In these short-term periods, plants would not be able to acclimate to the new radiation conditions because acclimation may take years to occur (Lappalainen *et al.*, 2008) and can be also influenced by other environmental factors such as water availability (Arróniz-Crespo *et al.*, 2011).

There are two studies which make exception to the statement above (occurrence of DNA damage under enhanced UV-B and lack of damage under ambient UV-B). First, Boelen *et al.* (2006) did not find any DNA damage in four Antarctic mosses exposed to artificially enhanced UV-B. This could have occurred because of an insufficient UV-B dose applied (but only irradiance and not dose was mentioned in the paper), unusually effective protection and repair mechanisms in the species used, or the influence of environmental factors that protected mosses from DNA damage, such as desiccation (Turnbull *et al.*, 2009). Second, Turnbull & Robinson (2009) did find DNA damage in three Antarctic mosses exposed to only ambient UV-B levels, and in two of them a positive association between CPDs accumulation and incident UV-B radiation was demonstrated. The increase in CPDs was probably due to an imbalance between DNA damage and repairing, given the extremely severe environmental conditions the mosses had to withstood, especially low temperature (-18.2°C to 4.9°C in the period studied).

Values of thymine dimers found in PAB samples of *J. cordifolia* (1.7-7.2 dimers Mbp^{-1}: Fig. 1) were relatively low in comparison with the range found in mosses exposed to similar UV-B enhancements under field (1-13 dimers Mbp^{-1}: Lud *et al.*, 2002; Boelen *et al.*, 2006) or controlled (59-68: Lud *et al.*, 2002) conditions. Values in *J. cordifolia* were also low in comparison with those reported for cyanobacteria, phytoplankton and macroalgae (5-40: Häder & Sinha, 2005). The only values near those obtained for *J. cordifolia* in the present study were those found in *Sanionia uncinata* outdoors (0-7.1: Lud *et al.*, 2003) and *Bryum pseudotriquetrum*, *Fontinalis antipyretica* and *J. cordifolia* itself in the laboratory (3.5-6: Otero *et al.*, 2006; Chapter 6). It must

be taken into account that these values depend on the species used and the experimental conditions applied (mainly the UV-B dose, but also temperature, proportions of other wavelengths, etc.), so that it is difficult to compare the different values in terms of UV-B tolerance of the species.

Effect of UV-A and PAR on DNA damage

In our study, a slight, almost negligible, DNA damage was detected in PA samples (0-1.5 dimers Mbp^{-1}), and even less in P samples (0-0.9). Thus, although not completely specific to PAB samples, the DNA damage found in our study was by far mainly produced in these samples, as occurs with other physiological variables in *J. cordifolia*, in particular the accumulation of UV-absorbing compounds (Chapter 7). This kind of slight DNA damage was also found in four Antarctic mosses exposed to UV-A controls under field conditions, with values around 1 dimer Mbp^{-1} (Boelen *et al.*, 2006). In our experiment, it is very unlikely that DNA damage, although slight, has been caused by UV-A, because the variable measured to evaluate DNA damage was the amount of thymine dimers, and UV-A-induced damage never produces dimers but is mediated through oxidative processes mostly involving guanine (Ravanat *et al.*, 2001). PAR can also cause DNA alterations mediated through oxidative damage (Sinha & Häder, 2002), but, as in the case of UV-A, it does not lead to the production of thymine dimers. Therefore, the low amount of thymine dimers detected in P and PA samples might be due to the residual UV-B present in these regimes (see Table 2).

The difference in DNA damage between P and PA samples was very low but significant, and could be related with the slightly higher physiological damage caused by UV-A with respect to PAR (for example, a higher decrease in the maximum quantum yield of photosystem II: Chapter 7). This could have resulted in a slightly higher vitality and a consequent higher efficiency of protection and repair mechanisms in P samples. More studies are needed to precisely differentiate the effects of PAR and UV-A on

the physiology of *J. cordifolia*.

Diel changes in DNA damage

The period of the day significantly influenced DNA damage on both measurement days (global effect: $P<0.001$), although more clearly in the second than in the first (Fig. 1). Lud *et al.* (2002) also found that, in an Antarctic moss, DNA damage was slightly different on two consecutive days of measurement, but showing similar patterns (as occurred in our study).

The significantly highest ($P<0.001$) amount of thymine dimers was found at the end of the high-PAR plus UV period, whereas the amounts detected in the rest of periods were not, in general, significantly different between them. In particular, the apparent increase of thymine dimers in the first period of low PAR with respect to darkness was not significant in any of the measurement days. The increase in DNA damage at the end of the high-PAR plus UV period occurred more clearly in PAB samples than in PA and P samples (Fig. 1). This would mean that enhanced UV-B radiation was the factor determining diel changes. The residual presence of UV-B in the P and PA regimes (Table 2) may explain the rough coincidence of the diel changes in PAB samples with those found in P and PA samples, as occurred with the presence of thymine dimers in these two last samples.

Two facts were especially remarkable regarding the diel changes in DNA damage in *J. cordifolia*. First, the cumulative effect of UV-B on DNA damage, which determined that damage did not appear in the first hours of high PAR plus UV-B but at the end of this period, after 7 h of UV-B exposure. This pattern of variation is similar to that found in the moss *Sanionia uncinata*, which only began to show a significant DNA damage around midday, after some hours of exposure to artificially enhanced UV-B outdoors (Lud *et al.*, 2002, 2003). A similar pattern was found in the terrestrial alga *Prasiola crispa* in the Antarctic (Lud *et al.*, 2001). Thus, it may be suggested that, in the first hours of UV-B exposure, repair mechanisms

could counteract UV-B-induced DNA damage, but at the end of the period of high PAR plus UV-B there would be an imbalance between damage and repair leading to an increase in thymine dimers. Thus, the amount of thymine dimers in a specific moment of the day may be the result of the balance between damage and repair (Lud *et al.*, 2002). The second important point regarding diel changes is that, after UV-B was switched off, the amount of thymine dimers steeply decreased, recovering the values measured in darkness (the first and last measurements in the day were never significantly different). This would mean that, once UV-B-induced damage ceased, the repairing mechanisms of the liverwort could easily revert it to a more or less basal state, always within the frame provided by the UV-B levels applied in the present study. Photoreactivation could occur rapidly at the end of the day, even under the low-PAR applied to the samples, because 1) this process is apparently saturated at relatively low photon levels (Krizek, 2004), and 2) plants exposed to photoreactivating radiation can eliminate the majority of damage within hours or even minutes (Britt, 2004). As a consequence, DNA damage did not accumulate during consecutive days. The quick repairing after UV-B radiation had been found in mosses (Lud *et al.*, 2002) and an alga (Lud *et al.*, 2001) outdoors.

This is the first time in which the diel variation in DNA damage in response to artificial changes in photosynthetic, UV-A and UV-B radiations is evaluated in a liverwort. DNA damage (amount of thymine dimers) was significantly stronger in PAB than in P and PA samples. This corroborates that DNA damage is a considerably specific biomarker of artificially enhanced UV-B, under both laboratory and field conditions. The diel changes in DNA damage in *J. cordifolia* consisted of a significant increase in thymine dimers at the end of the period of high-PAR plus UV, once a certain amount of UV-B had been accumulated, and a subsequent quick and complete DNA repair after UV-B cessation and the instauration of a low-PAR level. These diel changes occurred much more clearly in PAB samples than in PA and P samples, and thus the differences in DNA damage were mainly due to UV-B. The low amount of thymine dimers produced by *J. cordifolia* and its dynamicity in DNA repairing indicated that UV-B had little

effect on DNA damage in this species, which is well adapted to the UV-B levels applied.

Once established that DNA damage in *J. cordifolia* responds to diel UV-B changes under laboratory conditions, it would be interesting to test if DNA damage in this species also shows diel variations under ambient UV-B and, in particular, if damage increases around midday in response to accumulated UV-B.

CHAPTER 9

DIEL CHANGES IN PHYSIOLOGICAL DAMAGE AND PROTECTION MECHANISMS OF AN AQUATIC LIVERWORT UNDER AMBIENT RADIATION IN THE FIELD

9.1. ABSTRACT

We assessed the diel changes in the physiological and UV-protective responses of the leafy aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* to environmental factors during two consecutive days under field conditions. Photosynthetic (PAR), UV-A and UV-B radiations, together with water temperature, were monitorized as the main environmental variables, while the response variables were chlorophyll fluorescence (both modulated and direct), UV-absorbing compounds (differentiating the soluble and insoluble fractions, and in both cases analyzing both the global amount of UV-absorbing compounds and several individual compounds), and DNA damage. Variables related to photosynthetic performance (Φ_{PSII}, F_v/F_m, Performance Index) or photoprotection against high PAR (NPQ) showed clear diel changes in response to, presumably, ambient radiation (in particular, PAR). Φ_{PSII}, F_v/F_m, and PI showed an inverse relationship with respect to irradiance, whereas NPQ showed a direct relatioship. These changes were similar to those shown by the same liverwort exposed to an artificial day under laboratory conditions, indicating dynamic photoinhibition and protection of PSII from excess radiation through the xanthophyll cycle. Variables related to photoprotection against UV radiation through UV-absorbing compounds did not show clear diel changes. This contrasted with the results obtained in the laboratory, where at least some variables increased in response to enhanced UV-B. DNA damage could not be demonstrated in any liverwort sample, in line with other studies conducted in the field under ambient UV-B but not with field and laboratory studies using enhanced UV-B, in which DNA damage had been demonstrated. Manipulative experiments using lamps or filters are needed to discriminate the effects of each wavelength under field conditions.

RESUMEN

Se evaluaron los cambios en la fisiología y los mecanismos de protección frente a la radiación UV que se produjeron en respuesta a los factores ambientales en la hepática acuática *Jungermannia exsertifolia* subsp. *cordifolia*, a lo largo de dos ciclos diarios consecutivos en condiciones de campo. Como variables ambientales principales se monitorizaron las radiaciones fotosintética (PAR), UV-A y UV-B, además de la temperatura del agua. Las variables de respuesta de la planta fueron la fluorescencia de clorofilas (tanto modulada como directa), los CAUV (diferenciando las fracciones soluble e insoluble, y analizando en ambos casos la cantidad global de CAUV y varios compuestos individuales), y el daño al ADN. Las variables relacionadas con la función fotosintética (Φ_{PSII}, F_v/F_m, PI) o la fotoprotección frente a alta PAR (NPQ) mostraron ciclos diarios evidentes en respuesta, seguramente, a los cambios en la radiación natural (en particular a la PAR). Φ_{PSII}, F_v/F_m y PI tenían una relación inversa con respecto a la irradiancia, mientras que NPQ mostraba una relación directa. Estos cambios eran similares a los que se producían en la misma especie cuando se exponía a un día artificial en condiciones de laboratorio, e indicaban una fotoinhibición dinámica y una protección del PSII frente al exceso de radiación mediante el ciclo de las xantofilas. Las variables relacionadas con los CAUV no mostraron cambios diarios claros, lo que contrastaba con los resultados obtenidos en el laboratorio, donde al menos algunas variables aumentaban en respuesta a un suplemento de UV-B. No se encontró daño al ADN en ninguna muestra de la hepática, en concordancia con otros estudios de campo que utilizaban niveles naturales de UV-B, mientras que sí se habían encontrado daños en otros estudios de campo y laboratorio en los que se suministraba un suplemento de UV-B. Se apunta la necesidad de emprender estudios de campo en los que se manipule la radiación, mediante lámparas o filtros, para discriminar los efectos de cada longitud de onda.

9.2. INTRODUCTION

As it was said in the Introduction of Chapters 7 and 8, a number of field studies have assessed the changes in the responses of bryophytes to UV in diverse temporal scales: interannual (Núñez-Olivera *et al.*, 2009), seasonal (Núñez-Olivera *et al.*, 2009; Lappalainen *et al.*, 2010), day-to-day (Newsham *et al.*, 2002; Newsham, 2003; Newsham *et al.*, 2005) and daily (Green *et al.*, 2000; Conde-Álvarez *et al.*, 2002; Lud *et al.*, 2002, 2003; Green *et al.*, 2005; Lappalainen *et al.*, 2010). Under field conditions, the responses to UV can be modified by the interference of environmental factors other than UV, such as temperature, water availability or other radiation wavelengths. Thus, laboratory studies have also been conducted to prevent this problem and to separate the effects of the different factors (see Chapters 7 and 8). However, this kind of studies are not directly extrapolable to field conditions, and this may limit their ecological meaning. Hence, a combination of both types of studies may provide a more solid background to establish a cause-effect relationship between UV radiation and the bryophyte responses to this environmental factor.

In the specific studies on diel changes mentioned above, modulated chlorophyll fluorescence has been analyzed as one of the response variables (Green *et al.*, 2000, 2005; Conde-Álvarez *et al.*, 2002; Lud *et al.*, 2002, 2003), given that this powerful technique has revealed extremely useful to assess the effects of many different adverse factors (Maxwell & Johnson, 2000). However, the direct fluorescence technique obtaining OJIP curves and derived variables such as the Performance Index (PI) has also been found to be sensitive to adverse factors such as excess light, temperature, drought, etc. (Strasser *et al.*, 2000). Thus, both chlorophyll fluorescence techniques were applied in the present study. To our knowledge, this is the first time that direct fluroescence is applied to bryophytes.

Other variables have been measured to evaluate diel responses of bryophytes to UV, such as photosynthetic pigments, DNA damage and UV-absorbing compounds. With respect to UV-absorbing compounds, their

measurement was exclusively based on global variables such as the bulk UV absorbance of the soluble fraction of plant extracts (Conde-Álvarez *et al.*, 2002; Lud *et al.*, 2003; Green *et al.*, 2005; Lappalainen *et al.*, 2010), without considering either the insoluble fraction or the responses of individual compounds. The differentiation between the soluble (mainly vacuolar) and the insoluble (cell wall-bound) fractions may be important because the cell wall-bound compounds would provide a more spatially uniform UV screen than the intracellular compounds (Clarke & Robinson, 2008). In addition, the analysis of individual UV-absorbing compounds represents a useful complement to the global estimation of UV protection provided by the bulk UV absorbance, since each compound may respond to UV in a different manner (Morales *et al.*, 2010).

It is also remarkable that there is only one study on the diel changes in the responses to UV radiation in liverworts (Conde-Álvarez *et al.*, 2002), given that the remaining studies have dealt with mosses (Green *et al.*, 2000, 2005; Conde-Álvarez *et al.*, 2002; Lud *et al.*, 2002, 2003; Lappalainen *et al.*, 2010). Moreover, the only study devoted to liverworts used a thalloid one (Conde-Álvarez *et al.*, 2002), so that leafy liverworts have never been studied regarding this issue.

The aim of this study was to assess the diel changes in the physiological and UV-protective responses of the leafy aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* to environmental factors during two consecutive days under field conditions. In particular, photosynthetic, UV-A and UV-B radiations, together with water temperature, were monitorized. The response variables were chlorophyll fluorescence (both modulated and direct), UV-absorbing compounds (differentiating the soluble and insoluble fractions, and in both cases analyzing both the global amount of UV-absorbing compounds and several individual compounds), and DNA damage. Our field study would be appropriate to complement the laboratory studies previously carried out using the same liverwort (see Chapters 7 and 8).

9.3. MATERIALS AND METHODS

Plant material and experimental site

Samples of the foliose aquatic liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Vána (hereafter *J. cordifolia*) were monitorized *in situ* for two consecutive days (20-21 July 2011) at the stream Lavieja (1280 m a.s.l., 42º03'00'' N, 02º35'30'' W), in La Rioja (northern Spain). Samples were submerged and exposed to full sun. The vegetation of the sampling zone is an open shrubland, mainly composed by *Cytisus scoparius* L., with scattered beech trees (*Fagus sylvatica* L.).

Environmental data

Water temperature was measured *in situ* every 30 min during the sampling period (Testo 106 thermometer, TestoAG, Lenzkirch, Germany). Photosynthetic (PAR), UV-A and UV-B irradiances were continuously measured using a quantum sensor (LI-190SA, LI-COR, Lincoln, NE, USA) and a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland). The biologically effective UV irradiance (UV_{BE}) was calculated using the spectrum of Flint & Caldwell (2003). The accumulated doses (in kJ m^{-2}) of PAR, UV-A, UV-B and UV_{BE} along several times of the day were also calculated.

Physiological measurements

Chlorophyll *a* fluorescence was measured *in situ* using two instruments: the portable pulse amplitude modulation fluorometer MINI-PAM (Walz Instruments, Effeltrich, Germany) and AquaPen-P AP-P 100 (Photon Systems Instruments, Brno, Czech Republic).

Minimal and maximal fluorescence (F_0 and F_m) were measured with MINI-PAM using the saturation pulse method, following Schreiber *et al.* (1995) and Núñez-Olivera *et al.* (2004), in samples dark-adapted for 20 min. The maximum quantum yield of PSII was given by the ratio F_v/F_m, where $F_v = F_m - F_0$, and the effective quantum yield of photosynthetic energy conversion of PSII (Φ_{PSII}) by the ratio $(F_m'- F_t)/F_m'$. The apparent electron transport rate through PSII (ETR) was calculated as: ETR = Φ_{PSII} x PFD x 0.5 x 0.84, where PFD is photon flux density. Quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined as $(F_m - F_m')/F_m'$. The curve Φ_{PSII} *vs.* PFD along the day was fitted to a negative exponential curve and the curve ETR *vs.* PFD along the day was fitted to Jassby and Platt's hyperbolic tangent equation.

The OJIP curve was obtained with the AquaPen and was used to analyze each chlorophyll fluorescence transient and to obtain the Performance Index (PI: Strasser *et al.*, 2000). In bried, in samples dark-adapted for 20 min, chlorophyll fluorescence transients were induced by a saturating PFD of 3000 μmol m^{-2} s^{-1} and the relative fluorescence was recorded at different frequencies between 10 μs to 2 s. The relative variable fluorescence was calculated as $V_t = (F_t - F_0) / (F_m - F_0)$. Cardinal points of the OJIP curve and derived parameters were calculated with the Fluorpen 2.0 software, based on Strasser *et al.* (2000). Starting from these data, the Performance Index (PI) was obtained as a multi-parametric expression of the three independent steps contributing to photosynthesis: absorption of light energy, trapping of excitation energy, and conversion of excitation energy to electron transport.

For the analysis of UV-absorbing compounds, samples were collected and the surface area of the prostrate apices was measured *in situ* (LI-COR LI-3000 area meter, Lincoln, NE, USA). Immediately, samples were frozen in liquid N_2 and transported to the laboratory. Then, samples were processed as described in Chapter 3. The bulk UV absorbance of both the soluble

compounds (SUVAC), presumably mainly located in the vacuole, and the insoluble cell wall-bound compounds (WUVAC), was determined by spectrophotometry (Perkin-Elmer λ35 spectrophotometer, Perkin-Elmer, Wilton, CT, USA) as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of surface area. Using HPLC (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA), seven hydroxycinnamic acid derivatives were analyzed, five in the soluble fraction and two additional ones in the insoluble fraction. The soluble compounds were: *p*-coumaroylmalic acid, caffeoylmalic acid (or phaselic acid), feruloylmalic acid, 5''-(7'',8''-dihydroxycoumaroyl)-2-caffeoylmalic acid, and 5''-(7'',8''-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid. These will be hereafter referred to as C1 to C5, respectively. The insoluble compounds were *p*-coumaric and ferulic acids, which will be referred to as C6 and C7, respectively.

DNA damage was evaluated by detection of thymine dimers (Sinha *et al.*, 2001). Samples were collected, immediately frozen in liquid N_2 and transported to the laboratory. Once there, samples were processed as in Chapter 3. Calibration of DNA damage was made using UV-C irradiated DNA of the plasmid pBSK. We also checked that non-irradiated DNA of salmon sperm showed no DNA damage, and that DNA samples of the liverwort that had been exposed to UV-B in a previous experiment under laboratory conditions (see Chapter 8) did show DNA damage.

Most of the variables (F_v/F_m, NPQ, OJIP curves, PI, SUVAC, WUVAC, the concentrations of C1 to C7, and DNA damage) were measured at five different moments of the day on the two consecutive days: in the darkness (just before dawn), and around 11:00, 14:00, 17:00 and 20:00 h (local hour, 2-h delayed with respect to solar hour). Φ_{PSII} was measured every 30 min since 06:30 to 20:30. Chlorophyll fluorescence variables were measured in three replicates, except Φ_{PSII}, which was measured in one replicate in continuous. SUVAC, WUVAC, and the concentrations of C1 to C7 were measured in five replicates, and samples for DNA damage in three replicates.

Statistical analysis

The global effect of the period of the day on the responses of each physiological variable was tested using a one-way analysis of variance (ANOVA), once proved that the data met the assumptions of normality (Shapiro-Wilks test) and homoscedasticity (Levene test). In the case of significant differences, means were then compared by Tukey's test. If the data did not meet those assumptions, Kruskal-Wallis tests were applied. In this case, and when significant differences occurred, means were compared by Mann-Whitney tests. Bivariate correlations (Pearson's coefficients) between all the physiological and environmental variables were obtained.All the statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

9.4. RESULTS

Changes in the environmental variables (photosynthetic, UV-A, UV-B, and UV_{BE} irradiances, together with water temperature) that were measured in the study site on both sampling days are shown in Fig. 1. The first sampling day was considerably sunny, with only one marked cloudy episode in the afternoon. However, the second day was very cloudy, especially from midday on, and only in the morning the sun alternated with clouds. All the radiation variables varied concomitantly on both sampling days. On the first day, irradiances showed maximum values around solar noon and minimum values both at dawn and dusk. However, on the second day, irradiances showed very low values along all the day except, approximately, in the 4-h period before solar noon, when several peaks and valleys, depending on cloudiness, were recorded. Water temperature showed a similar trend to that pointed out for irradiances, but somewhat delayed, especially on the first day. On the first day, low temperature values were recorded in the morning, and then values increased until mid-afternoon and then progressively decreased. Changes in water temperature were attenuated in the second day, in line with the more slight changes in irradiances. Maximum temperatures occurred in the morning, and lower temperatures were observed in the rest of the day. All the irradiances (photosynthetic, UV-A, UV-B, and UV_{BE}) were highly intercorrelated (at least $P<0.01$). Water temperature was significantly correlated with UV-A, UV-B, UV_{BE} irradiances ($P<0.05$), and with the photosynthetic, UV-A, UV-B, and UV_{BE} accumulated doses ($P<0.01$).

On both sampling days, the daily variation of Φ_{PSII} was, apparently, totally inverse to the changes shown by the irradiances, and thus Φ_{PSII} showed, on the first day, low values in the central hours of the day and high values in the morning and evening, and on the second day, peaks and valleys around solar noon and, again, high values in the morning and evening (Fig. 2). Φ_{PSII} values were higher on the second than on the first sampling day, except in the first hours of the morning and the last hours of

the evening, in which values were similar on both sampling days. Putting together all the values of Φ_{PSII}, ETR and PFD recorded along both sampling days, the relationship between Φ_{PSII} and PFD fitted very significantly with a negative exponential function, whereas the relationship between ETR and PFD fitted equally significantly with a hyperbolic tangent function (Fig. 2).

Diel changes of PI, as derived from the OJIP fluorescence transients, are shown in Fig. 3. On the first measurement day, PI showed the highest values before dawn, then significantly declined to a plateau for most of the day, and finally significantly increased before dusk, although not reaching the maximum values shown before dawn. On the second day, values were homogeneous for most of the day, except a significant decrease in the morning followed by a subsequent increase and recovery of the initial values.

Diel changes in F_v/F_m and NPQ, together with those of Φ_{PSII} when using only five measurements per day, were stronger on the first than on the second sampling day (Fig. 3). On the first day, F_v/F_m showed rather similar changes to PI: F_v/F_m reached its maximum values before dawn, then progressively and significantly declined until the end of the afternoon, and finally significantly increased before dusk, although without a complete recovery of the values shown before dawn. Φ_{PSII} showed a similar trend to F_v/F_m, but with stonger and clearly significant changes along the day: it decreased until solar noon and then increased until the last measurement before dusk, completely recovering the values shown before dawn. NPQ followed a neatly inverse trend to Φ_{PSII}. On the second day, F_v/F_m hardly showed diel changes, whereas Φ_{PSII} significantly decreased in the sunny morning of the day and then significantly increased, and, again, NPQ showed inverse changes to Φ_{PSII}. Several chlorophyll fluorescence variables were intercorrelated: F_v/F_m and Φ_{PSII} were positively correlated between them ($P<0.05$) and negatively correlated with NPQ (at least $P<0.01$). PI was positively correlated with Φ_{PSII} ($P<0.05$) and negatively with NPQ.

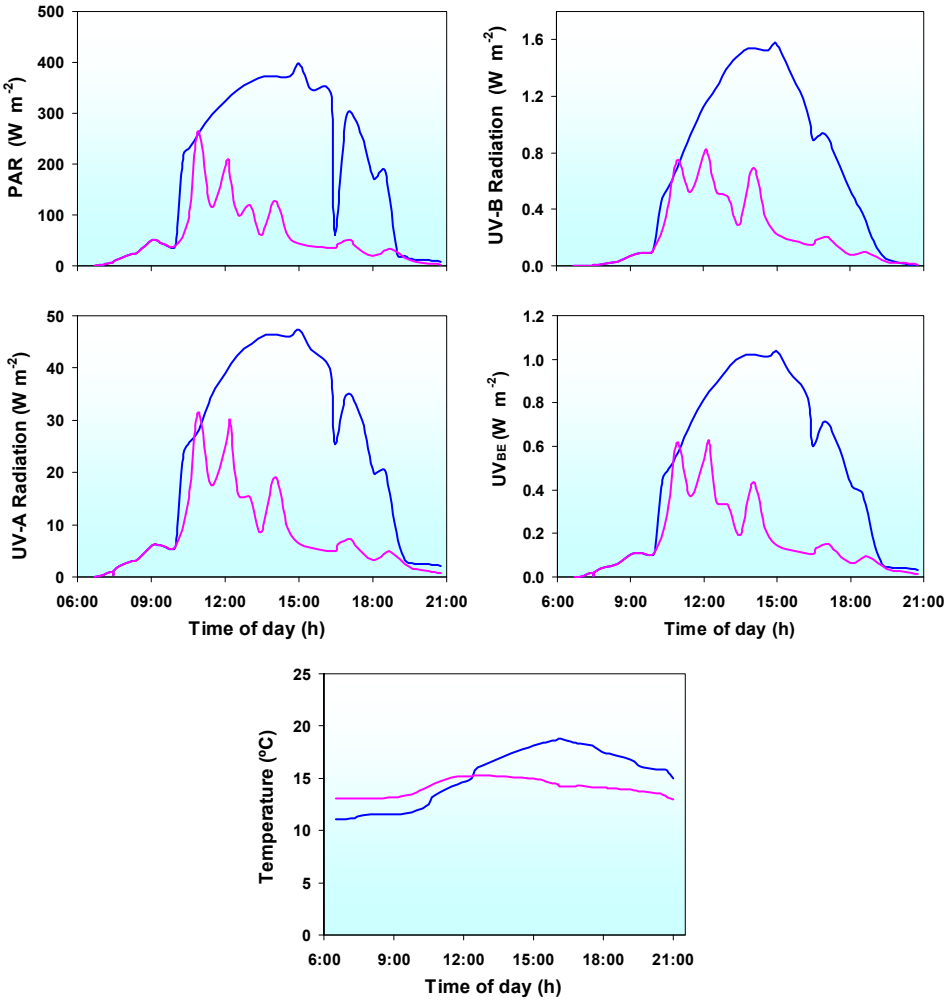

Fig. 1. Diel changes in the environmental variables measured on two consecutive days (20-21 July 2011) in the study site: photosynthetic (PAR), UV-A, UV-B, and UV_{BE} (biologically effective UV) irradiances, and water temperature. The first day of measurement is shown in blue and the second one in pink.

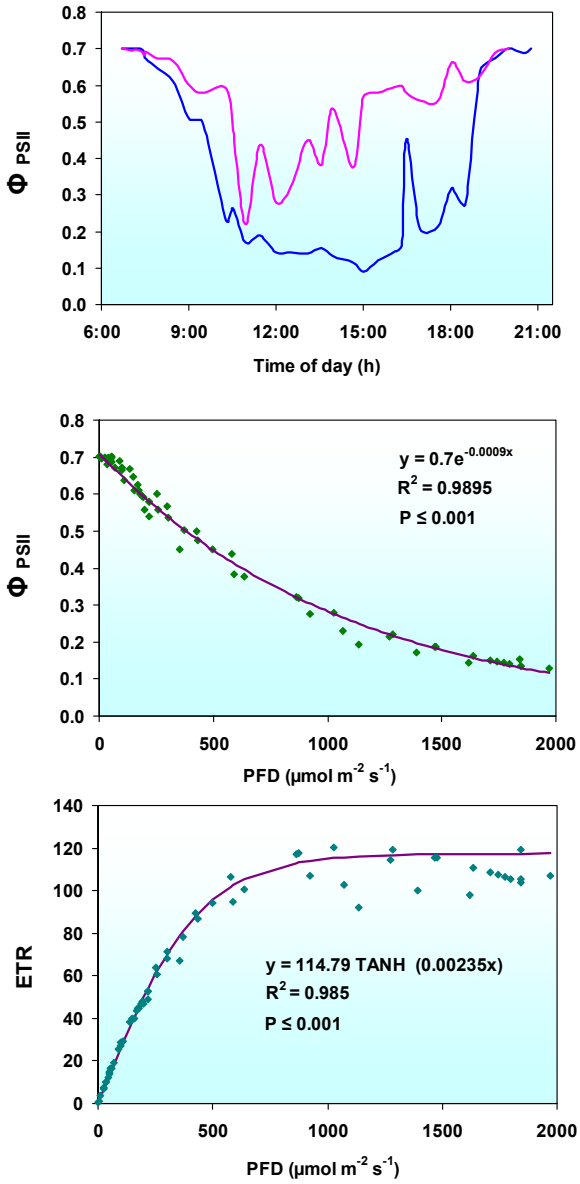

Fig. 2. Diel changes in the effective quantum yield of PSII (Φ_{PSII}) of *Jungermannia exsertifolia* subsp. *cordifolia*, measured continuously on two consecutive days (top; first day in blue and second day in pink), together with the fitted curves Φ_{PSII} *vs.* PFD (middle) and ETR *vs.* PFD (bottom). The respective equations and determination coefficients are shown for fitted curves.

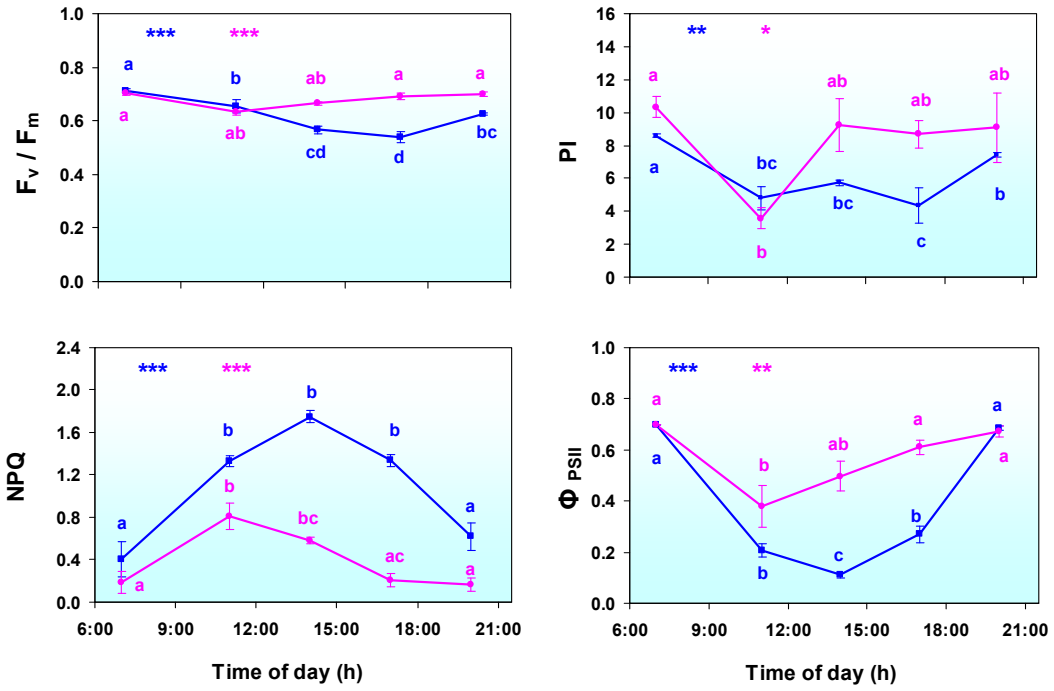

Fig. 3. Diel changes in four chlorophyll fluorescence variables (F_v/F_m, maximum quantum yield of PSII; NPQ, non-photochemical quenching; PI, performance index; and Φ_{PSII}, effective quantum yield of PSII) of *Jungermannia exsertifolia* subsp. *cordifolia*, measured in five different periods of the day on the two consecutive measurement days (first day in blue and second day in pink). Means ± SE are shown. For each variable, the overall effect of the period of the day is shown for each measurement day (first day in blue and second day in pink). This effect was tested by either a one-way ANOVA or a Kruskal-Wallis test (***P < 0.001, **P < 0.01, *P < 0.05, NS non-significant). For each measurement day, different letters mean significant differences at least at P<0.05.

With respect to the variables related to UV-absorbing compounds, the global spectrophotometric variables from both the soluble and insoluble fractions (SUVAC and WUVAC) showed slight or non-existent diel changes on both sampling days (Fig. 4-5). Regarding the concentrations of the individual UV-absorbing compounds from the soluble fraction on the first sampling day (Fig. 4), C1, C2 and C5 showed rather stable values along the day but significantly increased before dusk; C3 decreased in the second half of the day, and C4 significantly increased in the morning, then remained stable, and again significantly increased before dusk. Changes in the soluble UV-absorbing compounds on the second sampling day were much slighter than on the first day (Fig. 4): C1 showed rather similar changes to those shown in the first day, with a significant increase before dusk, whereas C3 showed stable values along the day until a significant increase before dusk (as C1); C2, C4 and C5 did not show any change. C1, C2 and C5 were positively intercorrelated (at least $P<0.05$), C5 was positively correlated with C4 ($P<0.01$), and C4 with C2 ($P<0.01$). The insoluble UV-absorbing compounds C6 and C7 (Fig. 5) showed even slighter diel changes than the soluble ones on both sampling days. On the first day, both compounds showed ups and downs along the day, whereas on the second day they did not show any change. C6 and C7 were positively intercorrelated ($P<0.05$). There was no correlation between chlorophyll fluorescence variables and the variables related to UV-absorbing compounds.

There were some significant correlations between physiological and environmental variables. Irradiances were positively correlated with NPQ ($P<0.001$) and negatively with F_v/F_m and Φ_{PSII} ($P<0.01$). PI was only correlated (negatively) with photosynthetic and UV-A irradiances ($P<0.05$). Water temperature was negatively correlated with F_v/F_m ($P<0.01$) and C3 ($P<0.05$), and positively with NPQ ($P<0.05$).

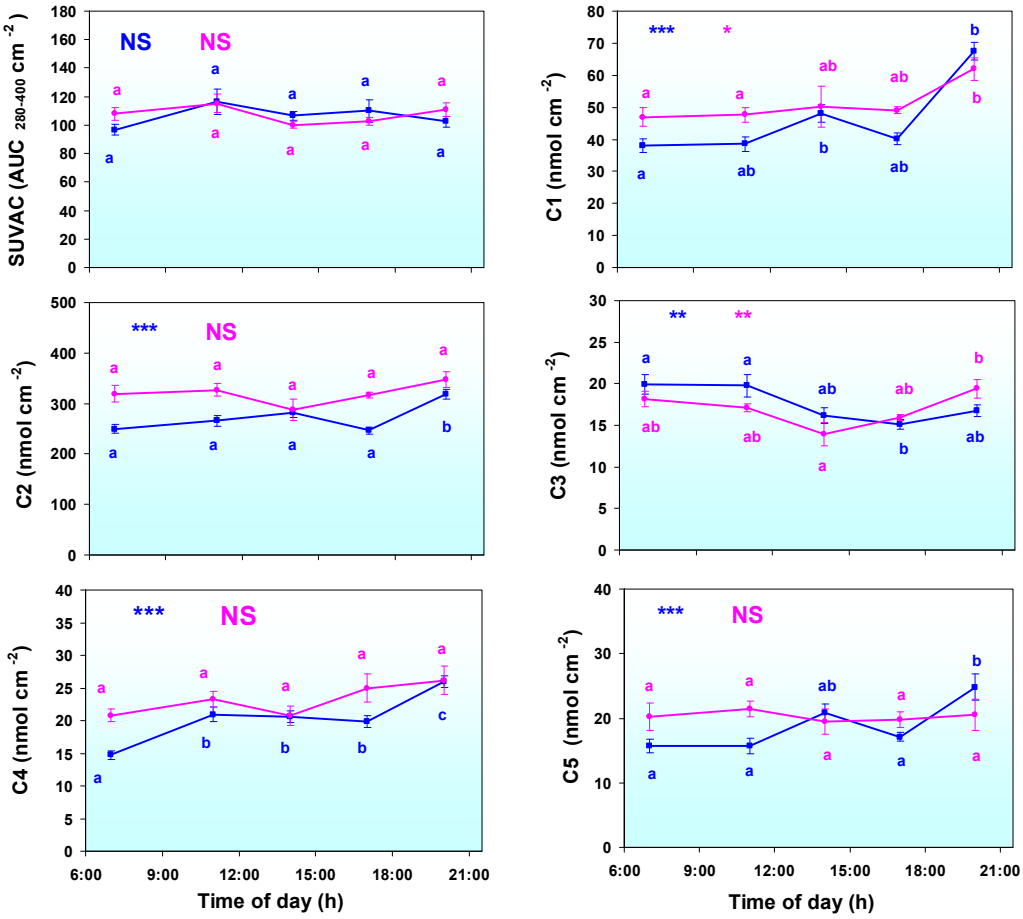

Fig. 4. Diel changes in the bulk levels of methanol-soluble UV-absorbing compounds (SUVAC) and the concentrations of the five individual UV-absorbing compounds from the soluble fraction (C1 to C5, see names in the text) of *Jungermannia exsertifolia* subsp. *cordifolia*, measured in five different periods of the day on the two consecutive measurement days (first day in blue and second day in pink). Means ± SE are shown. For each variable, the overall effect of the period of the day is shown for each measurement day (first day in blue and second day in pink). This effect was tested by either a one-way ANOVA or a Kruskal-Wallis test (***P < 0.001, **P < 0.01, *P < 0.05, NS non-significant). For each measurement day, different letters mean significant differences at least at P<0.05.

DNA damage was not detected in any of the samples analyzed (Fig. 6). It is remarkable that we took some precautions to prevent any methodological error in the detection of damage in the samples of *J. cordifolia* from this experiment. Simultaneously with the analysis of these samples, 1) we analyzed DNA damage in UV-C-irradiated DNA from a plasmid (the usual procedure for damage calibration); 2) we checked that unirradiated DNA samples of salmon sperm did not show any damage; 3) we demonstrated damage in DNA samples of *J. cordifolia* both irradiated with UV-C and irradiated with UV-B in a previous experiment under laboratory conditions (see Chapter 8). These additional results corroborated the lack of DNA damage in the liverwort samples from the present field experiment.

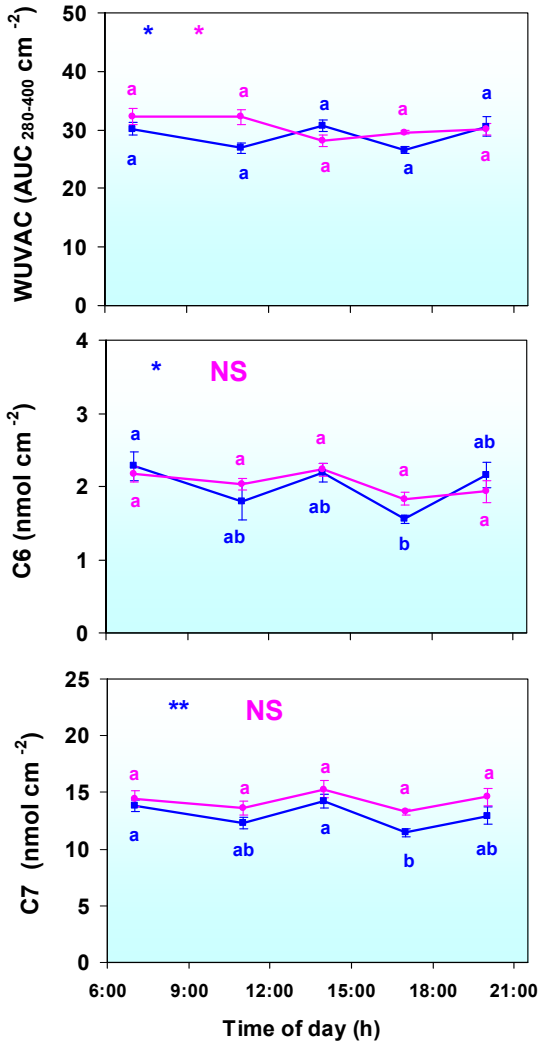

Fig. 5. Diel changes in the bulk levels of cell wall-bound insoluble UV-absorbing compounds (WUVAC) and the concentrations of the two individual UV-absorbing compounds from the insoluble fraction (C6 and C7, see names in the text) of *Jungermannia exsertifolia* subsp. *cordifolia*, measured in five different periods of the day on the two consecutive measurement days (first day in blue and second day in pink). Means ± SE are shown. For each variable, the overall effect of the period of the day is shown for each measurement day (first day in blue and second day in pink). This effect was tested by either a one-way ANOVA or a Kruskal-Wallis test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS non-significant). For each measurement day, different letters mean significant differences at least at $P<0.05$.

9.5. DISCUSSION

The two measurement days were very different between them, because the first one was predominantly sunny and the second one almost totally cloudy. This accidental meteorological environment provided an adequate frame to compare the pysiological changes of *J. cordifolia* under contrasted environmental conditions. These rapid meteorological changes, even in the mid of the summer, are more frequent in mountain zones (Körner, 2003) than in lowland regions (Núñez-Olivera *et al.*, 2006). Radiation changes were more drastic and dynamic than changes in water temperature (Fig. 1). This was expected because 1) radiation can be easily modified by the cloudy episodes that can occur along the day, and 2) water has a high buffering capacity. This capacity made water temperature more predictable than radiation, and caused a delay in the temperature changes with respect to changes in radiation variables. Thus, water temperature was better correlated with the accumulated radiation doses than with the instantaneous irradiances. To sum up, both radiation and water temperature showed clear variations between the two measurement days and, especially the radiation variables, within each of the days. This variability provided a good experimental situation to analyze the dinamicity of the responses of the different physiological variables of the liverwort.

Consistent diel changes occurred in the chlorophyll fluorescence variables (Φ_{PSII}, F_v/F_m, NPQ, PI), especially in the first measurement day, when radiation changes were more marked. Probably, these changes were mainly dependent on the variations in PAR, which might be a more decisive factor than the different UV irradiances, as derived from the findings achieved in manipulative experiments of UV radiation under both field and laboratory conditions, either on bryophytes (see Chapter 7) or tracheophytes (Núñez-Olivera *et al.*, 2006). Loss of photosynthetic efficiency of PSII (denoted by Φ_{PSII} decline) was found when PAR irradiance increased, either during the central hours of the day (on the first measurement day) or during transient episodes within a day (on the second measurement day).

Similar losses of photosynthetic efficiency of PSII, and concomitant Φ_{PSII} declines, were found along most of the day on the first measurement day with respect to the second one, because of the higher PAR levels in the second day. As a consequence, Φ_{PSII} showed a significant negative exponential relationship with PFD (Fig. 2). The Φ_{PSII} declines described above may be the result of increased dissipation of excessively absorbed energy by thermal radiation. This would depend on the increased activity of the xanthophyll cycle, as shown by the increase in NPQ, which would serve to protect PSII from excess radiation (Demmig-Adams & Adams, 1996). This is strongly supported by the high negative correlation between NPQ and Φ_{PSII} and their inverse variation (Fig. 3). Nevertheless, the midday increase in NPQ could be also indicative of energy dissipation through alternative mechanisms to the xanthophyll cycle (Maxwell & Johnson, 2000). The transient decreases in Φ_{PSII} represent a general feature of photosynthesis in natural environments and are considered to be a down-regulation that maintains a balance between light-driven electron flow and requirements for reducing power (Núñez-Olivera *et al.*, 2006).

F_v/F_m is a good index of photoinhibition and its decrease is generally attributed to damage in PSII (Maxwell & Johnson, 2000). Thus, its diel changes, with a decrease in the afternoon and an incomplete recovery at dusk, suggest that dynamic photoinhibition occurred to the liverwort in this study, as was also shown under laboratory conditions (Chapter 7). Photoinhibition can be mainly attributed to high PAR, but, at least to a certain extent, also to high UV radiation, because the decrease in F_v/F_m is a usual response to enhanced UV-B in *J. cordifolia* (Otero *et al.*, 2006; Martínez-Abaigar and Núñez-Olivera, 2011) and other bryophytes (Montiel *et al.*, 1999; Conde-Álvarez *et al.*, 2002). PI varied along the day similarly to F_v/F_m, and provided much similar information, but PI changes were stronger than F_v/F_m changes, so that the former may be more sensitive to high radiation stress than the latter, as occurs regarding other stresses (Thach *et al.*, 2007).

The diel changes in chlorophyll fluorescence variables that have been found here were mostly congruent with those found in other bryophytes (Green et al., 2000; Conde-Álvarez et al., 2002; Lud et al., 2002, 2003) and tracheophytes (Veit et al., 1996; Núñez-Olivera *et al.*, 2006). These findings suggest that *J. cordifolia* in this experiment was well adapted to its environmental niche and that the environmental conditions experienced during the experiment caused dynamic photoinhibition but were not so adverse to cause chronic damage.

In *J. cordifolia*, ETR increased with increasing PFD until saturation, fitting well with a hyperbolic tangent function (Fig. 2). Saturation occurred at a relatively high PFD level, as it was found under laboratory conditions, but showing 2-fold higher values (see Martínez-Abaigar *et al.*, 2003, for comparative laboratory values). Thus, ETR was higher under field than under laboratory conditions. This was in line with the higher Φ_{PSII} in the field than in the laboratory for similar PDF levels: in the laboratory, Φ_{PSII} showed values around 0.20 for 500 μmol m^{-2} s^{-1} PFD (Chapter 7), whereas in the field it reached 0.45 (the present study). On the other hand, *J. cordifolia* showed relatively low NPQ values with respect to other bryophytes, which would indicate and adaptation to shade conditions (Marschall & Proctor, 2004; Proctor & Smirnoff, 2011). However, the relatively high PFD levels required for ETR saturation in our species did not correspond with that adaptation. This discrepancy could be due to the different experimental conditions used by Marschall & Proctor (2004) and Proctor & Smirnoff (2011), who did their measurements in short periods under laboratory conditions. Another plausible hypothesis for this discrepancy would be that, in truly aquatic species such as *J. cordifolia* under field conditions, high radiation stress would not be accompanied by hydric stress, and thus NPQ can be maintained at a low level because the species does not need an excessive protection.

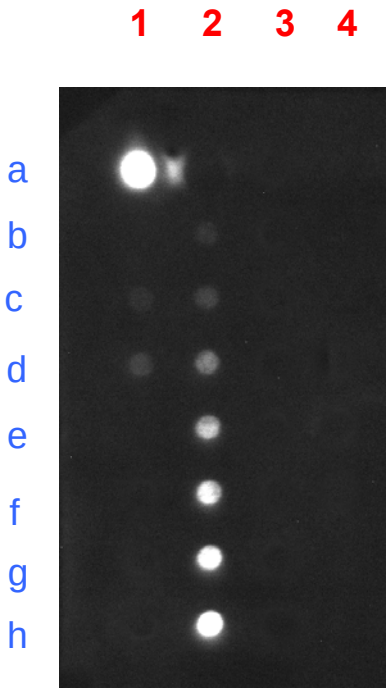

Fig. 6. Results of the immunoassay performed to determine DNA damage (presence of thymine dimers). The dot blot shows: in column 1 (lanes f-h), unirradiated control DNA samples of salmon sperm, using 1, 2 or 3 µg DNA per dot, respectively; in column 2 (lanes a-h), increasing amounts (0, 0.5, 1, 2, 3, 4, 5 and 10 ng) of DNA from plasmid pBSK irradiated with UV-C (calibration column); in column 1 (lane a), a 250-ng DNA sample from *Jungermannia exsertifolia* subsp. c*ordifolia* irradiated with UV-C; in column 1 (lanes c-d), 1 and 2-µg DNA samples of the liverwort from a previous laboratory experiment in which they had been irradiated with UV-B and had shown damage; in columns 3 and 4 (lanes a-h), 1-µg DNA samples of the liverwort corresponding to at least one replicate for each sampling time on each sampling day.

The bulk UV absorbance of both the soluble (SUVAC) and the insoluble cell wall-bound compounds (WUVAC) did not show clear diel changes in any of the two measurement days (Fig. 4-5). With respect to the individual compounds, their diel changes were somewhat more marked in the first than in the second measurement day (Fig. 4-5), which could indicate that diel changes were induced, to a certain extent, by the higher radiation of the first day with respect to the second. However, most of the individual UV-absorbing compounds did not show clear diel changes, and thus their response to the radiation increase in the central hours of the day on the first measurement day, was weak or non-existent. These findings strongly contrasted with the significant diel changes shown by the bulk UV absorbances, C6 and C7 under laboratory conditions (Chapter 7). These variables increased at the end of the high-PAR plus UV period which was applied within an artificial day, and these changes were mainly attributed to enhanced UV-B. We did not expect strong diel changes in UV-absorbing compounds under field conditions (i.e., ambient UV-B levels), because most of them had not responded to enhanced UV-B in the laboratory (Otero *et al.*, 2006; Arróniz-Crespo *et al.*, 2008). Nevertheless, we did expect that C1 responded to diel changes in ambient UV-B, because this compound is a good biomarker of UV-B under different conditions (Otero *et al.*, 2006; Núñez-Olivera *et al.*, 2009). Surprisingly, the most frequent change in the individual UV-absorbing compounds that was found in the present study was the significant increase at the end of the day which occurred in C1, C2, C4 and C5 on the first measurement day and in C1 and C3 on the second day. We cannot offer a good explanation for these changes.

Quick changes in the bulk UV absorbance of the soluble fraction of bryophytes had been previously reported under field conditions, in response to either UV-B increases caused by Antarctic ozone depletion (Newsham *et al.*, 2002; Newsham, 2003; Newsham *et al.*, 2005) or artificially enhanced UV-B using lamps (Lappalainen *et al.*, 2010). However, no clear daily pattern in variables related to UV-absorbing compounds had been shown in all these

studies or in the one conducted by (Conde-Álvarez *et al.*, 2002) using a thalloid liverwort exposed to full solar spectrum (as in our study). Thus, diel changes in UV-absorbing compounds may be more difficult to be demonstrated under field than under laboratory conditions. This can occur not only due to the interference of other environmental factors, that may obscure the radiation effects, but also to complex physiological processes that take place simultaneously within the plant. Among others, these processes can be the interconversion of some UV-absorbing compounds into others (given that they share the same metabolic pathway), the change of location of the compounds from the vacuole to the cell wall, or the changing levels of substrate and activity of enzymes of phenolic biosynthesis in response to environmental or internal factors (Liu *et al.*, 1995). Thus, it may not be so strange that the variables related to UV-absorbing compounds have not shown more clear and explicable diel changes under field conditions. Another additional explanation to the lack of diel changes might be that submerged aquatic bryophytes would not need high levels of protecting UV-absorbing compounds, because the water column would act as a protectant itself (Frost *et al.*, 2005). This may not be the cause in our study, because the stream water was oligotrophic and with a low organic matter content, and under these conditions UV-B radiation may easily reach the liverwort samples. In addition, the samples were submerged just a few cm because the discharge was relatively low, in line with the sampling period (mid-summer).

It can be interesting to note that there was no correlation between chlorophyll fluorescence variables and the variables related to UV-absorbing compounds. This lack of correlation could mean that photosynthetic performance and photoinhibition protection are physiological processes determined by different environmental factors to those affecting UV protection through UV-absorbing compounds. In addition, both types of processes may occur at different temporal scales, with chlorophyll fluorescence-related processes showing a higher dynamicity than the

synthesis and accumulation of UV-absorbing compounds.

DNA damage was not detected in any of the samples analyzed at any time on any measurement day. This result was reliably obtained because we took additional methodological precautions. Thus, there was a lack of DNA damage in the liverwort samples under field conditions, and thus *J. cordifolia* did not show diel changes in DNA damage. This contrasted with the results obtained in a diel cycle conducted in the laboratory under artificially enhanced UV-B, because DNA damage increased at the end of a period of high-PAR plus UV radiation, although damage was completely repaired after UV cessation (see Chapter 8). Several additional studies have demonstrated DNA damage in bryophytes under artificially enhanced UV-B in the laboratory (Lud *et al.*, 2002; Otero *et al.*, 2006; Turnbull *et al.*, 2009; see Chapters 5 and 6) or the field (Lud *et al.*, 2002, 2003). This is in line with the target character of DNA with respect to UV-B (Jansen *et al.*, 1998). In contrast with this, and in line with our results in the present experiment, exposure to natural ambient UV-B levels under field conditions did not result in DNA damage (Lud *et al.*, 2002, 2003; Boelen *et al.*, 2006; Núñez-Olivera *et al.*, 2009, 2010). This could be due to the efficiency of protection and repairing mechanisms in bryophytes under these conditions. The only study in which DNA damage was found in samples exposed to ambient UV-B was performed in Antarctica (Turnbull & Robinson, 2009), probably because repair mechanisms were severely limited by low temperature.

All the radiation variables (photosynthetic, UV-A, UV-B, and UV_{BE}) were highly intercorrelated, so that we cannot perfectly discriminate the effects of each one separately. Water temperature was also correlated with some radiation variables, which further complicates the separation of the effects of each environmental variable. However, on the basis of previous results obtained in the laboratory (see Chapters 7 and 8), we can attribute the physiological changes in the liverwort to the variations in one or another environmental variable. In particular, dynamic changes in

chlorophyll fluorescence variables were probably mainly due to changes in the photosynthetic radiation environment, mainly PAR (Núñez-Olivera *et al.*, 2006; Chapter 7), although the discrimination between the different wavelengths is difficult. Manipulative experiments using lamps or filters, to respectively provide either enhanced UV-B or ambient solar radiation deprived of UV-B to the samples, should be needed to discriminate the effects of each wavelength. This kind of manipulative studies have been very rarely conducted in stream bryophytes (Rader & Belish, 1997), due to the logistic problems inherent to such a dynamic and changing ecosystem as a stream.

In conclusion, in *J. cordifolia* under field conditions, variables related to photosynthetic performance (Φ_{PSII}, F_v/F_m, PI) or photoprotection against high PAR (NPQ) showed clear diel changes in response to, presumably, ambient radiation (in particular, PAR). Φ_{PSII}, F_v/F_m, and PI showed an inverse relationship with respect to irradiance, whereas NPQ showed a direct relatioship. These changes were similar to those shown by the same liverwort exposed to an artificial day under laboratory conditions, indicating dynamic photoinhibition and protection of PSII from excess radiation through the xanthophyll cycle. Variables related to photoprotection against UV radiation (the bulk UV absorbance and individual compounds from both the soluble and insoluble cell wall-bound fractions of the liverwort) did not show clear diel changes. This contrasted with the results obtained in the laboratory, where at least the bulk UV absorbances of both the soluble and insoluble fractions, together with some compounds of the insoluble fraction, increased in response to enhanced UV-B. DNA damage could not be demonstrated in any liverwort sample and thus did not show any diel cycle. This is in line with other studies conducted in the field under ambient UV-B, that could not either detect DNA damage, but not with field and laboratory studies using enhanced UV-B, in which DNA damage has been demonstrated.

CHAPTER 10

SEASONAL AND INTERANNUAL CHANGES OF KAEMPFEROL 3,7-O-DIGLUCOSIDE IN AN AQUATIC MOSS UNDER AMBIENT RADIATION IN THE FIELD

10.1. ABSTRACT

We studied the relationships between environmental variables and the concentration of the flavonol kaempferol 3,7-*O*-diglucoside in the aquatic moss *Bryum pseudotriquetrum* in an unforested headwater stream over a three year period. Neither environmental variables or kaempferol concentration showed significant interannual variations. However, most environmental variables (water temperature, stratospheric ozone, and photosynthetic, UV-A and UV-B radiation) showed distinct seasonal variations, as well as kaempferol concentration, which showed higher values in spring to autumn than in winter. We could not discriminate between the relative influence of the temperature and the three spectral regions of solar radiation that were measured (PAR, UV-A and UV-B) on kaempferol concentration, but UV radiation might be the most convincing factor influencing kaempferol. Thus, kaempferol 3,7-*O*-diglucoside could contribute to an adequate photoprotection in *Bryum pseudotriquetrum* in periods of high radiation.

RESUMEN

Se estudiaron las relaciones entre las variables ambientales y la concentración del flavonol kaempferol 3,7-*O*-diglucósido en el musgo acuático *Bryum pseudotriquetrum* en la zona supraforestal de un arroyo de cabecera durante un periodo de tres años. Ni las variables ambientales ni el kaempferol mostraron variaciones interanuales significativas. Sin embargo, la mayoría de las variables ambientales (temperatura del agua, ozono estratosférico y radiaciones fotosintética, UV-A y UV-B) mostraron variaciones estacionales, así como la concentración de kaempferol, que era mayor desde la primavera hasta el otoño que en el invierno. No se pudo discriminar el efecto relativo que ejercían la temperatura y las tres regiones espectrales que se midieron (PAR, UV-A y UV-B) sobre la concentración de kaempferol, pero la radiación UV parecía ser el factor determinante más convincente. Por lo tanto, el kaempferol 3,7-*O*-diglucósido podría contribuir a una adecuada fotoprotección en *B. pseudotriquetrum* en periodos de alta radiación.

10.2. INTRODUCTION

Aquatic bryophytes play important ecological roles in peatlands, lakes and headwaters, and are good bioindicators of diverse types of water pollution (Ah-Peng & Rausch de Traubenberg, 2004). These facts emphasize the relevance of their study. In particular, bryophytes are frequently the most abundant primary producers in headwaters, and have importance in nutrient cycles and food webs, supporting periphyton and providing a refuge, and occasionally food, for macroinvertebrates, amphibia and fish (Stream Bryophyte Group, 1999). Bryophyte domination in headwater streams is primarily based on their tolerance to several stress and disturbance factors, such as low temperature, ice formation, abrasion by turbulent water and suspended solids, substrate movement, seasonal desiccation, nutrient scarcity, CO_2 limitation in the stagnant parts of alkaline streams, and high photosynthetic and UV irradiances in open streams (Glime, 1984; Glime & Vitt, 1984; Slack & Glime, 1985).

Aquatic bryophytes are perennial, and may thus exhibit changing physiological characteristics throughout the year. Seasonal physiological changes may be caused by a complex interaction between environmental factors (hydrology, light availability, temperature, water chemical composition, or pollution) and internal factors, such as genetics, age, morphology, and physiological condition (Martínez-Abaigar & Núñez-Olivera, 1998). Among environmental factors, the reduction in water availability during dry periods is likely the most important one, given that desiccation may result in pigment alterations and reduction in vitality (Martínez-Abaigar *et al.*, 1994; Peñuelas & Vallcorba, 1988). Another important factor is the dramatic seasonal variation in light availability in forested streams with a deciduous canopy, since both canopy closure and opening lead to strong physiological changes (Martínez-Abaigar *et al.*, 1994; Ylla *et al.*, 2007). The influence of temperature can be also relevant and, for example, it has been demonstrated that growth rates depend on temperature in different aquatic bryophytes (Glime, 1987; Kelly & Whitton, 1987). The

availability of mineral nutrients, such as nitrogen and phosphorus, may be less important physiologically, because bryophytes have, in general, low nutrient requirements and can therefore grow well in nutritionally poor waters (Glime & Vitt, 1984; Martínez-Abaigar *et al.*, 2002).

The seasonal effects of UV radiation have been studied in diverse aquatic phototrophs, such as marine macroalgae (Abdala-Diaz *et al.*, 2006; Aguilera *et al.*, 2002), lake phytoplankton (Scott *et al.*, 2009), and flowering plants from rivers (Germ *et al.*, 2002, 2006), but only rarely in aquatic bryophytes in headwater streams (Núñez-Olivera *et al.*, 2009). UV radiation represents only about 6% of solar radiation, but may have harmful effects on photosynthetic organisms, such as alterations in DNA, photosynthesis, growth and development (Jansen *et al.*, 1998). Some protective mechanisms (accumulation of UV-absorbing compounds and antioxidants, protein and DNA repair mechanisms) can counteract these negative effects. Among aquatic environments, headwater stream ecosystems might be particularly vulnerable to the damaging effect of UV radiations, which are more intense at high altitudes (Björn *et al.*, 98), and penetrate the oligotrophic and shallow waters typical of headwater streams more readily (Frost *et al.*, 2005). Furthermore, the low temperatures which prevail for most of the year in headwaters may limit the development of protection and repair mechanisms of plants (Giordano *et al.*, 2003). The impact of UV radiations on plants is of growing concern because the anthropogenic depletion of the stratospheric ozone layer has led to increased fluxes of solar UV-B radiation (280-315 nm) being received at the Earth's surface (McKenzie *et al.*, 2007). At northern mid-latitudes, ozone losses were around 6% in 1997-2000 relative to 1980 levels, which might result in a UV-B increase of up to 12% (McKenzie *et al.*, 2003).

Bryophytes in headwaters may show seasonal physiological changes (mainly alterations in pigment composition and photosynthesis rates) when they suffer desiccation or are exposed to drastic light changes when living under a dense forest canopy (Martínez-Abaigar *et al.*, 1994; Peñuelas & Vallcorba, 1988; Ylla *et al.*, 2007). The study of continuously submerged

bryophytes in open streams could help reveal the influence of other environmental factors (such as UV radiation exposure) on bryophyte physiology in the absence of desiccation. Furthermore, the variables used to demonstrate seasonal changes have been growth (Kelly & Whitton, 1987), photosynthetic pigments (Martínez-Abaigar *et al.*, 1994; Peñuelas & Vallcorba, 1988), photosynthetic metabolism (photosynthesis rates or net primary production: Davey & Rothery, 1996; Ylla *et al.*, 2007), percent coverage (Roberts *et al.*, 2007; Sherwood *et al.*, 2000), mineral nutrition (Ellwood *et al.*, 2007; Martínez-Abaigar *et al.*, 2002; Núñez-Olivera *et al.*, 2001). However, seasonal variations in the concentration of individual UV-absorbing compounds have been little studied (Núñez-Olivera *et al.*, 2009). Finally, some of the abovementioned studies follow a seasonal sampling pattern (often obtaining only four measurements a year or less), and/or only covered one year of sampling.

The aim of this study was to relate, in the long-term (three years) and under field conditions, both seasonal and interannual variations in ambient UV radiation with changes in the concentration of the flavonol kaempferol 3,7-*O*-diglucoside, one of major flavonoids (Webby *et al.*, 1996) and a potential UV-protective compound, in the moss *Bryum pseudotriquetrum* from an unforested headstream. This species was selected because it is common and abundant in the studied zone, and thus provides plenty of biomass for analysis, and because the accumulation of UV-absorbing compounds, one of the putative protection mechanisms against UV radiation, is significantly higher than in other moss species (Arróniz-Crespo *et al.*, 2004). Among the environmental factors, we were particularly interested in studying the effect of UV radiation, due to its importance both *per se* and because of the UV increase at the Earth's surface as a consequence of the anthropogenic depletion of the stratospheric ozone layer (McKenzie *et al.*, 2003, 2007). Thus, together with water temperature, sample depth and photosynthetically active radiation (PAR), UV-A and UV-B radiations and stratospheric ozone were assessed.

10.3. MATERIALS AND METHODS

Samples of *Bryum pseudotriquetrum* were collected at the first-order stream Lumbreras, which is located in the upper basin of the River Iregua (La Rioja, northern Spain) within the limits of the Natural Park of Sierra Cebollera. The stream flows mainly over sandstones and quartzites (Purbeck-Weald facies, Jurassic-Cretaceous). The vegetation of the sampling zone is a subalpine shrubland dominated by *Vaccinium myrtillus*, *Juniperus communis* subsp. *alpina* and *Calluna vulgaris*, and intermingled with scattered *Pinus sylvestris*. The sampling site was located at 1903 m altitude and had the following coordinates: 42º00'30'' N, 02º38'40'' W. All the samples grew under similar irradiance conditions (exposed to full sun because of the absence of an overhead canopy) and were collected from a rocky waterfall with a continuous discharge throughout the year. Samples were collected around noon on a monthly basis between December 2004 and December 2007. Samples could not be collected in some winter and spring months, because of heavy snow cover. On each collection day, five samples (replicates) were collected. Samples were rinsed in the stream water, stored in ice-covered polythene bottles and transported to the laboratory in a portable icebox (temperature < 5ºC). The material was then rinsed again with stream water and 2-cm green healthy apices were selected for analysis.

The flavonol kaempferol 3,7-*O*-diglucoside was by high-performance liquid chromatography (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA), following the separation and detection method described by Arróniz-Crespo *et al.* (2006). Its quantification was made by calibration curves with the commercial external standard kaempferol (Sigma-Aldrich, St. Louis, MO, USA). Concentration was expressed as nmol per unit of DM.

On each sampling day, we determined local air and water temperature and sample depth below the water surface. On those days in which sampling river water was impossible, water temperature was estimated using the regression equation between air and water temperature

at the collection site. Photosynthetically active radiation (PAR), and UV-A and UV-B irradiances, were continuously measured at Valdezcaray (La Rioja, 42º15'37'' N, 2º58'04'' W, 2138 m), 35 km away from the sampling site, using appropriate sensors (Skye SKP-215, SKU-420 and SKU-430, Skye Instruments Ltd., Powys, UK). Given that all the radiation variables were strongly correlated ($P<0.001$), gaps in these data were filled with data generated starting from global radiation data from the meteorological observatory of Ventrosa (La Rioja, 42º10'34'' N, 2º50'44'' W, 1565 m), belonging to the Meteorological Net of the Gobierno de La Rioja (http://www.larioja.org/npRioja/default/defaultpage.jsp?idtab=442821). This was the nearest official observatory to the sampling zone (24 km). Daily data of total stratospheric ozone were obtained from the Total Ozone Mapping Spectrometer (TOMS) webpage (http://toms.gsfc.nasa.gov/) over the sampling period.

For the analysis of the radiation variables (as daily doses of PAR, UV-A and UV-B) and total stratospheric ozone, we used the mean for each sampling day and the two previous days. This period seems sufficient to find responses in MEUVAC levels (Jansen *et al.*, 1998; Newsham, 2003; Newsham *et al.*, 2002; Sullivan *et al.*, 2007). We also tested a 7–day period but did not find any difference. We additionally obtained the daily doses of UV-A, UV-B, and weighted UV-B (Plant Damage action spectrum) for the sampling site using a model (Engelsen & Kylling, 2005), but the temporal variation of these data was similar to that found in real data from Valdezcaray and Ventrosa.

Kaempferol concentration and environmental variables were examined to determine whether the temporal sequence of values deviated significantly from a random pattern, using the test of the number of runs of consecutive values above or below the median (runs analysis). The effects of year and season (winter: December–February; spring: March–May; summer: June–August; autumn: September–November) on the variables were tested using a two-way analysis of variance (ANOVA), once it had been proved

that the data met the assumptions of normality and homoscedasticity. For each year, the effect of the season on the physiological variables was tested using a one-way ANOVA. In the case of significant differences, means were then compared by Tukey's test. Bivariate correlations (Pearson's coefficients) between all the variables used were obtained. All statistical procedures were performed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

10.4. RESULTS

Changes in environmental variables along the sampling period are shown in Fig. 1. Their ranges were: 1803–12998 kJ m^{-2} d^{-1} for PAR dose, 209–1357 kJ m^{-2} d^{-1} for UV-A dose, 3.3–34.3 kJ m^{-2} d^{-1} for UV-B dose, 261–389 DU for total stratospheric ozone, and -1.6–15.8 °C for water temperature. All these variables showed evidence of a non-random temporal pattern (at least $P<0.05$ in runs analysis), exhibiting clear annual cycles in the three years studied, with the highest values in summer months in all of them except ozone, whose maxima were found in March-April. Accordingly, the effect of season was significant in all these environmental variables (Table 1). All the radiation variables were positively pair-correlated ($P<0.001$), and also with water temperature ($P<0.001$) and ozone ($P<0.01$). Samples were submersed between 0 (water level) and 5 cm depth for most of the studied period. This variable did not show annual cycles in the runs analysis or a significant effect of the season in the ANOVA (Table 1). The main effect of the year, and the interactions between year and season, were not significant for any environmental variable (Table 1).

Kaempferol 3,7-*O*-diglucoside changes over the sampling period are shown in Fig. 2, and seasonally-grouped changes in Fig. 3. Its range was 2.90–28.27 nmol mg^{-1} DM. This variable showed a random temporal pattern (runs analysis) over the sampling period, although the season affected it significantly in the ANOVA (Table 1). It showed relatively high values in June–November 2005, April–October 2006 and April–July 2007, whereas decreasing values were found in the transition from autumn to winter and in winter itself. Unfortunately, no values were available for this variable in the winters of 2005 and 2006.

All the radiation variables (PAR, UV-A and UV-B) and temperature were positively correlated with the concentration of kaempferol 3,7-*O*-diglucoside ($P<0.01$). The main effect of year was negligible (Table 1).

Table 1. Main effects of the year and season on the environmental variables and in the concentration of kaempferol 3,7-*O*-diglucoside of *Bryum pseudotriquetrum* (two-way ANOVA). ***P<0.001, **P<0.01, *P<0.05, NS non-significant.

	Year	**Season**
PAR dose	NS	***
UV-A dose	NS	***
UV-B dose	NS	***
Total ozone	NS	***
Water temperature	NS	***
Sample depth	NS	NS
Kaempferol	NS	*

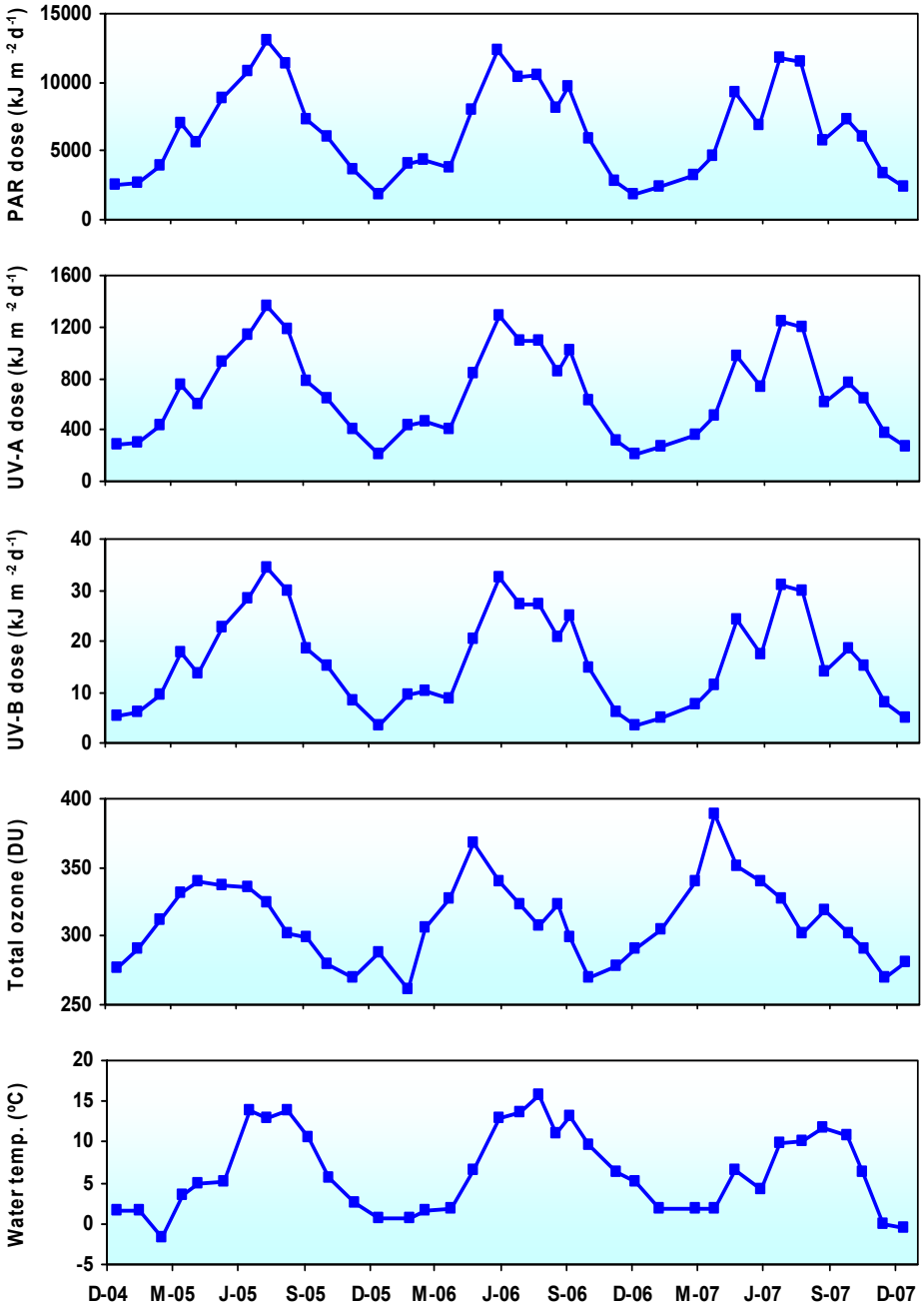

Fig. 1. Changes in environmental variables along the sampling period (December 2004 – December 2007). Daily doses of photosynthetic (PAR), UV-A and UV-B radiation, together with total stratospheric ozone and water temperature, are shown. For radiation variables and ozone, means for each sampling day and the two previous days are shown. D, December. M, March. J, June. S, September.

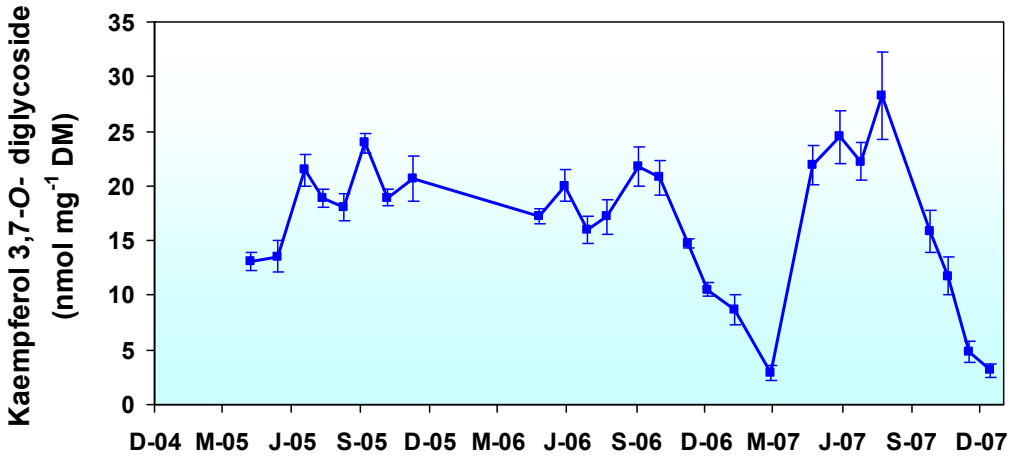

Fig. 2. Changes in the concentration of kaempferol 3,7-*O*-diglucoside in *Bryum pseudotriquetrum* in the period studied (December 2004 – December 2007). Means ± SE (n = 5) are shown. D, December. M, March. J, June. S, September.

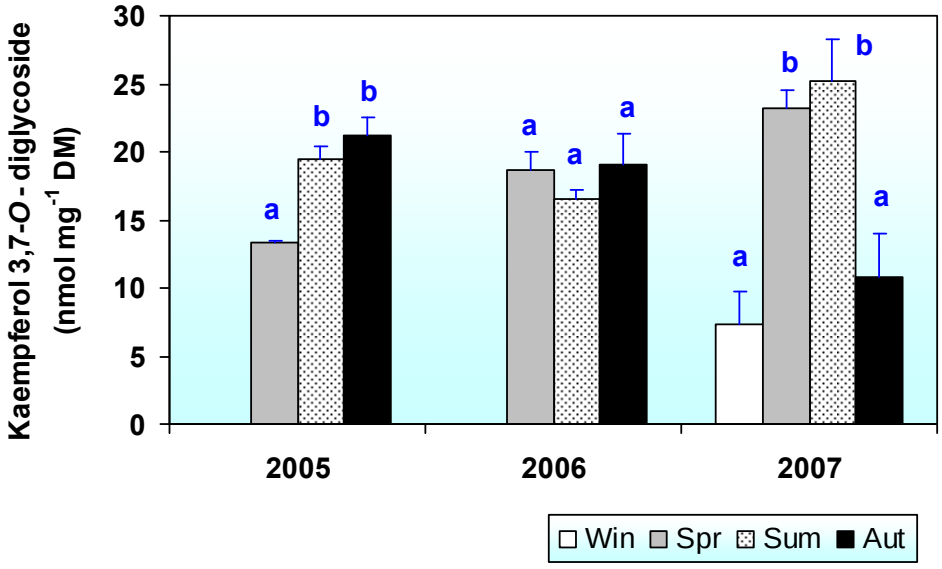

Fig. 3. Seasonally-grouped changes in the concentration of kaempferol 3,7-*O*-diglucoside of *Bryum pseudotriquetrum* in the period studied (December 2004 – December 2007). Winter, December-February; spring, March-May; summer, June-August; autumn, September-November. Different letters indicate significant seasonal differences for each year (at least $P<0.05$: one-way ANOVA and Tukey's test).

10.5. DISCUSSION

Environmental parameters did not differ between the three years studied, as shown by the ANOVA (Table 1), whereas most environmental variables showed clear annual cycles, demonstrated by both runs analysis and ANOVA. Water temperature and daily doses of PAR, UV-A and UV-B showed, in general, high values in summer, low values in winter, and intermediate values in spring and autumn. This annual variation and the positive correlations found between these variables were expected, because of their cyclic character. In particular, the strong correlation between PAR and UV was consistent with previous studies (Sobolev, 2000). Stratospheric ozone also showed clear seasonal cycles and positive correlations with radiation variables and temperature. The values of ozone (261-389 DU) and UV radiation (for example, 3.3-34.3 kJ m^{-2} d^{-1} for UV-B dose) found in our study fall in between the wide ranges pointed out for 17 stations throughout the world (201-495 for ozone and, roughly, 2.1-65.6 for UV-B dose: Häder *et al.*, 2007a). The positive correlation between ozone and UV may be surprising, because it would have been expected that low ozone values would be coupled with high UV-B levels and vice-versa. However, it must be taken into account that ozone maxima are usually reached in March-May and minima in October-November, whereas UV peaks around the summer solstice and then decreases, in a more or less parallel manner to ozone (Häder *et al.*, 2007a). Therefore, for long study periods like ours, the main factors influencing the annual variability of UV are usually solar elevation and cloudiness, whereas the ozone changes may have a smaller influence (Seckmeyer *et al.*, 2008).

Samples were collected between the water level and 5 cm depth for most of the studied period. Water levels varied little throughout the year in the rocky waterfall and changes in depth of the moss samples did not follow an annual cycle. Sherwood *et al.* (2000) also reported relatively constant depth values in a stream from September to July. At those shallow depths, and given that the streams in our zone of study have low conductivity and dissolved organic matter (Núñez-Olivera *et al.*, 2001), it can be assumed that

the attenuation of radiation with depth would not be very important (Frost *et al.*, 2005). Thus, the influence of water depth in determining the radiation climate experienced by the moss was probably not relevant in the present study.

The concentration of kaempferol 3,7-*O*-diglucoside in *Bryum pseudotriquetrum* showed little interannual physiological variations, probably because the environmental conditions were similar from year to year. In contrast, kaempferol showed clear seasonal changes, with higher values in spring to autumn than in winter, and it was positively associated with radiation levels and temperature, which also varied seasonally in a clear manner.

This is one of the first times that the level of an individual UV-absorbing compound shows a direct association with radiation levels in bryophytes. Previously, some hydroxycinnamic acid derivatives (in particular, *p*-coumaroylmalic acid) showed similar associations in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* (Núñez-Olivera *et al.*, 2009). In higher plants, kaempferol derivatives are generally induced by UV radiation (Dixon & Paiva, 1995). The double glycosylation of kaempferol 3,7-*O*-diglucoside may have a decreasing effect on its antioxidative activity, but in turn may favour its solubility and accumulation in the vacuole (Rice-Evans *et al.*, 1997; Sroka, 2005). Thus, it could be postulated that the main function of this compound in *B. pseudotriquetrum* could be passive UV absorption, as shown in other plant species (Rozema *et al.*, 2002). Flavonoids in general may play a protective role against high UV levels in other species of the same genus, given that total flavonoids in *Bryum argenteum* from Antarctica increased with decreasing ozone and, presumably, increasing UV (Markham *et al.*, 1990). In *Bryum pseudotriquetrum*, radiation changes would be crucial for the development of photoprotection mechanisms based on the accumulation of flavonoids, and these compounds could contribute to an adequate photoprotection in periods of high radiation.

We cannot discriminate between the relative influence of the temperature and the three spectral regions of solar radiation that were measured (PAR, UV-A and UV-B) on kaempferol concentration, since all these variables were strongly correlated. However, UV radiation might be the most convincing factor influencing kaempferol, in the light of the studies that have previously related the UV exposure with the accumulation of UV-absorbing compounds (Arróniz-Crespo *et al.*, 2008a; Dunn & Robinson, 2006; Martínez-Abaigar *et al.*, 2003; Newsham, 2003; Newsham *et al.*, 2002; Núñez-Olivera *et al.*, 2009; Snell *et al.*, 2007; Taipale & Huttunen, 2002). This kind of protection may be important for at least some bryophytes, given their structural simplicity (unistratose leaves) and the consequent lack of structural protection against UV, such as thick cuticles, epidermis and hairs.

Conclusions

The general conclusion of the present Doctoral Thesis is that we have progressed in the understanding of the ecophysiological responses of aquatic bryophytes to UV-B radiation, considering both the damage suffered and the protection mechanisms developed, and paying particular attention to the proteccion provided by the UV-absorbing compounds (UVACs). It is remarkable that we have studied UV-B responses 1) trying to improve the methodology for the evaluation of UVACs; 2) using diverse response variables; 3) applying different experimental conditions: enhanced UV-B in the laboratory *vs.* ambient UV-B in the field; 4) considering different temporal scales of variation (daily, monthly, seasonally, yearly); and 5) experimenting with both liverworts and mosses, that represent the two main bryophyte groups. All this may contribute to acquire a more global perspective on the effects of UV-B radiation on bryophytes, an important aspect in the context of the UV-B enhancement that is occurring in the Biosphere as a consequence of stratospheric ozone depletion.

The specific conclusions of this Doctoral Thesis are as follows:

1. We have analytically differentiated the UVACs present in the soluble and insoluble fractions of bryophytes, which correspond to the two main cell compartments where UVACs can be located: vacuoles and cell walls, respectively. In addition, in each fraction we have analyzed both the bulk UV absorbance (which is indicative of the global UV-B protection) and the concentration of individual compounds, both hydroxycinnamic acids and

flavonoids. Each fraction housed different compounds and thus may represent a different kind of UV-B protection. Also, each compound responded to UV-B in a specific manner. These aspects may contribute to properly evaluate the UV-B protection provided by UVACs in bryophytes.

2. The traditional method to extract cell wall-bound UVACs, the alkaline digestion of cell walls, was better than any of the enzymatic digestions assayed in the aquatic liverwort *Jungermannia cordifolia*, both when those compounds are to be evaluated globally and individually. The alkaline digestion was more efficient and rapid than the enzymatic ones, and was not particularly aggressive because the compounds analyzed conserved their molecular integrity.

3. As response variables to UV-B, we have used the sclerophylly index (as an indirect measurement of growth), photosynthetic pigments (chlorophyll, chlorophyll a/b ratio, and the components of the xanthophyll cycle), diverse chlorophyll fluorescence variables (F_v/F_m, Φ_{PSII}, ETR, NPQ, PI), soluble and insoluble UVACs (both globally and individually, see Conclusion 1), and DNA damage (as the amount of thymine dimers).

4. The responses of the physiological variables were, in general, stronger in the laboratory than in the field, given that the samples in the laboratory were exposed to enhanced UV-B to simulate stratospheric ozone depletion, whereas samples in the field only received ambient UV-B. In addition, PAR levels in the laboratory were lower than in the field, and thus the UV-B/PAR ratio was notably different. Therefore, extrapolation of laboratory results to the field is not recommendable.

5. The most consistent and relevant responses to enhanced UV-B in the laboratory were a decrease in F_v/F_m (an unspecific symptom of plant stress) and increases in DNA damage, the bulk UV absorbances (especially in the soluble fraction) and the concentration of certain individual UVACs, such as *p*-coumaroylmalic and *p*-coumaric acids from the liverwort *Jungermannia cordifolia* and two kaempferols from the moss *Bryum pseudotriquetrum*. These compounds merit further investigation as potential UV-B biomarkers. In the field, UVACs and DNA damage did not react to daily UV-B variations, so that these variables were not useful as UV-B biomarkers considering this temporal scale under natural conditions.

6. Chlorophyll fluorescence variables showed similar diel changes under both laboratory and field conditions. F_v/F_m, Φ_{PSII} and PI decreased at midday and subsequently recovered during the afternoon and evening, whereas NPQ showed the contrary trend. These changes, togeteher with the increase in zeaxanthin at midday, revealed a dynamic photoinhibition and the protection of PSII through the activation of the xanthophyll cycle, which was attributed mainly to the daily PAR variations.

7. Each response variable showed its own dynamicity and temporal scale of variation, which could also be influenced by the different experimental conditions applied in the laboratory and the field. Taking all this into account, and in general, 1) chlorophyll fluorescence variables and the xanthophyll cycle showed rapid responses in the laboratory and the field, exhibiting clear diel cycles under both conditions; 2) UVACs also showed rapid responses in the

laboratory (and thus diel cycles), whereas in the field they showed seasonal but not diel changes; 3) DNA damage varied quickly in the laboratory, showing diel cycles, whereas in the field no damage could be detected; and 4) the sclerophylly index varied after one month of exposure to enhanced UV-B in the laboratory, given that growth needs a longer response period than other physiological processes.

8. Liverworts and mosses seemed to show different mechanisms to cope with high UV-B levels. The bulk UV absorbance of the soluble fraction in the liverwort *Jungermannia cordifolia* was higher than that of the insoluble fraction, whereas the mosses *Bryum pseudotriquetrum* and *Fontinalis antipyretica* showed the contrary trend. In addition, the soluble and insoluble fractions of the liverwort were responsive to UV-B, whereas in the mosses only the soluble fraction was responsive. These differerences might have ecological and phylogenetic implications: 1) if the insoluble cell wall-bound fraction would constitute a more efficient UV-B screen than the soluble vacuolar one, mosses as a group would be more competitive than liverworts in UV-B-rich environments; and 2) these ecophysiological differences between liverworts and mosses could be an additional evidence of the phylogenetic distance between both groups, that nowadays is considered to be deeper than previously thought.

CONCLUSIONES

La conclusión general de la presente Tesis Doctoral es que se ha progresado en la comprensión de las respuestas ecofisiológicas de los briófitos acuáticos a la radiación UV-B, considerando tanto los daños sufridos como los mecanismos de protección desarrollados, y prestando una atención especial a la protección conferida por los compuestos absorbentes de UV (CAUV). Resulta destacable que se han estudiado las respuestas a la radiación UV-B 1) intentando mejorar la metodología para la evaluación de los CAUV; 2) utilizando diversas variables de respuesta; 3) aplicando distintas condiciones experimentales: suplemento de UV-B en el laboratorio *vs.* niveles naturales de UV-B en el campo; 4) considerando diferentes escalas temporales de variación (diarias, mensuales, estacionales, interanuales); y 5) experimentando con hepáticas y musgos como principales grupos de briófitos. Todo ello puede contribuir a la obtención de una perspectiva más global acerca de los efectos de la radiación UV-B sobre los briófitos, un aspecto importante en el marco del aumento de UV-B que está ocurriendo en la Biosfera como consecuencia de la degradación de la capa estratosférica de ozono.

Las conclusiones específicas de esta Tesis Doctoral son las siguientes:

1. Se han diferenciado analíticamente los CAUV presentes en las fracciones soluble e insoluble de los briófitos, que corresponden a los dos compartimentos celulares donde principalmente se pueden localizar: las vacuolas y las pareces celulares, respectivamente. Además, en cada fracción se han analizado tanto la absorbancia UV global (indicativa de la protección global frente a la radiación

UV-B) y la concentración de compuestos individuales, tanto ácidos hidroxicinámicos como flavonoides. Cada fracción albergaba CAUV diferentes y por lo tanto podría representar un tipo distinto de protección frente a la radiación UV-B. Así mismo, cada compuesto respondía de manera específica a la radiación UV-B. Estos aspectos pueden contribuir a una mejor evaluación de la protección que proporcionan los CAUV frente a la radiación UV-B en los briófitos.

2. El método tradicional para extraer los CAUV unidos a la pared celular, la digestión alcalina de la paredes, era mejor que cualquiera de las digestiones enzimáticas que se ensayaron en la hepática *Jungermannia cordifolia*, tanto cuando esos CAUV se evaluaron globalmente como individualmente. La digestión alcalina era más eficiente y rápida que las enzimáticas, y no era particularmente agresiva porque los CAUV analizados conservaron su integridad molecular.

3. Como variables de respuesta a la radiación UV-B, se han utilizado el índice de esclerofilia (como medida indirecta del crecimiento), los pigmentos fotosintéticos (clorofila, cociente clorofila *a/b* y componentes del ciclo de las xantofilas), diversas variables de fluorescencia de clorofilas (F_v/F_m, Φ_{PSII}, ETR, NPQ, PI), los CAUV solubles e insolubles (tanto globalmente como individualmente: ver Conclusión 1), y el daño al ADN (cantidad de dímeros de timina).

4. Las respuestas de las variables fisiológicas fueron, en general, más intensas en el laboratorio que en el campo, ya que en el laboratorio se aplicaba a las muestras un suplemento artificial de UV-B para simular la degradación de

ozono estratosférico, mientras que en el campo las muestras recibían tan sólo niveles naturales de UV-B. Además, los niveles de PAR en el laboratorio eran menores que en el campo, por lo que la proporción UV-B/PAR era notablemente diferente. Por lo tanto, no resulta recomendable la extrapolación de los resultados de laboratorio a condiciones de campo.

5. Las respuestas más consistentes y relevantes al suplemento de UV-B en el laboratorio fueron un descenso de F_v/F_m (un síntoma inespecífico de estrés) y aumentos en el daño al ADN, la absorbancia UV global (especialmente en la fracción soluble) y la concentración de ciertos CAUV individuales, como los ácidos *p*-cumaroilmálico y *p*-cumárico en la hepática *Jungermannia cordifolia*, y dos tipos de kaempferol en el musgo *Bryum pseudotriquetrum*. Estos compuestos merecen una investigación más amplia como biomarcadores potenciales de radiación UV-B en briófitos. En el campo, los CAUV y los daños al ADN no respondieron a las variaciones diarias de UV-B, por lo que estas variables no resultaban útiles como biomarcadores de UV-B en esta escala temporal en condiciones naturales.

6. Las variables de fluorescencia de clorofilas mostraron ciclos diarios similares en condiciones de laboratorio y campo. F_v/F_m, Φ_{PSII} y PI disminuían a mediodía y posteriormente se recuperaban durante la tarde, mientras que NPQ mostraba una tendencia contraria. Estos cambios, junto con el aumento de la concentración de zeaxantina a mediodía, revelaban una fotoinhibición dinámica y una protección del PSII mediante la activación del ciclo de las xantofilas, lo que se atribuyó principalmente a las variaciones diarias de PAR.

7. Cada variable de respuesta mostró su propia dinamicidad y escala temporal

de variación, que podían estar influidas también por las distintas condiciones experimentales aplicadas en el laboratorio y el campo. Integrando todo ello, y en general, 1) las variables de fluorescencia de clorofilas y el ciclo de las xantofilas respondían rápidamente en el laboratorio y el campo, y mostraban ciclos diarios evidentes en ambas situaciones; 2) los CAUV también mostraron respuestas rápidas en el laboratorio, y por lo tanto ciclos diarios, mientras que en el campo se registraron cambios estacionales pero no diarios; 3) el daño al ADN variaba rápidamente en el laboratorio y describía ciclos diarios, mientras que en el campo no se pudo detectar; y 4) el índice de esclerofilia variaba únicamente después de un mes de exposición a un suplemento de UV-B en el laboratorio, dado que el crecimiento necesita un periodo mayor de respuesta que otros procesos fisiológicos.

8. Las hepáticas y los musgos parecían mostrar diferentes mecanismos para enfrentarse a altos niveles de UV-B. La absorbancia UV global de la fracción soluble de la hepática *Jungermannia cordifolia* era mayor que la de la fracción insoluble, mientras que en los musgos *Bryum pseudotriquetrum* y *Fontinalis antipyretica* ocurría lo contrario. Además, en la hepática, ambas fracciones eran sensibles a la radiación UV-B, pero en los musgos solamente lo era la fracción soluble. Estas diferencias pueden tener implicaciones ecológicas y filogenéticas: 1) si la fracción insoluble, ligada a la pared celular, constituyese un filtro UV-B más eficiente que la fracción soluble vacuolar, los musgos como grupo serían más competitivos que las hepáticas en ambientes ricos en radiación UV-B; y 2) estas diferencias ecofisiológicas entre hepáticas y musgos podrían ser una prueba más de la distancia filogenética entre ambos grupos, que actualmente se considera mayor de lo que se pensaba anteriormente.

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Los mejores momentos

- “Espera que me reaccione yo”..Paula Gómez Villaescusa. Enero/2010. (Primera hora del Lunes).

- “La leche vale pa to”. Paula Febrero/2010. (Resuspendiendo bacterias para congelarlas).

- “Ya no voy a beber más cervezas, prefiero cubatas”. Yo. Febrero/2010-9:45am.

- “¡¡¡AAA!!!, es peor tener fiesta” Paula. Marzo/2010-10:10am. (Tras un puente de tres días).

- “Se la va a meter debajo de la mesa y tracataca” Marian del Castillo 5/10/2011 (Limpiando la pulpa de la uva).

- “Eso no se autoclavó bien, porque se autoclavó mal”. Marian.

- “Te voy a dejar porque tengo el teléfono en la oreja”. Marian. 2012 (Se lo dijo a la familia por teléfono para hablar mejor con ellos por teléfono).

- “¡¡¡Es verdad!!! La Cumarina se echa a pelo” Marian. 7-12-2011 Puente de la constitución, (Poniendo viales de Vacuolas con nuestra amiga HPLC).

- “Esto tuyo háztelo mirar”. Marian. (Cualquier día).

- “Yo no gasto más de lo normal y… esto se vacía”. Laura Monforte López. 23/12/2010. (Antes de las vacaciones).

- “La becaria soy yo… y me lo como todo” Laura. 2011. (A alguien había que reñir).

- “Cuando se borran es mejor poner el número” Laura. 10-10-2011. (Poniendo eppendorf).

- “No es que eso este mal, es que no lo hemos hecho bien” Laura. 23/01/2012. (Con la tesis de Gabi).

- “¿Necesitáis algo? Pregunto yo. Y responde Marian. “Un chico que nos abanique”. Y contesta Laura a lo lejos, “NO… uno que nos dé calor”. 26/10/2011.

- “Algo está mal seguro… pero que ha sido inconsciente” Belén Fadrique. 2011. (Cuando no sale algo en el laboratorio).

- “Para mandar primero hay que tener una opinión”. Belén. Abril/2011

- “Me vengo aquí el domingo y me las cepillo” Yo. 25/01/2012. (Preparando las gráficas bonitas de la tesis).

- “Aquí hay más de lo que es mío” Rafa.

- “Si es que te lo doy todo” Rafa. 13/01/2012. (Con los dímeros de timina).

- “¿Vamos a tomar café? Dice Fidel. - “No, primero vamos a limpiar las bolas”. Contesta Laura. A lo que Fidel responde, - “Lo primero es lo primero”.