UNIVERSIDAD DE LA RIOJA

TESIS DOCTORAL

Título
Ultraviolet-absorbing compounds in bryophytes: phylogeny, ecology and biomonitorization
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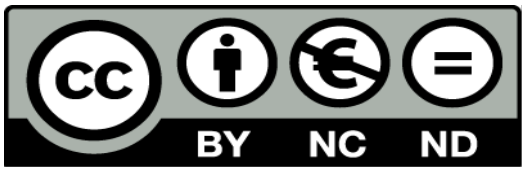
Titulación
Departamento
Agricultura y Alimentación
Curso Académico
2013-2014



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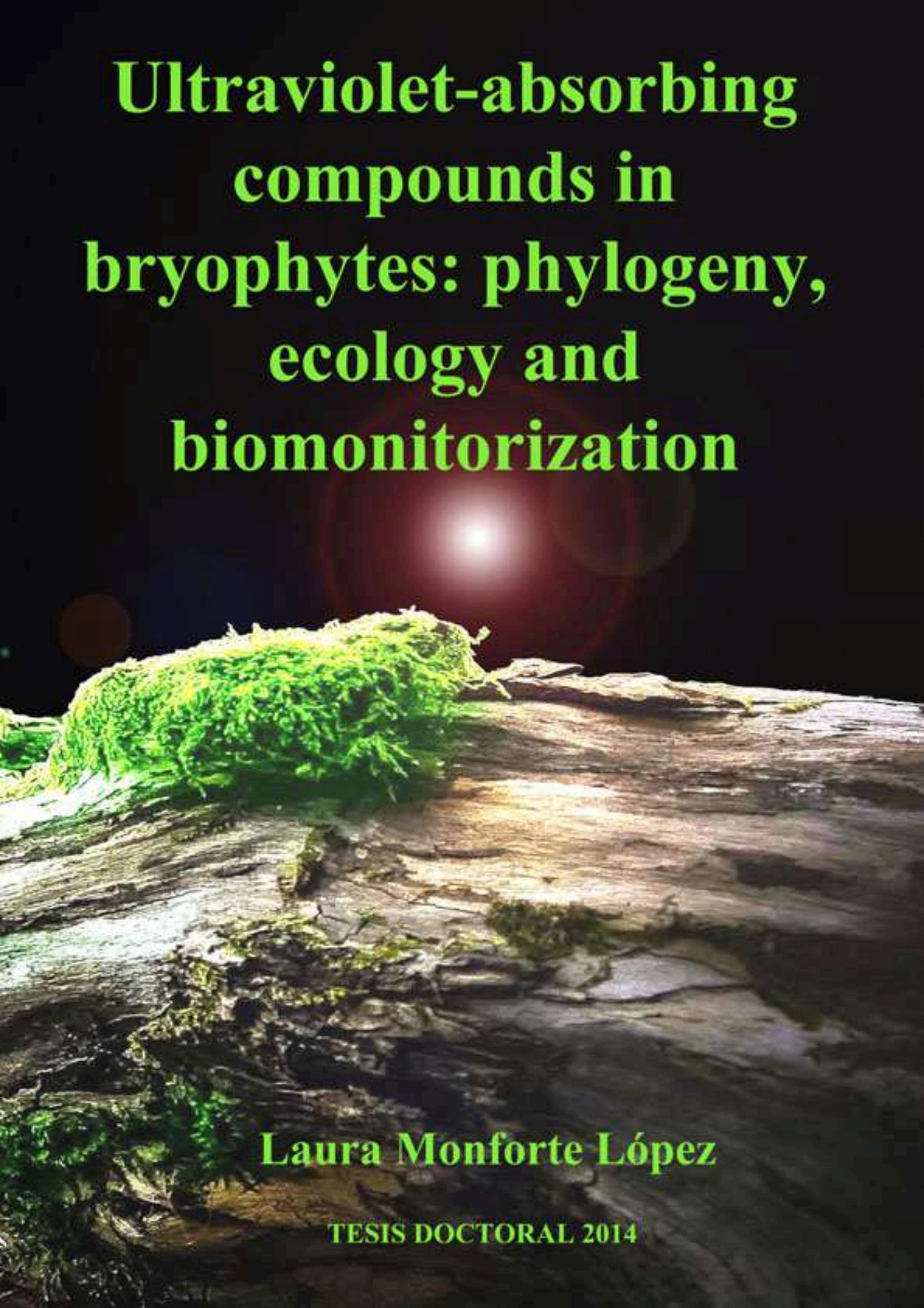

Laura Monforte López

TESIS DOCTORAL 2014

UNIVERSIDAD DE LA RIOJA

DEPARTAMENTO DE AGRICULTURA Y ALIMENTACIÓN

Ultraviolet-absorbing compounds in bryophytes: phylogeny, ecology and biomonitorization

Memoria presentada por:

LAURA MONFORTE LÓPEZ

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

(Programa de Doctorado "Ecosistemas Agrícolas Sostenibles")

Fdo. LAURA MONFORTE LÓPEZ

2014

UNIVERSIDAD DE LA RIOJA

DEPARTAMENTO DE AGRICULTURA Y ALIMENTACIÓN

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

INFORMAN:

Que la Memoria titulada "**Ultraviolet-absorbing compounds in bryophytes: phylogeny, ecology and biomonitorization**" ha sido realizada en las Áreas de Fisiología Vegetal y Botánica de la Universidad de La Rioja bajo nuestra dirección por LAURA MONFORTE LÓPEZ, Licenciada en Ciencias Ambientales. Considerando que se encuentra concluida, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

Y para que conste, expedimos el presente informe en Logroño, a 18 de Junio de dos mil catorce.

Fdo. Javier Martínez Abaigar Fdo. Encarnación Núñez Olivera

A mis padres

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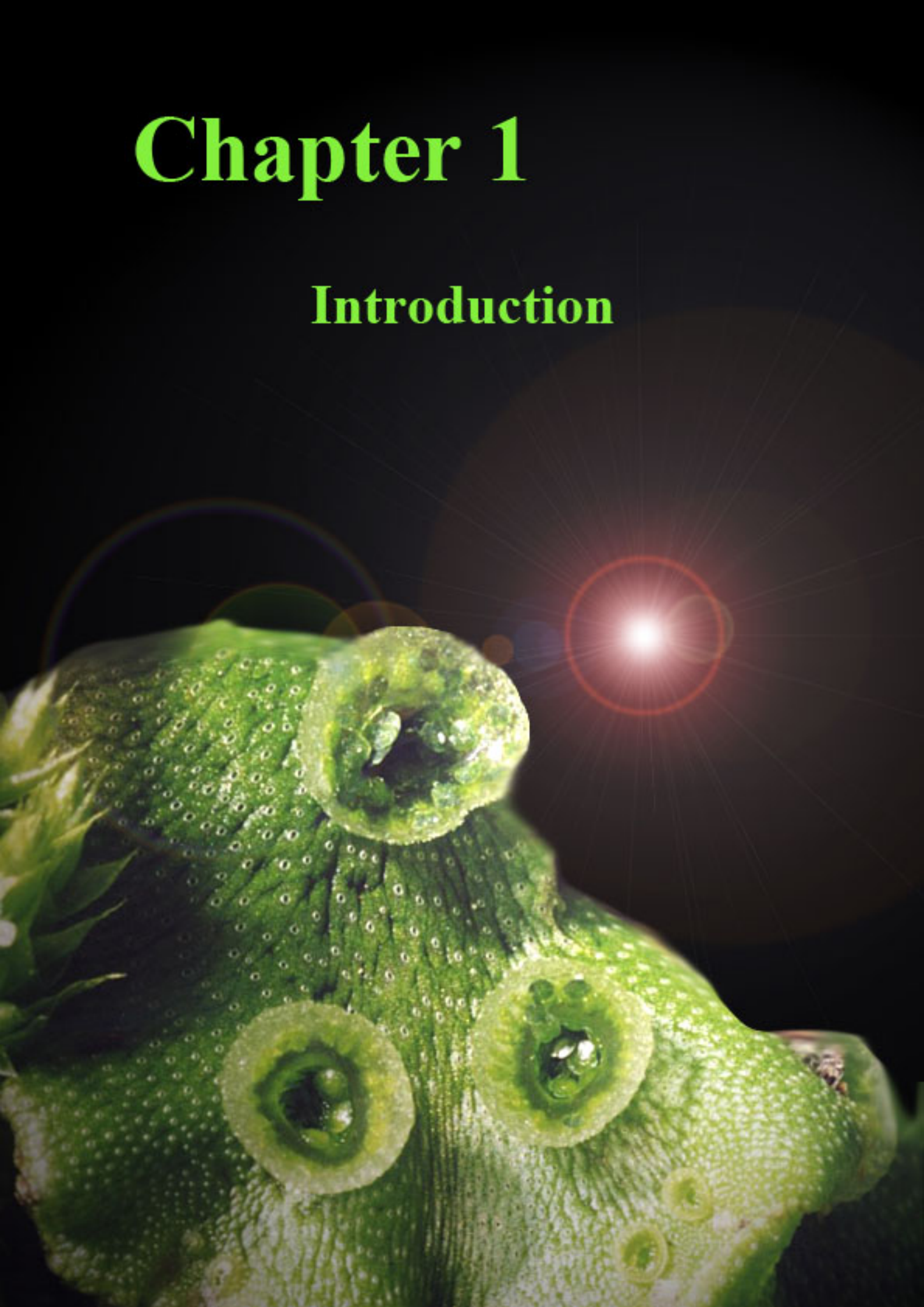
Chapter 1

Introduction



1.- INTRODUCTION

Bryophytes

Bryophytes are photosynthetic organisms with a diplobiontic life-cycle characterized by a marked alternation of generations in which a haploid phase gives rise, following fertilization, to a diploid phase that eventually undergoes meiosis to regenerate haploid cells. The transition from haploid to diploid generation is characterized by fusion of gametes, yielding a zygote. The reverse transition is marked by meiosis, resulting in spore formation. The gametophyte bears the sex organs and the sporophyte holds the sporangium. However, unlike tracheophytes, in which the dominant life cycle stage is the diploid sporophyte, bryophytes are characterized by dominant, perennial gametophytic stages, with relatively small, unbranched, monosporangiate sporophytes, that remain permanently attached to the maternal gametophytes. Bryophytes can be either dioecious or monoecious. In dioecious bryophytes, male and female sex organs are borne on different gametophyte plants. In monoecious bryophytes, both are borne on the same plant.

Traditionally, bryophytes comprise three main lineages (Shaw & Goffinet, 2000): liverworts (Division Marchantiophyta), mosses (Bryophyta), and hornworts (Anthocerotophyta). The major bryophyte lineages differ from one another in a variety of attributes, most conspicuously in the architecture of the vegetative gametophyte body and the sporophyte. The sporophytes of many moss species do not continue to grow after spore production and are essentially annual. Liverwort sporophytes are especially short-lived and generally persist for weeks to months. The sporophytes of hornworts are potentially indeterminate in life span, but in nature they rarely persist for more than a year (Shaw *et al.*, 2011).

Bryophytes lack typical organs of vascular plants (root, stem, leaf), thick cuticle, epidermis, functional stomata, and trichomes, although exhibit a wide range of structural complexity. Attachment of a bryophyte to the substrate is by simple filaments called rhizoids. Likewise, many bryophytes have an axial structure with expansions resembling stems and leaves, but not comparable in complexity to these organs, and are called caulidia and phyllidia, respectively.

Liverworts have been divided according to gametophyte growth form: complex thalloids, where the thallus typically has both photosynthetic and storage tissues, with dorsal pores (not stomata); simple thalloids, that lack significant tissue differentiation; and the leafy liverworts, typically with two rows of lateral leaves and a row of ventral amphigastria (often lacking). Several smaller groups fall outside this simple classification (Crandall-Stotler, 2009). In mosses, gametophytes are always leafy. Their growth forms (orthotropic, with major axes perpendicular to the substrate, and plagiotropic, with principal axes parallel to the substrate) are associated with two major moss types. The acrocarpous mosses are characterized by sparsely or unbranched stems, grow upright and the archegonia are formed terminally on the main stems. Pleurocarpous, in contrast, have archegonia borne on short lateral branches, so that sporophytes occur laterally along the gametophyte stems. All hornworts have thalloid gametophytes that harbor endosymbiotic cyanobacteria of the genus *Nostoc* (Villarreal *et al.*, 2010). Despite their relatively limited species diversity, hornworts are of particular phylogenetic interest because they appear to be the sister group to tracheophytes (Qiu *et al.*, 2006).

Bryophytes lack a fully developed vascular system for the transport of water and nutrients, which drastically limits their size. In addition, they cannot develop complex supporting or conducting tissues because, unlike tracheophytes, bryophytes always lack lignin (Popper, 2008) although can produce lignans (Ligrone *et al.*, 2008). Physiologically, bryophytes are poikilohydrous. This characteristic represents not only a limitation but also a successful life strategy, since bryophytes combine poikilohydry with the capacity of maintaining latent life after desiccation (Oliver *et al.*, 2005). Thus, 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 tracheophytes) in the colonization of certain harsh environments, such as bare rocks, protosoils or tree barks, and their ability to survive cold and dry conditions is unknown in other principal plant groups (Glime, 2007). The hydration state in most bryophytes is dependent on ambient humidity. However, many species have evolved structures that modify water uptake and storage, limiting water loss. As a consequence, bryophytes may differ greatly in their evaporation properties (Rice *et al.*, 2001).

Given that desiccation tolerance and other ecophysiological traits also vary among bryophytes (Proctor, 2009), different species exhibit a broad range of ecological amplitudes, including aquatics which prevail in, for example, mountain streams, deep lakes and certain wetlands.

Globally, bryophytes are highly diverse, are present in most environments, and play a significant ecological role in many terrestrial and freshwater ecosystems. Moreover, bryophytes contribute to a significant proportion of the production and biomass in a variety of ecosystems (Vanderpoorten & Goffinet, 2009).

Ultraviolet radiation and its effects on photosynthetic organisms

Ultraviolet (UV) radiation is a minority component (about 6%) of the total solar radiation reaching the Earth surface, and represents a natural environmental factor that has been involved in the appearance of diverse adaptive changes in organisms through the development of life (Cockell & Knowland, 1999). The CIE (Commission Internationale d'Eclairage) divided UV in three wavelength categories: UV-A (315-400 nm) is the most abundant UV component and the less hazardous one; UV-B (280-315 nm) provokes diverse biochemical and physiological responses, including some nocive effects; and UV-C (<280 nm) is extremely harmful but absent at ground level due to stratospheric oxygen, ozone and other atmospheric gases absorption (Hollósy, 2002). Both UV-A and UV-B radiation play also a regulatory role on the physiology of plants (Hideg *et al.*, 2013). In the last decades, the study of UV effects on photosynthetic organisms has received considerable attention, especially because the anthropogenic reduction of stratospheric ozone has led to an increase in UV-B in the biosphere (since only UV-B and not UV-A is absorbed by stratospheric ozone). These effects are highly dependent on wavelength, and different biological weighting functions have been conceived to calculate the biologically effective UV (UV_{BE}). UV_{BE} 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 UV have dealt with UV-B. 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.

UV irradiance at the Earth surface 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 the ozone layer is recovering, models predict an increase of UV radiation at ground level due to changes in other climatic factors such as cloud cover (McKenzie *et al.*, 2011).

In photosynthetic organisms, UV radiation has traditionally been considered as a stress factor. However, in last years it has been suggested that it is also an environmental regulator controlling gene expression to activate protective and repair mechanisms, mediated by the UV-B specific photoreceptor UV RESISTANCE LOCUS (uvr8) (Jenkins, 2009; Hideg *et al.*, 2013; Morales *et al.,* 2013). Anyway, an excess of 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; Hollósy, 2002). Also, DNA alterations, oxidative damage, and changes in mineral absorption can occur (Tuteja *et al.*, 2009). 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 have developed a number of mechanisms directed to minimize the penetration of UV, prevent oxidative stress and repair the damage caused (Jansen *et al*., 1998): accumulation of UV-absorbing compounds (UVACs: 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 UV on photosynthetic organisms have been studied mainly in terrestrial plants, especially of agricultural interest (Jansen *et al.,* 1998; Caldwell *et al*., 2007), and in marine phytoplankton and macroalgae (Day & Neale, 2002; Häder *et al*., 2007).

Bryophytes and UV radiation

In the last decade, bryophytes are acquiring increasing importance in the context of UV research and are mentioned in recent reviews (Björn, 2007; Caldwell *et al.*, 2007; Häder *et al.*, 2007; Newsham & Robinson, 2009). To our knowledge, around 93 papers containing original data have been published on this topic (Table 1.1). The bryophyte responses have been analysed in terms of diverse variables, such as the photosynthetic pigment composition, the rates of net photosynthesis and dark respiration, some variables of chlorophyll fluorescence, especially the maximum quantum yield of PSII (F_v/F_m), the sclerophylly index (the quotient between the dry mass and the shoot area), the level of UVACs, DNA damage, protein concentration, length growth, and morphological symptoms (both macro- and microscopic).

The results obtained are diverse, and contradictory results have been reported, since UV has been found either to stimulate, depress or have no effect on the bryophyte performance (Johanson *et al.*, 1995; Björn *et al.*, 1998; Searles *et al.*, 1999; Phoenix *et al.*, 2001). The increase in UVACs, the most usual response of vascular plants to enhanced UV (Searles *et al.*, 2001a), has been manifested also in bryophytes in laboratory conditions (Fabón et al., 2010, 2012a, 2012b) but less frequently in field conditions (Markham *et al.*, 1998; Arróniz-Crespo *et al.*, 2006, 2011). The effect of UV seems to depend on the species considered, the experimental design, and other additional factors such as temperature, water availability, etc. The water status of bryophytes and their desiccation tolerance is one of the factors influencing decisively on UV effects (Takács *et al.*, 1999; Sonesson *et al.*, 2002; Lappalainen *et al.*, 2008; Turnbull *et al.*, 2009).

The research has focused mainly on terrestrial and semiaquatic bryophytes from Antarctic habitats and circumpolar heathlands and peatlands. In particular, a certain number of studies have been conducted on bryophytes from circumpolar bogs, most of them under long-term field conditions (Searles *et al.*, 1999, 2001b, 2002; Robson *et al.*, 2003), concluding that ambient UV levels seemed to affect morphogenic rather than production processes. In Arctic bogs, UV 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 UV-B (Gehrke *et al.*, 1996), but in a two-year experiment biomass production did not change (Gehrke, 1998).

Table 1.1. 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
Arróniz-Crespo *et al.* (2011)	*Hylocomium splendens* (M), *Polytrichum commune* (M), *Barbilophozia lycopodioides* (L)	T	F, S, VLo	C, Fl, G, 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

Reference	Used species	Ambient	Type of experiment	Variables used
Clarke & Robinson (2008)	*Bryum pseudotriquetrum* (M), *Ceratodon purpureus* (M), *Schistidium antarctici* (M)	T	F, N, M	Mt2
Clarke *et al.* (2012)	*Ceratodon purpureus* (M), *Bryoerythrophyllum recurvirostre* (M), *Bryum pseudotriquetrum* (M), *Schistidium antarctici* (M)	T	L, N, VLo	G
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
Fabón *et al.* (2010)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	L, S, M	A, Mt2, Fl, Sc
Fabón *et al.* (2011)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	L, S, Sh	A
Fabón *et al.* (2012a)	*Jungermannia exsertifolia* subsp. *cordifolia* (L)	A (R)	L, S, Sh	Mt2, Fl, PP

Reference	Used species	Ambient	Type of experiment	Variables used
Fabón *et al.* (2012b)	*Fontinalis antipyretica* (M), *Bryum pseudotriquetrum* (M)	A (R)	L, S, M	A, Mt2, Fl, Sc
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
Gerotto *et al.* (2012)	*Physcomitrella patens* (M)	T	L, ?, Sh	Fl, Mt1
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

Reference	Used species	Ambient	Type of experiment	Variables used
Hespanhol *et al.* (2014)	*Andreaea heinemannii* subsp. c*rassifolia* (M), *Andreaea heinemannii* subsp. *heinemannii* (M), *Andreaea rothii* subsp. *falcata* (M), *Andreaea rothii* subsp. *rothii* (M), *Andreaea rupestris* (M), *Grimmia decipiens* (M), *Grimmia horrida* (M), *Grimmia laevigata* (M), *Grimmia montana* (M), *Grimmia orbicularis* (M), *Grimmia pulvinata* (M), *Grimmia ramondii* (M), *Grimmia reflexidens* (M), *Grimmia tergestina* (M), *Grimmia trichophylla* (M), *Racomitrium aciculare* (M), *Racomitrium affine* (M), *Racomitrium aquaticum* (M), *Racomitrium elongatum* (M), *Racomitrium heterostichum* (M), *Racomitrium lanuginosum* (M), *Racomitrium macounii* subsp. *macounii* (M), *Racomitrium obtusum* (M)	T	F, N, Sh	Mt2
Hooijmaijers & Gould (2007)	*Isotachis lyallii* (L), *Jamesoniella colorata* (L)	T	F, N, ?	Fl, Mt2, PP
Hughes *et al.* (2006)	*Drepanocladus sp.* (M)	T	F, N, Sh	Z

Reference	Used species	Ambient	Type of experiment	Variables used
Hui *et al.* (2013)	*Bryum argenteum* (M)	T	L, S, Sh	Fl, PP, Ox, PS1, PS2, U
Hui *et al.* (2014)	*Bryum argenteum* (M), *Didymodon vinealis* (M)	T	L, S, Sh	Fl, PP, Mt1, Mt2, U
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

Reference	Used species	Ambient	Type of experiment	Variables used
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

Reference	Used species	Ambient	Type of experiment	Variables used
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
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

Reference	Used species	Ambient	Type of experiment	Variables used
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

Reference	Used species	Ambient	Type of experiment	Variables used
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
Núñez-Olivera *et al.* (2010)	*Bryum pseudotriquetrum* (M), *Fontinalis antipyretica* (M)	Semi 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
Ren *et al.* (2010)	*Distichium inclinatum* (M), *Encalypta alpine* (M)	T	L, S, VSh	Fl
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

Reference	Used species	Ambient	Type of experiment	Variables used
Schipperges & Gehrke (1996)	*Hylocomium splendens* (M), *Sphagnum fuscum* (M)	T, P	F-L, S, M- VLo	G, H, P
Schroeter *et al.* (2012)	*Bryum argenteum* (M)	T	F, N, Sh	G, Fl, P, PP
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

Reference	Used species	Ambient	Type of experiment	Variables used
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
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

Other studies in Arctic bogs used modulated systems which provide UV supplements proportional to ambient UV levels (Niemi *et al.*, 2002a). Bryophytes from mountain streams have also been studied, because mountain streams might be particularly exposed to the effects of UV, because of high altitude (the biologically active UV increases 5-20% per 1000 m altitudinal increase: Björn *et al.*, 1998) and relatively low depths where UV can easily reach the organisms (Frost *et al.*, 2005). The effects of UV on bryophytes from mountain streams have recently been reviewed (Martínez-Abaigar & Núñez-Olivera, 2011).

Studies under both field and controlled conditions have been conducted, using lamps to increase UV-B simulating stratospheric ozone depletion or filters to study the effects of current UV levels. In addition, field studies using natural gradients of UV in the temporal or spatial scales have also been carried out. Studies of this type are, for example, temporal variations in the Antarctic during the occurrence of "ozone hole" (Newsham *et al.*, 2005; Robinson *et al.*, 2005), seasonal and interannual variations of UV levels (Núñez-Olivera *et al.*, 2009), or spatial variations with altitude (Arróniz-Crespo *et al.*, 2006; Harris, 2009).

The factors influencing the UV responses of bryophytes can be divided into internal and environmental ones. With respect to internal factors, UV 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 UV effects (Martínez-Abaigar & Núñez Olivera, 2011). Interspecific differences in UV tolerance may be based on the degree of development of repairing and protecting mechanisms, such as the accumulation of UVACs (Martínez-Abaigar *et al.*, 2003). Also, a direct relationship between UV tolerance and desiccation tolerance has been suggested in bryophytes (Takács *et al.*, 1999; Csintalan *et al.*, 2001; Turnbull *et al.*, 2009). Another internal factor is the tissue age (Arróniz-Crespo *et al.*, 2008b).

Among environmental factors, a low culture temperature (2ºC) has been shown to enhance the adverse effects of UV in the UV-sensitive *Fontinalis antipyretica*, but not in the more UV-tolerant *Jungermannia exsertifolia* subsp. *cordifolia* (Núñez-Olivera *et al.*, 2004).

Other growth conditions, such as the levels of mineral nutrients (Martínez-Abaigar *et al.*, 2008), the presence of heavy metals (Otero *et al.*, 2006), the PAR level and the proportions UV/PAR, the previous light acclimation to sun or shade conditions

(Núñez-Olivera *et al.*, 2005), or the previous acclimation to high UV levels (Martínez-Abaigar *et al.*, 2009), may influence the responses to UV.

UV-absorbing compounds and their use as biomonitors of UV in bryophytes

UV-absorbing compounds (UVACs) are mainly phenolic derivatives that include a large and diverse group of molecules with a hydroxyl group attached to an aromatic ring. These include simple phenols, such as cinnamic acids, and polyphenols, such as flavonoids. The accumulation of UVACs is the most common response of vascular plants and bryophytes to UV (Searles *et al.*, 2001a; Newsham & Robinson, 2009). There is not a simple direct relationship between this accumulation and UV tolerance, but UVACs can reduce UV 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 UVACs, both in bryophytes and other photosynthetic organisms, has been the bulk UV absorbance. In bryophytes, both the bulk level of UVACs (global UV absorbance) and the individual specific compounds can be measured in two cell compartments (Schnitzler *et al.*, 1996; Fabón *et al.*, 2010): the soluble fraction (mainly located in the vacuoles) and the insoluble fraction (bound to the cell walls). Each fraction may have a different composition, may respond differently to UV, and may represent a different protection mechanism against UV effects (Fabón *et al.*, 2010). It has been postulated that the cell wall-bound fraction would represent a more efficient UV screen than the vacuolar fraction (Clarke & Robinson, 2008). This analytical differentiation between soluble and insoluble fractions has rarely been considered in bryophytes until recently (Fabón *et al.*, 2010, 2012a, 2012b; Soriano *et al.*, 2013; Hespanhol *et al.*, 2014).

In bryophytes, an increase in UV absorbance with increasing UV has been much more frequently found in liverworts than in mosses. In addition, the absorption spectra in the UV band are notably different in both groups (Arróniz-Crespo *et al.*, 2004; Otero *et al.*, 2008). The apparently different behaviour of both bryophyte groups with respect to the accumulation of methanol-extractable UVACs 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 UVACs could have been one of the factors favouring their success in the colonization of land, an environment with higher UV levels than the aquatic habitat.

Another critical point regarding UVACs in bryophytes is that the measurement of the bulk UV absorbance might be insufficient to assess the effects of UV because each individual compound may respond to UV 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; Fabón et al., 2010, 2012a). These studies make clear that, although all the individual compounds contribute to the bulk UV absorbance, their UV responses can be different: some of them may increase under enhanced UV 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 UV.

The relationships found between the concentrations of hydroxycinnamic acid derivatives in *Jungermannia exsertifolia* subsp. *cordifolia* and the spatial and temporal changes in UV (Núñez-Olivera *et al.*, 2009) make progress in the study of using bryophytes as UV bioindicators. This may take advantage of the well-known bioindication ability of 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 UV levels in northern Europe (Otero *et al.*, 2009). These studies, together with those of Ryan *et al.* (2009) and Snell *et al.* (2009) in the Antarctic, are the first ones using individual UVACs of bryophytes in UV 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 UV bioindication purposes, an adequate selection of both variables

and species must be carried out, and the use of hydroxycinnamic acid derivatives in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* has been proposed for this aim (Martínez-Abaigar & Núñez-Olivera, 2011). In particular, one of its hydroxycinnamic acid derivatives, *p*-coumaroylmalic acid, is specifically induced by enhanced UV in laboratory studies under different PAR levels, and correlates well with the temporal changes in UV under field conditions. In addition, a chemically similar UVAC (*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). Thus, this species is worth to be further studied in this context.

Chapter 2

Objectives

2.- OBJECTIVES

The general objective of the present Doctoral Thesis was to study the significance of ultraviolet-absorbing compounds (UVACs) in bryophytes, in relation with phylogenetic, ecological and biomonitorization aspects. This general objective can be divided into several more specific ones:

1. To carry out a phylogenetic study to know if the different evolutionary lineages of bryophytes (mainly liverworts and mosses), had the same compartmentation of UVACs between the soluble (mainly vacuolar) and insoluble (bound to the cell wall) fractions. This may have evolutionary importance since bryophytes, and in particular liverworts, were the first plants colonizing land, where they had to cope with substantially higher levels of UV radiation than those experienced in the primitive waters.

2. To study the influence of environmental factors on UVACs accumulation and compartmentation. In this way, we quantified different environmental variables: sun exposure, orientation, the Ellenberg indicator values for light, humidity, temperature, continentality, pH and nitrogen, and several other bryological attributes.

3. To analyse UVACs in herbarium samples of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* from all over Spain, evaluating their usefulness in the reconstruction of past UV levels and applying novel aspects such as: 1) the use of collection periods within the year presumably longer to those previously used; and 2) the use, for the first time in bryophytes, of both the soluble and insoluble fractions of UVACs for biomonitorization.

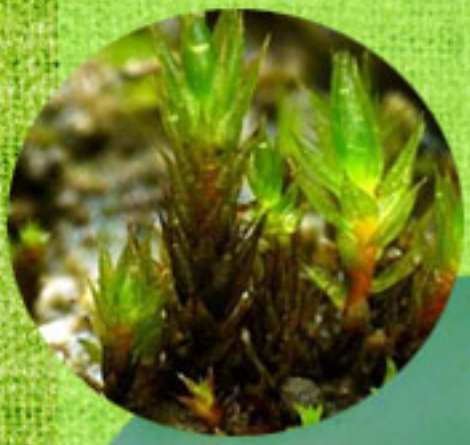
4. To evaluate the influence of environmental factors on the physiology of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* across a wide latitudinal and altitudinal gradient in streams of the main mountain chains of Spain. The physiological variables measured were: UVACs (differentiating the soluble and insoluble fractions, and analysing both the bulk levels and individual compounds), the maximum quantum yield of PSII (F_v/F_m), sclerophylly index and DNA damage.

5. To compare the physiological results obtained in fresh samples of *Jungermannia exsertifolia* subsp. *cordifolia* with those derived from the herbarium samples of the same species, which were collected in the same mountain chains of Spain considering similar latitudinal and altitudinal gradients. This comparison may help assess the influence of sample storage on the integrity of UVACs, as well as the influence of stratospheric ozone degradation on the UVACs of bryophytes.

Chapter 3

Compartmentation of ultraviolet absorbing compounds in liverworts and mosses: linking UV, phylogeny and ecology






3.1.- ABSTRACT

In the conquest of land, photosynthetic organisms had to cope with substantially higher levels of UV radiation than those experienced in the primitive aquatic environment. Given that bryophytes (in particular, liverworts) represent the water-to-land transition from algal ancestors to tracheophytes, the study of how bryophytes cope with UV radiation may be evolutionarily important. The main response of bryophytes to UV is the accumulation of UV-absorbing compounds (UVACs), that can be located either in the vacuoles (soluble fraction, SUVACs) or bound to the cell walls (insoluble fraction, IUVACs). This compartmentation is important because the cell wall fraction may provide a more efficient UV protection to the cell than the vacuolar fraction. In this context, our aim was to study whether the levels and compartmentation of UVACs were different between the two most important evolutionary lineages of bryophytes, liverworts and mosses, and to evaluate the phylogenetic and ecological implications of this difference.

We analysed by spectrophotometry the levels and compartmentation of UVACs in 87 bryophytes (22 liverworts, 64 mosses and 1 hornwort), and also measured the sclerophylly index of the samples (the ratio of dry mass *vs.* surface area). In general, liverworts had higher levels of SUVACs and lower levels of IUVACs than mosses, and vice-versa. Exceptions to this general rule appeared in species with peculiar UVAC compositions or compartmentations, such as *Atrichum undulatum*, *Bartramia pomiformis*, and species of *Bryum*, *Porella* and *Frullania*. The difference between liverworts and mosses is an additional evidence of the phylogenetic distance between both groups, and thus UVACs compartmentation represents a new ecophysiological trait that could have been evolutionarily important in the colonization of new UV-rich environments after the conquest of land by plants, because mosses would have been more competitive in sunny UV-rich environments. UVAC levels and compartmentation were also useful to differentiate acrocarpous and pleurocarpous mosses, and also peculiar mosses as *Sphagna*, but not the main evolutionary lineages in liverworts or the different Orders of mosses and liverworts.

We tested the relationships between UVACs accumulation and compartmentation, and diverse environmental variables including orientation, sun exposure, and the Ellenberg indicator values for light, temperature, moisture, reaction and nitrogen, as well as several other bryological attributes. There were not clear and solid relationships between UVACs and the environmental variables, in particular those determining the UV amount received by the samples. Thus, UVACs may be constitutive and privative of each species, and may not be usually induced by the natural ambient UV levels.

3.2.- INTRODUCTION

In photosynthetic organisms, an excessive UV exposure may induce a number of harmful effects, such as DNA damage, oxidative damage, and diverse alterations in photosynthesis, which may globally lead to alterations in growth and development (Jansen *et al.*, 1998). Nevertheless, these effects have mainly been found in experiments using unreal and exaggerated UV levels, and thus UV, in particular UV-B, has gone from being considered for decades a generic stressor to become a specific regulator (Jansen & Bornman, 2012), just another type of wavelengths stimulating the adaptation of organisms.

Diverse responses of tracheophytes to UV seem to be protection and repair mechanisms against its harmful effects: production of structural protections, such as hairs, thick cuticles and multilayered epidermis, and accumulation of UV-absorbing compounds (UVACs). Chemically, UVACs are mainly phenolic compounds, mostly flavonoid and hydroxycinnamic acid (HCA) derivatives. Structural protections and UVACs can reduce UV penetration and, consequently, damage to the potential targets in the cells, such as chlorophyll, photosynthetic enzymes and DNA. Other protection and repair mechanisms are antioxidant systems and DNA-repairing systems, such as photolyases.

The most usual response of plants to UV radiation, in particular UV-B, is the accumulation of UVACs (Searles *et al.*, 2001b; Newsham & Robinson, 2009). In tracheophyte leaves, UVACs are found in most plant cell compartments, including trichomes, waxes, cell walls, cytosol, vacuole, endoplasmic reticulum, chloroplast, nucleus and small vesicles (Bornman *et al.*, 1997; Zhao & Dixon, 2010). UVACS can be translocated between the different compartments, frequently from the soluble pool in the vacuole to be covalently bound to the cell wall (Fischbach *et al.*, 1999; Kaffarnik *et al.*, 2006). Also, since flavonoids and HCAs are synthesized in the endoplasmic reticulum, they are released in small vesicles that can migrate to different compartments, such as the vacuole and the cell wall (Agati *et al.*, 2012). Compartmentation is crucial to fully understand the diverse functions of UVACs: UV screening, insect attraction, pathogen defense, antioxidants, etc. (Agati & Tattini, 2010; Agati *et al.*, 2012). For example, flavonoids in the cell wall of epidermal cells may

absorb efficiently UV-B wavelengths, whereas vacuolar flavonoids might constitute an antioxidant system (Agati *et al.*, 2012).

The phylogenetic-evolutionary perspective of UVACs and their compartmentation in relation to UV radiation has been little studied (Cockell & Knowland, 1999). However, in the conquest of land, photosynthetic organisms had to cope with substantially higher levels of UV radiation than those experienced in the primitive aquatic environment. The structurally simple bryophytes were the first true plants that colonized land (Qiu *et al.*, 2007; Zobell *et al.*, 2010), although the early colonization of terrestrial environment by plants such as liverworts could be favoured by the previous presence of other organisms, such as symbiotic fungi (Bidartondo *et al.*, 2011) or cyanobacteria. In any case, the three different evolutionary lineages of bryophytes (liverworts, mosses and hornworts), that nowadays are considered strongly supported monophyletic groups (Qiu *et al.*, 2007), played a key role in land colonization. Liverworts are considered to be the earliest diverging land plants, then mosses evolved, and finally hornworts, that constitute the sister group to tracheophytes. Being the first true plants colonizing land, it is a challenging question, evolutionarily relevant, how bryophytes managed to cope with UV, and whether UVACs compartmentation was important in that context.

Bryophytes have logically been much less studied regarding UV radiation than tracheophytes, but, surprisingly, Wolf *et al.* (2010) concluded that the model moss *Physcomitrella patens* was more capable of surviving UV-B stress than *Arabidopsis*. One of the bases of this UV tolerance, given that bryophytes lack the structural UV defences typical of tracheophytes, might be the accumulation of UVACs (Newsham & Robinson, 2009). Regarding this, it is important to note that, as in tracheophytes, UVACs are compartmented in bryophytes, although much more simply. Basically, UVACs can be mainly located in the vacuoles or in the cell walls (Verhoeven & Liefveld, 1997; Fabón *et al.*, 2010, 2012a, 2012b; Newsham, 2010). This can be an essential differentiation, because cell wall-bound UVACs are more effective in UV protection than vacuole compounds, given that they form a continuous screen that protects the whole cell content (Clarke & Robinson, 2008).

In spite of the importance of UVACs compartmentation in bryophytes, only a few works have studied this issue. In their pioneer study, Clarke & Robinson (2008), they showed that among three mosses from the Antarctic, *Bryum pseudotriquetrum* had almost equal concentrations of soluble (vacuolar) and insoluble (cell wall-bound) UVACs (respectively, SUVACs and IUVACs), whereas *Ceratodon purpureus* and *Schistidium antarctici* showed a predominant (up to 9 times higher) concentration of cell wall-bound UVACs. Afterwards, Fabón *et al.* (2010) analyzed this trait for the first time in a liverwort (*Jungermannia exsertifolia* subsp. *cordifolia*) in mid-latitudes, finding that the concentration of SUVACs was about 2.5-fold higher than those of IUVACs. Lappalainen (2010) in subarctic *Pleuorozium schreberi* and *Polytrichum juniperinum*, Fabón *et al.* (2012b) in *Bryum pseudotriquetrum* and *Fontinalis antipyretica*, Soriano *et al.* (2013) in 8 species of *Sphagnum* from Norway, and Hespanhol *et al.* (2014) in saxicolous mid-latitude mosses belonging to the genera *Andreaea*, *Grimmia*, and *Racomitrium*, confirmed that IUVACs were more abundant than SUVACs in all the mosses studied (up to 12-fold times higher), but no liverwort was analysed in any of those studies.

Being aware of the contrasted UVAC compartmentation between mosses and the only liverwort analysed to date, our first aim was to carry out a phylogenetic study to know if the different evolutionary lineages of bryophytes (mainly liverworts and mosses), had the same UVACs compartmentation. In addition, given that the different functions UVACs may assume in the cell depend on their compartmentation (Agati *et al.*, 2012), our second aim was to study the influence of environmental factors on UVACs accumulation and compartmentation. In this way, we quantified different environmental variables: sun exposure, orientation, the Ellenberg indicator values for light, humidity, temperature, continentality, pH and nitrogen, and several other bryological attributes. It was expected that these variables would influence UVACs accumulation and compartmentation.

3.3.- MATERIALS AND METHODS

Plant material

Samples of 87 bryophytes (22 liverworts, 64 mosses and 1 hornwort: Table 3.1) were collected in northern Spain, mostly in La Rioja (84 species). The remaining 3 species were collected in the Basque Country, a neighbouring region to La Rioja. The relative proximity of the samples collected allowed to complete sample collection in only a few days (between 17 and 25 June 2010), to prevent, as far as possible, the radiation differences between the samples caused by the different collection dates. Nomenclature of species followed Hill *et al.* (2006) for mosses and Ros *et al.* (2007) for liverworts and hornworts.

The material was stored in a small polythene container (each species separately) and transported to the laboratory in a portable icebox (temperature below 5ºC). Once in the laboratory, healthy green apices (1-3 cm depending on the species) were cut and cleaned in distilled water to eliminate all types of debris.

Sclerophylly Index (SI)

The surface area of 8-10 prostrate apices of each species was measured (LI-COR LI-3000 area meter), after blotting them with filter paper to remove excess water. Then, apices were weighed to obtain fresh mass (FM) and then dried (60ºC for 24 h) and re-weighed to obtain dry mass (DM). SI was calculated, in 5 replicates, as the quotient between DM and the surface area.

Analysis of UV-absorbing compounds (UVACs)

Apices were weighed (FM) and surface area was measured (see above). Then apices were frozen in liquid N_2 and stored in eppendorf tubes at -80ºC until analysis. UVACs were analyzed in the soluble fraction (SUVACs), mainly located in the vacuole, and in the insoluble fraction (IUVACs), bound to the cell walls. In both fractions, the bulk level of UVACs was analysed globally by spectrophotometry, following Schnitzler *et al.* (1996), Arróniz-Crespo *et al.* (2006) and Fabón *et al.* (2010).

Table 3.1. Data of the 87 bryophytes (22 liverworts, 64 mosses and 1 hornwort) used in this study. For each species, the following data are provided (for details on the codes used, see the text): Abb, abbreviation used in the two Principal Components Analyses conducted; Ord, taxonomical Order the species belongs to; Alt, Lat and Lon, respectively altitude, latitude and longitude where the sample was collected; Ori, orientation; Imm, immersion index; Exp, exposure index; L and T, Ellenberg indicator values for Light and Temperature, respectively; TJan and TJul, mean January and July temperatures (ºC), respectively; F, Ellenberg indicator value for Moisture; Pre, mean annual precipitation (mm); K, R, and N, Ellenberg indicator values for continentality, reaction (pH), and nitrogen, respectively; LF1, primary life form; sex, distribution of sex organs; Len, gametophore size (mm); Bio, major biome (main biogeographic element within Europe); Eli, eastern limit of the species; SI, sclerophylly index; SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UV-absorbing compounds (in terms of the area under the absorbance curve in the interval 280-400 nm, $AUC_{280-400}$, per unit of DM); I/S, the quotient between the bulk levels of IUVACs and SUVACs. Nomenclature of species followed Hill *et al.* (2006) for mosses and Ros *et al.* (2007) for liverworts and hornworts.

Taxon	Abb	Ord	Alt (m)	Lat (ºN)	Lon (ºE)	Ori	Imm	Exp	L	T	TJan (ºC)	TJul (ºC)	F	Pre (mm)	K	R	N	LF1	Sex	Len (mm)	Bio	Eli	SI (mg cm^{-2})	SUVAC ($AUC_{280-400}$ mg^{-1} DM)	IUVAC ($AUC_{280-400}$ mg^{-1} DM)	TUVAC ($AUC_{280-400}$ mg^{-1} DM)	I/S
Aneura pinguis (L.) Dumort.	*Ap*	Metz	1030	42.12	-2.64	2	2	1	8	6	3.3	14.2	8	1220	5	7	2	Mt	D	60	3	6	2.66 ± 0.09	27.61 ± 1.13	24.81 ± 1.67	53.21 ± 1.00	0.88 ± 0.09
Apometzgeria pubescens (Schrank) Kuwah.	*Au*	Metz	1220	42.15	-2.68	2	1	1	4	3	1.7	12.9	6	1457	6	7	3	We	D	35	4	6	2.60 ± 0.08	58.40 ± 8.09	36.50 ± 3.29	98.54 ± 13.50	0.60 ± 0.05
Barbilophozia hatcheri (A.Evans) Loeske	*Bh*	Jung	1150	42.19	-2.71	2	1	2	6	2	1.1	12.3	6	1287	6	2	2	We	D	40	2	6	1.57 ± 0.05	27.69 ± 0.65	24.71 ± 1.17	50.92 ± 1.52	0.87 ± 0.02
Chiloscyphus polyanthos (L.) Corda	*Cp*	Jung	50	43.37	-1.84	1	3	1	6	4	3.3	14.2	9	1230	6	6	4	At	M	30	5	6	1.77 ± 0.09	46.55 ± 2.64	22.65 ± 1.38	69.20 ± 3.85	0.49 ± 0.02
Conocephalum salebrosum Szweyk., Buczkowska & Odrzykoski	*Cs*	Marc	820	42.29	-2.59	4	2	2	7	3	3.1	15.0	7	1055	6	7	5	Mt	D	90	5	6	1.59 ± 0.24	27.05 ± 0.61	35.63 ± 0.89	61.79 ± 0.78	1.29 ± 0.02
Frullania tamarisci (L.) Dumort.	*Ft*	Pore	1163	42.19	-2.71	2	1	2	7	3	3.4	13.9	4	1339	4	5	2	Ms	D	80	5	2	3.55 ± 0.17	11.76 ± 0.74	49.80 ± 1.54	61.56 ± 2.28	4.25 ± 0.14
Jungermannia exsertifolia Steph. subsp. *cordifolia* (Dumort.) Váña	*Je*	Jung	1767	42.01	-2.63	5	3	3	7	2	1.9	12.6	9	1776	6	2	3	Ms	D	81	4	3	2.65 ± 0.08	45.04 ± 2.28	25.69 ± 1.33	71.98 ± 3.46	0.56 ± 0.01
Lophocolea bidentata (L.) Dumort.	*Lb*	Jung	745	42.29	-2.58	2	1	1	7	3	3.4	14.6	6	1094	5	5	3	We	M	60	7	3	2.16 ± 0.12	62.47 ± 1.94	45.24 ± 1.69	106.98 ± 2.17	0.70 ± 0.04
Lunularia cruciata (L.) Lindb.	*Lc*	Marc	947	42.21	-2.67	2	2	1	7	8	3.7	15.3	6	931	4	6	7	Mt	D	40	9	2	2.98 ± 0.27	15.99 ± 1.39	36.97 ± 1.00	52.96 ± 0.52	2.36 ± 0.26
Marchantia polymorpha L.	*Mp*	Marc	998	42.16	-2.66	4	2	3	8	6	3.2	14.7	6	998	5	5	5	Mt	D	100	5	6	2.62 ± 0.24	42.56 ± 2.41	40.63 ± 4.10	84.39 ± 4.26	0.94 ± 0.12
Marsupella sphacelata (Gieseke ex Lindenb.) Dumort.	*Ms*	Jung	1969	42.01	-2.65	3	3	3	8	3	1.3	11.9	7	2020	4	2	1	We	D	20	2	3	3.57 ± 0.18	35.70 ± 1.83	47.88 ± 1.78	83.58 ± 2.27	1.36 ± 0.09

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Pellia endiviifolia (Dicks.) Dumort.	*Pe*	Foss	591	42.31	-2.53	2	2	2	4	4	3.5	14.7	8	1097	5	9	4	Mt	D	50	8	6	0.74 ± 0.06	34.71 ± 4.09	19.78 ± 0.73	58.51 ± 1.95	0.50 ± 0.02
Pellia epiphylla (L.) Corda	*Pp*	Foss	1029	42.12	-2.64	2	2	1	4	4	3.3	14.3	8	1188	5	5	4	Mt	M	50	5	6	1.18 ± 0.07	131.07 ± 8.82	46.14 ± 2.50	179.28 ± 7.61	0.38 ± 0.03
Plagiochila porelloides (Torrey ex Nees) Lindenb.	*Po*	Jung	1181	42.19	-2.71	2	1	3	6	3	3.2	14.1	4	1252	5	7	4	Tf	D	60	5	6	1.59 ± 0.06	39.69 ± 2.00	29.47 ± 1.59	68.88 ± 2.29	0.76 ± 0.07
Porella arboris-vitae (With.) Grolle	*Pa*	Pore	900	42.15	-2.65	2	1	2	5	4	3.1	13.9	4	1545	5	7	2	Ms	D	80	9	2	3.78 ± 0.21	20.29 ± 1.10	90.42 ± 8.42	110.70 ± 9.19	4.45 ± 0.29
Porella platyphylla (L.) Pfeiff.	*Pl*	Pore	901	42.15	-2.65	4	1	2	5	3	3.5	15.2	4	968	5	6	3	Fa	D	50	5	6	4.51 ± 0.50	62.60 ± 3.87	31.72 ± 1.46	93.96 ± 3.28	0.51 ± 0.05
Radula complanata (L.) Dumort.	*Rc*	Radu	823	42.29	-2.59	2	1	1	7	3	3.4	14.5	5	1165	5	7	3	Ms	M	30	5	6	4.74 ± 0.44	62.10 ± 2.25	26.75 ± 0.40	88.84 ± 1.89	0.43 ± 0.02
Reboulia hemisphaerica (L.) Raddi	*Rh*	Marc	765	42.29	-2.58	2	1	2	7	4	3.5	14.5	7	1259	4	7	4	Mt	M	40	8	6	2.63 ± 0.13	27.65 ± 2.39	32.34 ± 3.17	59.99 ± 5.55	1.17 ± 0.01
Riccardia multifida (L.) Gray	*Rm*	Metz	1022	42.12	-2.64	2	2	1	7	4	3.1	13.8	8	1379	5	4	2	Mt	M	25	5	6	1.40 ± 0.05	119.64 ± 6.63	41.75 ± 0.80	161.39 ± 7.04	0.35 ± 0.02
Riccia beyrichiana Hampe ex Lehm.	*Rb*	Ricc	1329	42.03	-2.59	1	2	3	9	3	4.1	14.2	7	1402	3	2	3	St	M	25	5	2	3.24 ± 0.55	29.49 ± 1.37	76.97 ± 3.86	107.36 ± 4.80	2.53 ± 0.09
Scapania aspera Bernet & M.Bernet	*Sa*	Jung	928	42.17	-2.63	2	1	2	5	3	2.8	13.6	5	1439	4	9	2	We	D	60	5	3	3.18 ± 0.10	27.53 ± 0.85	21.97 ± 0.83	49.50 ± 1.24	0.80 ± 0.04
Scapania undulata (L.) Dumort.	*Su*	Jung	1030	42.12	-2.64	2	2	1	6	3	3.0	13.6	9	1382	5	5	2	Ms	D	100	5	3	2.80 ± 0.10	59.59 ± 3.87	31.09 ± 2.02	90.68 ± 5.82	0.52 ± 0.01
Abietinella abietina (Hedw.) M.Fleisch.	*Aa*	Hypn	937	42.17	-2.64	2	1	3	8	6	3.6	15.8	2	846	6	7	2	We	D	120	2	6	4.14 ± 0.09	5.15 ± 0.32	61.24 ± 4.15	66.56 ± 4.39	11.53 ± 0.52
Anomodon viticulosus (Hedw.) Hook. & Taylor	*Av*	Hypn	907	42.15	-2.65	2	1	2	4	3	3.6	15.1	4	1029	5	8	5	Mr	D	120	5	6	4.41 ± 0.20	11.31 ± 0.61	175.42 ± 6.80	191.59 ± 5.53	16.11 ± 1.05
Antitrichia curtipendula (Hedw.) Brid.	*Ac*	Hypn	1291	42.14	-2.68	2	1	2	6	3	2.0	12.5	4	1764	4	6	2	We	D	200	5	3	4.93 ± 0.38	8.01 ± 0.91	88.25 ± 3.40	95.24 ± 5.49	11.00 ± 0.68
Atrichum undulatum (Hedw.) P.Beauv.	*An*	Poly	713	42.28	-2.58	2	1	2	6	6	3.3	14.6	6	1121	5	4	5	Tf	M	70	5	6	2.78 ± 0.03	49.66 ± 18.48	34.17 ± 2.78	81.14 ± 17.51	0.81 ± 0.32
Aulacomnium palustre (Hedw.) Schwägr.	*Al*	Brya	1484	42.14	-2.69	2	2	3	7	2	3.1	14.0	7	1263	6	3	2	Tf	D	90	3	6	1.74 ± 0.08	16.85 ± 1.41	129.05 ± 11.52	155.79 ± 9.75	8.27 ± 0.20
Bartramia pomiformis Hedw.	*Bp*	Brya	1141	42.19	-2.70	2	1	2	5	3	2.9	13.8	5	1406	6	4	2	Tuft	M	65	5	6	4.47 ± 0.12	101.25 ± 4.74	138.58 ± 9.36	239.83 ± 9.94	1.38 ± 0.13

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Brachythecium rutabulum (Hedw.) Schimp.	*Bu*	Hypn	1135	42.19	-2.70	5	1	2	5	6	3.5	14.7	4	1058	5	6	6	Mr	M	120	7	3	1.84 ± 0.14	7.63 ± 0.65	111.95 ± 6.70	115.22 ± 6.05	14.45 ± 1.29
Bryum alpinum Huds. ex With.	*Ba*	Brya	1355	42.20	-2.72	4	2	3	8	6	3.2	13.3	7	1564	4	4	2	Tf	D	60	7	3	6.12 ± 0.44	22.98 ± 1.30	159.71 ± 9.20	175.87 ± 7.41	6.72 ± 0.76
Bryum pseudotriquetrum (Hedw.) P.Gaertn. et al.	*Bs*	Brya	1787	42.01	-2.63	4	2	2	7	6	3.2	14.1	7	1245	5	7	3	Tf	D	100	3	6	2.20 ± 0.12	50.41 ± 1.40	31.53 ± 1.71	81.94 ± 2.15	0.63 ± 0.04
Bryum weigelii Spreng.	*Bw*	Brya	1901	42.01	-2.64	5	2	3	8	2	0.7	11.7	8	1836	6	4	3	Tf	D	107	2	6	2.46 ± 0.21	119.93 ± 4.70	45.81 ± 1.10	165.07 ± 5.68	0.39 ± 0.02
Calliergonella cuspidata (Hedw.) Loeske	*Cc*	Hypn	1296	42.14	-2.68	2	2	2	8	3	3.4	14.5	7	1108	5	7	4	We	D	120	7	6	3.98 ± 0.12	7.80 ± 0.30	163.14 ± 17.75	174.38 ± 21.19	22.49 ± 3.34
Campylopus introflexus (Hedw.) Brid.	*Ci*	Dicr	1789	42.01	-2.63	2	1	3	8	6	3.6	14.8	2	1094	3	2	2	Tuft	D	50	7	2	4.03 ± 0.07	14.25 ± 0.94	57.01 ± 1.86	70.74 ± 2.86	4.01 ± 0.18
Ceratodon purpureus (Hedw.) Brid.	*Cu*	Dicr	1207	42.11	-2.63	2	1	3	8	6	3.4	14.6	2	1078	2	5	3	Tf	D	35	3	6	3.29 ± 0.09	25.62 ± 2.16	91.47 ± 3.19	112.45 ± 5.02	3.18 ± 0.04
Cinclidotus fontinaloides (Hedw.) P.Beauv.	*Cf*	Pott	885	42.26	-2.65	4	3	3	7	4	3.4	14.5	8	1135	5	8	4	At	D	120	8	3	4.83 ± 0.40	5.74 ± 0.60	29.31 ± 1.19	35.89 ± 1.71	4.98 ± 0.41
Cratoneuron filicinum (Hedw.) Spruce	*Cl*	Hypn	960	42.21	-2.67	2	2	1	7	6	3.7	15.1	7	1016	5	7	5	We	D	60	6	6	2.99 ± 0.13	10.11 ± 0.07	150.21 ± 2.08	156.95 ± 2.67	14.72 ± 0.12
Ctenidium molluscum (Hedw.) Mitt.	*Cm*	Hypn	903	42.15	-2.65	2	1	2	5	4	3.3	14.2	4	1216	5	8	2	Mr	D	60	5	3	3.57 ± 0.10	5.97 ± 0.55	112.68 ± 5.22	117.82 ± 5.57	19.68 ± 2.95
Cynodontium bruntonii (Sm.) Bruch & Schimp.	*Cb*	Dicr	1000	42.13	-2.65	2	1	1	4	4	2.9	13.9	3	1381	4	2	3	Cu	M	30	7	3	2.61 ± 0.11	21.92 ± 1.79	103.08 ± 5.10	125.96 ± 7.72	4.39 ± 0.02
Dichodontium palustre (Dicks.) M.Stech	*Dp*	Dicr	1786	42.01	-2.63	2	2	2	8	2	2.5	13.1	8	1521	6	2	2	Tf	D	90	4	3	2.62 ± 0.11	32.42 ± 1.17	171.20 ± 10.18	203.62 ± 10.61	5.29 ± 0.32
Dicranum scoparium Hedw.	*Ds*	Dicr	1150	42.19	-2.71	2	1	1	5	6	3.4	14.4	4	1150	5	4	2	Tuft	D	90	3	6	4.87 ± 0.10	16.22 ± 1.11	138.35 ± 11.60	158.61 ± 11.70	9.10 ± 1.28
Encalypta streptocarpa Hedw.	*Es*	Enca	1867	42.01	-2.64	2	1	2	5	6	3.2	14.4	5	1195	5	8	2	Tuft	D	60	5	5	3.62 ± 0.13	55.06 ± 4.01	80.86 ± 3.19	135.91 ± 5.82	1.50 ± 0.13
Fissidens grandifrons Brid.	*Fg*	Dicr	824	42.29	-2.59	4	2	1	8	8	4.9	15.7	8	875	2	9	4	Tuft	D	85	8	3	2.39 ± 0.18	25.94 ± 1.20	102.06 ± 14.52	127.99 ± 15.70	3.90 ± 0.40
Fontinalis antipyretica Hedw.	*Fa*	Hypn	1175	42.19	-2.71	5	3	1	8	6	3.3	14.3	9	1158	5	6	5	At	D	500	5	6	2.90 ± 0.15	5.74 ± 0.34	86.04 ± 6.31	89.17 ± 6.58	14.77 ± 1.54
Fontinalis squamosa Hedw.	*Fs*	Hypn	1327	42.08	-2.55	1	3	3	8	4	3.0	13.7	9	1409	4	2	3	At	D	400	7	2	4.31 ± 0.32	19.06 ± 0.93	50.22 ± 2.45	69.90 ± 3.81	2.57 ± 0.09
Grimmia decipiens (Schultz) Lindb.	*Gd*	Grim	1203	42.11	-2.63	2	1	3	9	4	3.5	13.8	1	1449	4	1	1	Cu	M	25	9	2	4.32 ± 0.12	10.23 ± 0.68	69.85 ± 5.13	80.08 ± 4.66	7.02 ± 0.85
Hedwigia stellata Hedenäs	*Hs*	Hedw	1203	42.11	-2.63	2	1	3	9	6	3.4	13.7	2	1520	5	2	1	Mr	M	50	7	3	4.56 ± 0.16	8.26 ± 0.43	33.17 ± 1.84	41.42 ± 2.10	4.03 ± 0.20

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Homalothecium lutescens (Hedw.) H.Rob.	*Hl*	Hypn	1149	42.19	-2.70	2	1	2	9	4	3.7	15.0	2	969	5	8	2	We	D	100	8	3	3.72 ± 0.09	13.30 ± 0.60	206.80 ± 5.56	220.10 ± 4.95	15.62 ± 1.12
Homalothecium sericeum (Hedw.) Schimp.	*He*	Hypn	1010	42.17	-2.68	3	1	1	8	3	3.5	14.7	2	1085	5	7	4	Mr	D	100	8	4	3.55 ± 0.17	15.62 ± 0.74	190.75 ± 10.45	206.37 ± 11.14	12.20 ± 0.27
Hookeria lucens (Hedw.) Sm.	*Hu*	Hook	1023	42.12	-2.64	2	2	1	2	3	3.4	13.9	7	1375	3	6	4	Ms	M	60	7	2	1.93 ± 0.16	6.91 ± 0.93	35.77 ± 1.24	41.92 ± 1.56	5.39 ± 0.72
Hylocomium splendens (Hedw.) Schimp.	*Hp*	Hypn	1162	42.19	-2.70	5	1	2	6	3	3.2	13.9	4	1271	6	5	2	We	D	175	3	6	2.84 ± 0.18	19.82 ± 1.67	241.09 ± 21.00	241.59 ± 13.29	11.23 ± 0.36
Hypnum cupressiforme Hedw.	*Hc*	Hypn	1293	42.14	-2.68	2	1	2	5	6	3.4	14.6	4	1098	5	4	4	Ms	D	60	6	6	1.72 ± 0.16	13.53 ± 0.08	102.33 ± 6.00	111.34 ± 8.70	7.71 ± 0.54
Isothecium alopecuroides (Lam. ex Dubois) Isov.	*Ia*	Hypn	779	42.29	-2.59	4	1	2	5	4	3.4	14.5	5	1159	6	6	5	De	D	50	5	3	3.64 ± 0.10	9.24 ± 0.32	87.64 ± 8.67	92.82 ± 11.16	9.00 ± 0.86
Leucobryum juniperoideum (Brid.) Müll.Hal.	*Lj*	Dicr	52	43.37	-1.84	1	1	1	7	4	3.9	15.2	6	1263	3	2	2	Cu	D	105	7	3	0.96 ± 0.04	5.56 ± 0.18	34.79 ± 2.86	43.28 ± 1.39	6.89 ± 0.07
Leucodon sciuroides (Hedw.) Schwägr.	*Ls*	Hypn	1008	42.16	-2.66	5	1	2	8	5	3.5	15.2	4	996	5	6	4	Mr	D	50	6	4	5.12 ± 0.24	4.06 ± 0.17	64.91 ± 4.48	72.10 ± 3.80	16.80 ± 0.87
Mnium hornum Hedw.	*Mh*	Brya	1032	42.12	-2.64	2	1	1	5	3	3.4	14.5	6	1129	4	3	4	Tf	D	70	7	3	3.34 ± 0.13	18.44 ± 0.83	79.70 ± 2.23	98.18 ± 3.10	4.33 ± 0.12
Mnium lycopodioides Schwägr.	*Ml*	Brya	1229	42.15	-2.68	2	1	1	5	3	-0.4	11.2	6	2198	6	7	3	Tf	D	70	4	6	2.94 ± 0.05	64.62 ± 7.36	67.22 ± 4.93	131.84 ± 9.54	1.07 ± 0.15
Neckera complanata (Hedw.) Huebener	*Nc*	Hypn	1146	42.19	-2.71	2	1	3	4	3	3.6	14.7	4	1104	5	7	4	Fa	D	50	5	3	2.59 ± 0.09	9.42 ± 0.53	100.31 ± 3.70	109.72 ± 4.14	10.71 ± 0.38
Neckera crispa Hedw.	*Nr*	Hypn	960	42.17	-2.63	1	1	2	3	3	3.0	14.1	4	1395	5	7	3	We	D	200	7	3	4.12 ± 0.09	5.35 ± 0.42	69.35 ± 3.55	77.22 ± 3.21	13.64 ± 0.73
Orthotrichum rupestre Schleich. ex Schwägr.	*Or*	Orth	1284	42.14	-2.68	2	1	2	8	2	2.9	13.0	3	1369	4	6	3	Cu	M	40	5	3	5.94 ± 0.44	11.91 ± 0.86	72.44 ± 3.83	86.65 ± 3.60	6.53 ± 0.82
Palustriella commutata (Hedw.) Ochyra	*Pc*	Hypn	588	42.31	-2.53	2	2	1	8	6	2.9	13.7	8	1318	5	8	2	We	D	100	5	6	1.05 ± 0.05	8.55 ± 0.58	145.32 ± 8.21	152.96 ± 10.28	16.17 ± 0.92
Palustriella falcata (Brid.) Hedenäs	*Pm*	Hypn	1873	42.01	-2.64	5	2	3	8	3	2.8	13.5	9	1389	5	6	2	We	D	100	5	6	3.64 ± 0.20	5.88 ± 0.36	183.74 ± 9.85	189.63 ± 10.13	31.41 ± 1.29
Philonotis calcarea (Bruch & Schimp.) Schimp.	*Pr*	Brya	1871	42.01	-2.64	2	2	3	8	2	2.7	13.6	7	1294	4	8	2	Tf	D	100	5	3	2.83 ± 0.07	14.54 ± 0.67	82.02 ± 4.49	96.57 ± 5.13	5.64 ± 0.09
Philonotis seriata Mitt.	*Ps*	Brya	735	42.29	-2.58	1	2	3	8	1	-0.1	11.0	7	1858	6	2	2	Tf	D	97	2	4	3.41 ± 0.08	28.30 ± 1.48	84.42 ± 3.06	112.73 ± 3.47	3.01 ± 0.18
Plagiomnium undulatum (Hedw.) T.J.Kop.	*Pu*	Brya	1177	42.19	-2.71	5	1	2	4	3	3.4	14.6	6	1099	5	6	5	Tf	D	150	7	3	2.95 ± 0.22	34.56 ± 1.63	55.06 ± 3.33	89.62 ± 4.62	1.59 ± 0.07

Taxon	Abb	Ord	Alt (m)	Lat (ºN)	Lon (ºE)	Ori	Imm	Exp	L	T	TJan (ºC)	TJul (ºC)	F	Pre (mm)	K	R	N	LF1	Sex	Len (mm)	Bio	Eli	SI (mg cm^{-2})	SUVAC (AUC$_{280-400}$ mg^{-1} DM)	IUVAC (AUC$_{280-400}$ mg^{-1} DM)	TUVAC (AUC$_{280-400}$ mg^{-1} DM)	I/S
Platyhypnidium riparioides (Hedw.) Dixon	*Pi*	Hypn	991	42.21	-2.67	2	3	1	4	3	3.4	14.6	8	1113	5	6	6	Ms	M	112	8	6	3.54 ± 0.18	17.50 ± 1.23	155.83 ± 4.09	173.32 ± 5.27	8.97 ± 0.45
Pleurochaete squarrosa (Brid.) Lindb.	*Pq*	Pott	419	42.45	-2.51	5	1	3	9	8	5.0	15.9	2	933	5	6	2	Tf	D	70	9	2	1.74 ± 0.03	20.44 ± 1.53	129.12 ± 5.97	149.56 ± 7.34	6.39 ± 0.27
Pleurozium schreberi (Willd. ex Brid.) Mitt.	*Ph*	Hypn	1176	42.19	-2.71	2	1	2	6	3	3.2	14.0	4	1253	6	2	2	We	D	120	5	6	4.60 ± 0.14	11.32 ± 0.66	137.56 ± 5.59	148.88 ± 5.29	12.43 ± 1.26
Pohlia wahlenbergii (F.Weber & D.Mohr) A.L.Andrews	*Pw*	Brya	1878	42.01	-2.64	2	1	1	6	6	3.2	14.2	7	1223	6	6	4	Tf	D	75	3	6	2.64 ± 0.04	37.47 ± 1.97	52.43 ± 2.29	89.90 ± 3.58	1.41 ± 0.07
Polytrichastrum formosum (Hedw.) G.L.Sm.	*Pf*	Poly	1154	42.19	-2.71	2	1	2	4	2	3.4	14.5	6	1164	5	2	3	Tf	D	100	5	6	6.69 ± 0.19	20.88 ± 1.13	62.84 ± 4.75	83.14 ± 4.72	3.14 ± 0.32
Polytrichum commune Hedw.	*Pn*	Poly	1500	42.14	-2.69	2	2	3	6	2	3.4	14.5	7	1349	6	2	2	Tf	D	250	3	6	5.60 ± 0.15	23.96 ± 0.31	56.14 ± 5.50	74.65 ± 1.33	2.12 ± 0.02
Polytrichum juniperinum Hedw.	*Pj*	Poly	1467	42.14	-2.69	2	1	3	8	2	3.4	14.4	4	1146		3	2	Tf	D	70	3	6	4.71 ± 0.23	14.98 ± 0.68	57.25 ± 1.02	72.23 ± 1.13	3.84 ± 0.20
Polytrichum piliferum Hedw.	*Po*	Poly	1495	42.14	-2.69	2	1	3	9	2	3.2	14.1	2	1238	5	2	1	Tf	D	45	3	6	8.24 ± 0.37	12.85 ± 0.80	47.12 ± 3.67	59.98 ± 3.87	3.72 ± 0.37
Pseudoscleropodium purum (Hedw.) M.Fleisch.	*Pe*	Hypn	1146	42.19	-2.70	2	1	2	6	4	3.4	14.5	4	1116	5	5	3	We	D	150	7	3	4.38 ± 0.37	5.38 ± 0.29	104.56 ± 3.26	109.95 ± 3.48	19.49 ± 0.74
Racomitrium aciculare (Hedw.) Brid.	*Ra*	Grim	1905	42.01	-2.64	2	2	3	6	3	2.9	13.5	7	1414	4	5	2	Tf	D	60	5	2	6.21 ± 0.41	13.05 ± 0.67	44.65 ± 1.50	57.71 ± 1.88	3.44 ± 0.16
Racomitrium elongatum Ehrh. ex Frisvoll	*Re*	Grim	1192	42.19	-2.71	4	1	3	8	3	2.4	13.4	9	1635	6	5	2	Tf	D	60	5	2	5.93 ± 0.21	5.45 ± 0.22	37.32 ± 2.86	42.84 ± 2.96	6.78 ± 0.50
Rhizomnium punctatum (Hedw.) T.J.Kop.	*Rp*	Brya	1026	42.12	-2.64	2	1	1	3	3	3.2	14.4	6	1170	4	4	4	Tf	D	70	5	6	2.34 ± 0.26	15.69 ± 0.51	50.99 ± 2.95	64.19 ± 2.13	3.10 ± 0.11
Rhytidiadelphus triquetrus (Hedw.) Warnst.	*Rt*	Hypn	1146	42.19	-2.70	2	1	1	7	3	3.3	14.2	4	1194	6	5	3	We	D	200	5	6	1.81 ± 0.04	4.52 ± 0.13	114.98 ± 8.00	127.48 ± 4.32	27.54 ± 1.44
Schistidium apocarpum (Hedw.) Bruch & Schimp.	*Sp*	Grim	1000	42.16	-2.66	2	1	3	4	6	3.4	14.5	3	1123	5	7	4	Tuft	M	50	6	6	3.89 ± 0.12	4.98 ± 0.32	45.96 ± 2.30	50.94 ± 2.32	9.38 ± 0.70

Taxon	Abb	Ord	Alt (m)	Lat (°N)	Lon (°E)	Ori	Imm	Exp	L	T	TJan (°C)	TJul (°C)	F	Pre (mm)	K	R	N	LF1	Sex	Len (mm)	Bio	Eli	SI (mg cm^{-2})	SUVAC (AUC$_{280-400}$ mg^{-1} DM)	IUVAC (AUC$_{280-400}$ mg^{-1} DM)	TUVAC (AUC$_{280-400}$ mg^{-1} DM)	I/S
Sphagnum fallax (H.Klinggr.) H.Klinggr.	*Sf*	Sphag	1504	42.14	-2.69	2	2	3	7	3	3.1	14.3	7	1306	6	2	3	Tf	D	200	5	3	0.65 ± 0.04	4.57 ± 0.58	10.81 ± 0.73	14.93 ± 1.10	2.24 ± 0.30
Sphagnum palustre L.	*Sl*	Sphag	1504	42.14	-2.69	2	2	3	6	4	3.1	13.9	6	1292	6	2	2	Tf	D	250	5	6	0.64 ± 0.03	3.71 ± 0.48	12.12 ± 0.52	15.64 ± 0.90	3.41 ± 0.45
Syntrichia ruralis (Hedw.) F.Weber & D.Mohr	*Sr*	Pott	1463	42.19	-2.75	4	1	3	9	6	3.6	15.4	2	879	5	6	4	Tf	D	50	6	6	3.85 ± 0.13	6.68 ± 0.64	88.20 ± 4.13	94.62 ± 5.35	13.34 ± 0.58
Thamnobryum alopecurum (Hedw.) Gangulee	*Ta*	Hypn	1196	42.16	-2.69	2	2	1	4	4	3.5	14.7	6	1128	4	7	6	De	D	80	7	3	3.65 ± 0.26	12.87 ± 0.99	126.16 ± 4.59	142.95 ± 2.39	10.23 ± 1.05
Thuidium tamariscinum (Hedw.) Schimp.	*Tt*	Hypn	771	42.29	-2.58	2	1	2	4	4	3.4	14.4	6	1160	4	4	4	We	D	140	7	3	2.72 ± 0.05	9.92 ± 0.49	145.37 ± 12.74	155.30 ± 12.34	14.83 ± 2.02
Tortella tortuosa (Hedw.) Limpr.	*To*	Pott	1023	42.17	-2.68	1	1	1	5	6	3.1	13.7	4	1378	6	8	2	Tuft	D	40	5	6	3.40 ± 0.22	12.42 ± 1.06	130.90 ± 7.67	143.32 ± 8.37	10.74 ± 0.78
Anthoceros punctatus L.	*At*	Anth	30	43.38	-1.83	3	1	3	7	6	4.7	14.9	8	1152	3	4	4	St	M	30	9	1	2.66 ± 0.32	52.49 ± 2.61	26.06 ± 1.10	78.55 ± 2.79	0.50 ± 0.03

Frozen apices were ground in a TissueLyser (Qiagen, Hilden, Germany), and then 5 ml of methanol:water:7 M HCl (70:29:1, v/v/v) was added for extraction (24 h at 4 ºC in the dark). The extract was centrifuged at 6000 *g* for 15 min to differentiate two fractions of UVACs. In the supernatant of the centrifugation, we measured the soluble compounds (SUVACs), and in the pellet, after alkaline digestion, the insoluble compounds (IUVACs) (Clarke & Robinson, 2008).

In the supernatant of the methanol extraction described above, the bulk level of SUVACs was measured as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM (using the ratio FM/DM obtained in the SI measurement) and per unit of surface area. This interval corresponds to the whole of UV-A and UV-B radiations. The pellet of the methanol extraction was hydrolysed with 2 ml 1 M NaOH for 3 h at 80ºC, acidified to pH 1.0 with 5.6 N HCl and extracted three times with ethyl acetate. After evaporation, the material was dissolved in 2 ml 100% methanol and the bulk level of IUVACs was measured in the same units previously described for the bulk level of SUVACs. A Perkin-Elmer λ35 spectrophotometer (Perkin-Elmer, Wilton, CT, USA) was used for these measurements, which were obtained in quintuplicate. The total bulk level of UVACs (TUVAC) was obtained as the sum of the bulk levels of SUVACs and IUVACs, and the quotient between the bulk levels of IUVACs and SUVACs (I/S) was also calculated.

Environmental data and bryological attributes

In situ, at the time of sampling, different geographical and environmental variables were obtained for each sample: altitude, latitude, longitude and orientation (GPS Oregon 550, Garmin, Olathe, Kansas, USA); immersion index; and exposure index. Orientation was divided into 5 categories, each one comprising 72º, with values from 1 to 5 corresponding to increasing sun exposure: 1, north orientation (from 324.0º to 35.9º); 2, northeast and northwest (from 288.0 to 323.9º and from 36.0º to 71.9º); 3, east and west (from 252.0º to 287.9º and from 72.0º to 107.9º); 4, southeast and southwest (from 216.0º to 251.9º and from 108.0º to 143.9º); and 5, south (from 144.0º to 215.9º).

Immersion index was categorized into 3 groups: 1 (terrestrial species), 2 (intermediate species), and 3 (typically aquatic species, growing covered by a presumably permanent layer of water). Exposure index was divided into 3 categories, taking into account the canopy and the topographical shading: 1 (shaded species, growing under a canopy or directly shaded by rocks or slopes), 2 (intermediate), and 3 (sun species, growing in full sun, not protected by any canopy or topographical shading).

In addition, different bryological attributes were obtained for each species. Ellenberg indicator values for Light (L), Temperature (T), Continentality (K), Moisture (F), Reaction or pH (R), and Nitrogen (N) were mainly obtained from Ellenberg *et al.* (1991; http://statedv.boku.ac.at/zeigerwerte/), and subsidiarily from Hill *et al.* (2007) and Delgado (2012). Ellenberg values have successfully been applied to bryoecological studies in recent years (Smart *et al.*, 2010; Muller *et al.*, 2012; Delgado & Ederra, 2013; Soriano *et al.*, 2013), and are highly reliable to show the ecological differences between species and to monitor environmental changes. L was divided into 9 categories, from 1 (plants in deep shade) to 9 (plant in full sun). T was categorized in 9 groups, from 1 (plants typical from extremely cold environments) to 9 (plants from extremely hot environments). K was divided into 9 categories, from 1 (eu-oceanic species) to 9 (eu-continental species). F was divided into 12 groups, from 1 (species from extremely dry environments) to 12 (normally submerged species). R was divided into 9 categories, from 1 (species from extremely acid environments, never found on weakly acid or basic substrata) to 9 (species from substrata with free calcium carbonate, mainly chalk and limestone). Finally, N was divided into 7 categories, from 1 (species from extremely infertile sites) to 7 (species often found in richly fertile places).

Other bryological attributes were obtained from Hill *et al.* (2007) and are referred to British bryophytes, but they can be extrapolable to other countries because they just represent ecological gradients (such as climatic attributes) or qualitative general features:

- TJan and TJul (ºC): mean January and July temperatures, respectively, in the hectads (10 km^2) where the species has been found in the British Isles.

- Pre (mm): mean annual precipitation of hectads where found.

- LF1: primary life form (organization of shoots into colonies). Among the 17 life forms recognized by Hill *et al.* (2007), in our study we have identified 11: aquatic trailing (At, attached to substrate and trailing in the water); cushion (Cu, dome-shaped colonies formed by variously-oriented shoots with a central origin); dendroid (De, sympodially branching shoots with stolons from which spring erect main shoots bearing branches above); fan (Fa, with shoots arising from vertical bark or rock, branching repeatedly in horizontal plane); mat, rough (Mr, with shoots creeping substratum, having numerous erect lateral branches); mat, smooth (Ms, with shoots that creep over substratum, having leafy branches that generally lie flat); mat, thalloid (Mt, with shoots that creep over substratum, composed of a layer of thalli); solitary thalloid (St, with solitary thalloid rosettes, forming a small patch rather than the more extensive growth of a thalloid mat); turf (Tf, with many loosely or closely packet vertical stems with limited branching); tuft (Tuft, forming loose cushions not necessarily of central origin); and weft (We, with loosely intertwining, usually richly branched layers).

- Sex: we have simplified the attributes in Hill *et al.* (2007), differentiating only dioecious (D) and monoecious (M) species. The "normally dioecious, rarely monoecious" species were included in the D species, whereas the "normally monoecious, rarely dioecious" species were included in the M species.

- Len: the height of the leafy shoot in acrocarpous mosses or the length of the shoot or thallus in pleurocarpous mosses and liverworts, expressed in mm.

- Bio: major biome, corresponding to the main biogeographic element within Europe. This attribute has been classified in 9 categories: 1 (arctic-montane, with a main distribution in tundra or above tree-line in temperate mountains), 2 (boreo-arctic montane, in tundra and coniferous forest zones), 3 (wide-boreal, from temperate zone to tundra), 4 (boreal-montane, main distribution in coniferous forest zone), 5 (boreo-temperate, in conifer and broadleaf zones), 6 (wide-temperate, from Mediterranean region to coniferous forest zone), 7

(temperate, in broadleaf forest zone), 8 (southern-temperate, in Mediterranean region and broadleaf forest zones), and 9 (Mediterranean-atlantic, in Mediterranean region and extending north in Atlantic zone of temperate Europe).

- Eli: eastern limit, which roughly represents a continentality gradient. This attribute has been categorized in six groups: 1 (oceanic, in atlantic zone of Europe, not or scarcely reaching east to Sweden, Germany or southern Spain); 2 (suboceanic, extending east to Sweden, central Europe or Italy); 3 (European, extending to more continental parts of Europe but not to Siberia); 4 (eurosiberian, with eastern limit between 60°E and 120°E); 5, Eurasian (extending across Asia to east of 120°E); and 6 (circumpolar, present in Europe, Asia and North America).

Data analysis

Bivariate correlations (Pearson's coefficients) between all the quantitative variables used, both environmental and physiological, were obtained. Altitude, latitude and longitude were excluded from this analysis, given that these data were obtained in a relatively small territory and did not represent true ecological features typical of each species.

Once proved the data met the assumptions of normality (Shapiro–Wilks's test) and homoscedasticity (Levene's test), the effects of the taxonomical Order (and other taxonomical arrangements), the liverwort structure, the major biome, the eastern limit, and the life form, on the UVAC variables were tested using one-way analysis of variance (ANOVA). In the case of significant differences, means were then 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. Student's *t* tests were applied when only two categories were to be compared (differences between mosses and liverworts, acrocarpous and pleurocarpous mosses, and dioecious and monoecious species).

The species used were ordinated by Principal Component Analysis (PCA) taking into account only the UVAC variables: the bulk levels of SUVACs, IUVACs and TUVACs per unit of both DM and area surface, as well as the quotient I/S. In addition, another PCA was carried out to ordinate species using UVAC variables, SI, and quantitative environmental variables and bryological attributes (including Ellenberg values). This second PCA allowed to globally explore the relationships between environmental and physiological variables. All the statistical procedures were performed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

3.4.- RESULTS AND DISCUSSION

Environmental characteristics and bryological attributes of the species studied

Table 3.1 shows a summary of the taxonomical, geographical, environmental and physiological variables, together with the bryological attributes, for the 87 bryophytes used in the present study. We studied 22 liverworts, 64 mosses and 1 hornwort, belonging to 19 Orders, 7 of liverworts, 11 of mosses and 1 of hornworts. In liverworts, the best represented Order was Jungermanniales (8 species), followed by Marchantiales (4), Metzgeriales and Porellales (3 each), Fossombroniales (2) and Radulales and Ricciales (1 each). In mosses, the best represented Order was Hypnales (26 species), followed by Bryales (12), Dicranales (7), Polytrichales (5), Grimmiales and Pottiales (4 each), Sphagnales (2), and Encalyptales, Hedwigiales, Hookeriales and Orthotrichales (1 each). This distribution meant that around 50% of the taxonomical Orders of mosses and liverworts were represented in our survey, on the basis of the classification by Shaw & Goffinet (2000). The only hornwort collected belongs to the Order Anthocerotales. With respect to the structure of the liverworts studied, 5 species were simple thalloids, 5 complex thalloids, 8 leafy with non-conduplicate leaves, and 4 leafy with conduplicate leaves. A total of 37 mosses were acrocarpous and 27 pleurocarpous.

Latitude and longitude gradients were narrow because the sampling design was conceived to prevent, as far as possible, the radiation differences between the samples caused by the different collection dates, and thus sampling was completed in a few days. Thus, latitude and longitude have been excluded in further data analyses. In this line, altitude was also excluded because we collected each species in the place better adapted to the quickness of the sampling and not always in its most typical altitude.

A total of 7 species (5 mosses and 2 liverworts), 8% of the total, showed a north orientation; 58 species (43 mosses and 15 liverworts), 68% of the total, showed northeast or northwest orientations; 3 species (1 moss, 1 liverwort and the only hornwort considered), 3% of the total, had east or west orientations; 10 species (7 mosses and 3 liverworts), 11% of the total, showed southeast or southwest orientations;

and finally 9 species (8 mosses and 1 liverwort), 10% of the total, showed a south orientation. These results roughly show that bryophytes in general, and liverworts in particular, prefer orientations with higher humidities (with some northern component).

Only 9% of the species (5 mosses and 3 liverworts) were typically aquatic, whereas 60% (41 mosses, 10 liverworts and the hornwort) were terrestrial and 31% (18 mosses and 9 liverworts) occupied intermediate more or less hygrophilous situations.

There were similar proportions of species among the three categories of the exposure index. A total of 31 species (25 mosses, 5 liverworts and 1 hornwort, accumulating 36% of the total of species) were sun-exposed, 26 species (17 mosses and 9 liverworts, 30% of the total) were shaded, and 30 species (22 mosses and 8 liverworts) grew in intermediate situations. Thus, liverworts preferred shaded environments, which is in line with their preference for more humid environments (Vanderpoorten & Goffinet, 2009).

Ellenberg values for light (L) varied from 4 to 9 for liverworts, with a mean value of 6.4, and from 2 to 9 for mosses, with the same mean value. Thus, ecological preferences for light were intermediate and very similar for both groups. The only hornwort showed a value of 7.

Ellenberg values for temperature (T) varied from 2 to 8 for liverworts (mean = 3.7) and from 1 to 8 for mosses (mean = 4.0). Again, the ecological preferences for temperature were intermediate (although slightly directed towards relatively cold temperatures) and similar for both groups. In relation with T, TJan varied from 1.1ºC to 4.1ºC in liverworts (mean = 3.0ºC), and from -0.4ºC to 5.0ºC (mean = 3.1ºC), whereas TJul ranged from 11.9ºC to 15.3ºC (mean = 14.0ºC) for liverworts and from 11.0ºC to 15.9ºC (mean = 14.1ºC) for mosses. These data, coming from real climatic data and not from general estimations, were congruent with the information derived from T values. The hornwort showed a value of 6 for T, 4.7ºC for TJan and 14.9ºC for TJul.

Ellenberg values for moisture (F) varied from 4 to 9 (mean = 6.5) for liverworts and from 1 to 9 (mean = 5.3) for mosses. Thus, the mosses studied showed a certain preference for drier environmental situations than the liverworts, in line with the general

ecological preferences of both groups (Vanderpoorten & Goffinet, 2009). However, this was not confirmed using the real data of annual precipitation corresponding to both groups, which were rather similar: 931-2020 mm (mean = 1295 mm) for liverworts and 846-2198 mm (mean = 1253) for mosses. The hornwort showed a F value of 8 and 1152 mm of annual precipitation.

Ellenberg values for continentality (K) varied from 3 to 6 (mean = 4.9) for liverworts and from 2 to 6 (mean = 4.9) for mosses, showing similar and intermediate values in both groups once more. The hornwort showed a value of 3. K was related with the Eli biogeographic element, which also showed broad continentality preferences for both liverworts and mosses: the most frequent element for the studied species was circumpolar, the broadest element, with 44 species (31 mosses and 13 liverworts), and the second most frequent was another broad element (European, with 26 species, 21 mosses and 5 liverworts).

Ellenberg values for reaction (R) varied from 2 to 9 (mean = 5.5) for liverworts and from 1 to 9 (mean = 5.1) for mosses. Ranges were very wide in both groups, mainly because of the lithological diversity of the territory where samples were collected. In the hornwort, R value was 4.

Ellenberg values for nitrogen (N) varied from 1 to 7 (mean = 3.2) for liverworts and from 1 to 6 (mean = 3.1) for mosses, showing that the species studied in both groups preferred relatively infertile environments. This is in line with the general preference of liverworts and mosses for this type of environment (Martínez-Abaigar & Ederra, 1992). N value for the hornwort was 4.

Regarding the primary life form (LF1), liverworts and mosses showed great differences. Shoots of most of the studied liverworts were organized in thalloid mats (8 species), smooth mats (5 species) and wefts (5 species), whereas mosses were mainly organized in turfs (24 species) and wefts (13 species).

With respect to the distribution of sex organs, most of the species were dioecious (15 liverworts and 54 mosses), whereas 7 liverworts, 10 mosses and the hornwort were monoecious. Gametophore size varied greatly, both in liverworts (from the 20 mm of *Marsupella sphacelata* to the 100 mm of *Marchantia polymorpha* and *Scapania undulata*) and in mosses (from the 25 mm of *Grimmia decipiens* to the 500 mm of *Fontinalis antipyretica*). The hornwort was relatively small (30 mm).

Finally, the best represented major biomes were intermediate ones: boreo-temperate (35 species, 23 mosses and 12 liverworts) and temperate (16 species, 15 mosses and 1 liverwort). In contrast, little represented biomes were boreo-artic montane (3 mosses and 2 liverworts) and mediterranean-atlantic (2 mosses, 2 liverworts and 1 hornwort).

In general, the Ellenberg values obtained were consistent with the fact that the bryophytes studied (both liverworts and mosses) were collected at mid-latitudes in a temperate biogeographical situation. Therefore, intermediate values were usually found as average in the different modalities, and it was rare to find extreme values in some Ellenberg categories. With respect to the only hornwort studied, no generality should be derived from the data obtained in the present study, because it would be necessary to study more hornwort species. On the other hand, it must be taken into account that Ellenberg values represent the overall ecological preferences of a determinate species, but they have not necessarily to coincide with the specific ecological situation of a determinate population of that species. Thus, each species has its particular ecological ranges, usually wider than those depicted by the Ellenberg values (which are not ranges but numbers) for a specific species. This conceptual difference may explain the differences sometimes found between the Ellenberg values and other indices which would render similar ecological information, such as the immersion index or the precipitacion (rendering information on moisture preferences, like F), TJan or TJul (rendering information on temperature preferences, like T), and the exposure index (rendering information on light preferences, like L).

Summary of physiological variables

Values of SI and UVAC variables are shown in Table 3.1. SI varied from 0.74 mg cm^{-2} (*Pellia endiviifolia*) to 4.74 (*Radula complanata*) in liverworts, and from 0.64 (*Sphagnum palustre*) to 8.24 (*Polytrichum piliferum*) in mosses. In general, mosses were more sclerophyllous than liverworts. The bulk level of SUVACs (in terms of $AUC_{280\text{-}400}$ mg^{-1} DM) varied from 11.76 (*Frullania tamarisci*) and 131.07 (*Pellia epiphylla*) in liverworts, and from 3.71 (*Sphagnum palustre*) and 119.93 (*Bryum weigelii*) in mosses. Liverworts showed higher values than mosses (the mean value for liverworts was 46.14, almost 2.5-fold higher than in mosses, whose mean value was

19.03). In contrast, the bulk levels of IUVACs (in the same units) varied between 19.78 (*Pellia endiviifolia*) and 90.42 (*Porella arboris-vitae*) in liverworts, whereas in mosses it varied between 10.81 (*Sphagnum fallax*) and 241.09 (*Hylocomium splendens*). The mean value in mosses was 93.94, almost 2.5-fold higher than in liverworts (38.18). These trends confirmed the suggestion of Fabón *et al.* (2010, 2012a, 2012b), based only on the data obtained in one liverwort and two mosses, that compartmentation of UVACs in liverworts and mosses was quite different. The bulk level of TUVACs (in the same units again) varied between 49.50 (*Scapania aspera*) and 179.28 (*Pellia epiphylla*) in liverworts, and between 14.93 (*Sphagnum fallax*) and 241.59 (*Hylocomium splendens*) in mosses. The mean value of the bulk level of TUVACs in mosses was higher than in liverworts. The quotient I/S varied between 0.35 (*Riccardia multifida*) and 4.45 (*Porella arboris-vitae*), whereas in mosses it varied between 0.39 (*Bryum weigelii*) and 31.41 (*Palustriella commutata*). The mean value of I/S in mosses (8.53) was 7-fold higher than the mean value in liverworts (1.21). More detailed statistical analyses for these variables are presented below.

Phylogenetic aspects: liverworts, mosses and hornworts

The samples of the 87 bryophytes were ordinated by Principal Components Analysis (PCA) on the basis of their UVACs, expressed both per DM and surface area. The first two axes accumulated 87.6% of the variance (55.1% for axis I and 32.5% for axis II). The plot generated with the first two axes is shown in Fig. 3.1.The most significant loading factors towards the positive part of axis I were the bulk levels of IUVACs and TUVACs, both per DM and surface area, together with I/S. Towards the negative part of axis I, the only significant loading factor was the bulk level of SUVACs per DM. For axis II, the most significant loading factors towards the positive part were the bulk level of SUVACs and TUVACs, both per DM and surface area. Towards the negative part of axis II, the only significant loading factor was I/S.

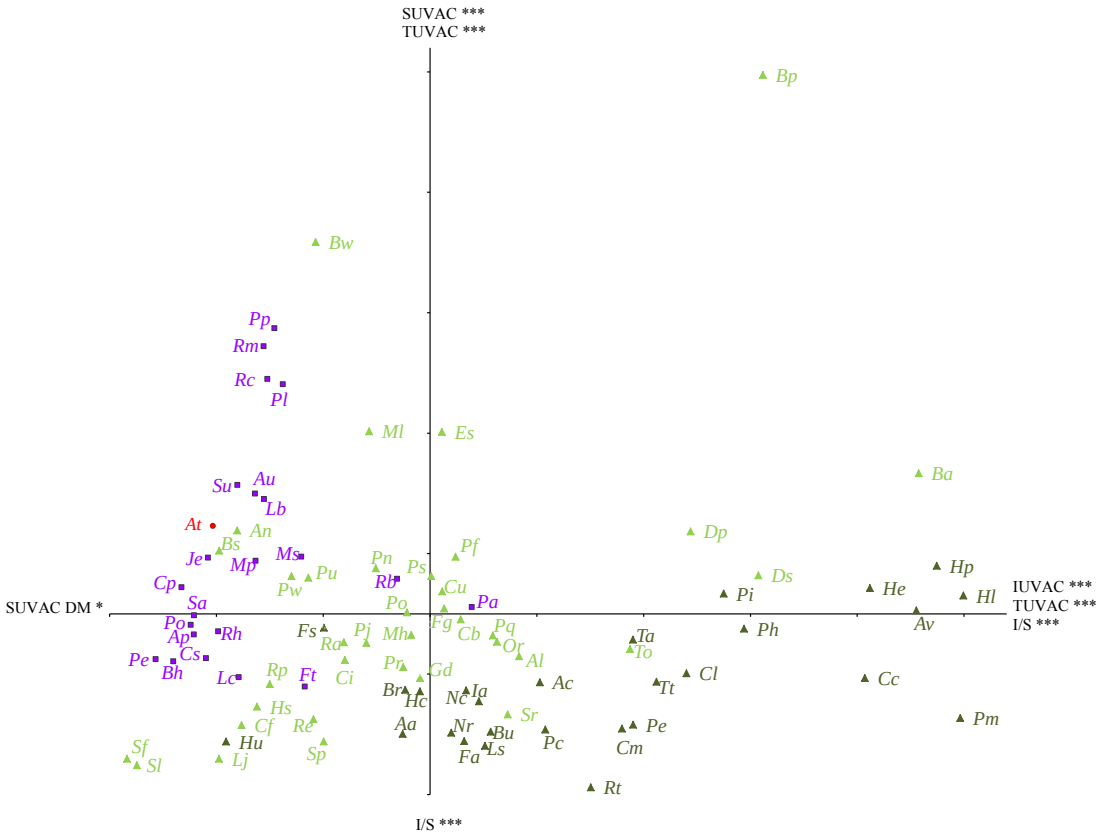


Figure 3.1. Ordination, through Principal Components Analysis (PCA), of the samples of the 87 bryophytes studied, on the basis of their UVACs. Liverworts are represented by purple quadrats, mosses by green triangles and the only hornwort by a red circle. Within mosses, acrocarpous mosses are represented by light green triangles and pleurocarpous mosses by deep green triangles. Significant loading factors for the positive and negative parts of each axis, together with their corresponding significance levels, are shown. ***, $p<0.001$; **, $p<0.05$; *, $p<0.01$. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UV-absorbing compounds, expressed per both dry mass (DM) and surface area. SUVAC DM, the bulk level of soluble UV-absorbing compounds, expressed per DM. I/S, the quotient between the bulk levels of IUVACs and SUVACs. For identification of species, see their codes in Table 3.1. Axis 1 is the horizontal one, and axis 2 is the vertical one. Each tic-mark on the axes represents one unit.

Consistent groups of liverworts and mosses could be differentiated in the PCA plot, mainly ordinated along axis I. Liverworts were placed towards the negative part of axis I, mainly in the fourth quadrant. They were characterized by high bulk levels of SUVACs per DM (the reason why liverworts were mainly placed towards the positive part of axis II), and low bulk levels of IUVACs and TUVACs (both per DM and surface area), together with low levels of I/S. Mosses appeared along the rest of axis I, showing high bulk levels of IUVACs and TUVACs, together with high levels of I/S. The only

hornwort analysed in our study appeared embedded within liverworts in the PCA plot. These differences between liverworts and mosses are shown in Fig. 3.2. Liverworts showed significantly higher bulk levels of SUVACs per DM, but lower bulk levels of IUVACs and TUVACs, together with lower levels of I/S, than mosses. Results of bulk levels of UVACs per surface area were similar to those obtained on a DM basis (data not shown).

These results confirmed the difference in UVACs compartmentation between mosses and liverworts that was suggested by Fabón *et al.* (2010, 2012a, 2012b) on the basis of the data obtained only in one liverwort and two mosses. Therefore, the two main evolutionary lineages of bryophytes, liverworts and mosses, have developed different alternatives to cope with UV radiation, with higher bulk levels of SUVACs in liverworts and higher bulk levels of IUVACs and TUVACs in mosses. Given that IUVACs may provide a more spatially uniform and effective UV screen than SUVACs (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. This matches well with the more shaded and humid environments preferred by liverworts in general, in comparison with mosses (Vanderpoorten & Goffinet, 2009).

The difference described above represents an additional difference between liverworts and mosses, in this case based on the UVACs compartmentation, a new ecophysiological trait maybe important evolutionarily in the colonization of new UV-rich environments after the conquest of land by plants. In this sense, mosses would be better qualified for this colonization than liverworts, although liverworts are considered the earliest diverging land plants (Zobell *et al.*, 2010), and thus their responses to UV radiation may have evolutionary importance in the water-to-land transition. On the other hand, this difference additionally supports the idea, derived from molecular taxonomy, of a higher phylogenetic distance between both bryophyte groups, although they share a similar life cycle. Qiu *et al.* (2007), using nucleotide sequences of seven chloroplast, mitochondrial, and nuclear genes from land plants and algae, found that liverworts, mosses, hornworts, lycophytes, monilophytes (ferns), seed plants, and angiosperms represented strongly supported monophyletic groups. In addition, liverworts represented the sister to all other land plants, and hornworts being sister to vascular plants.

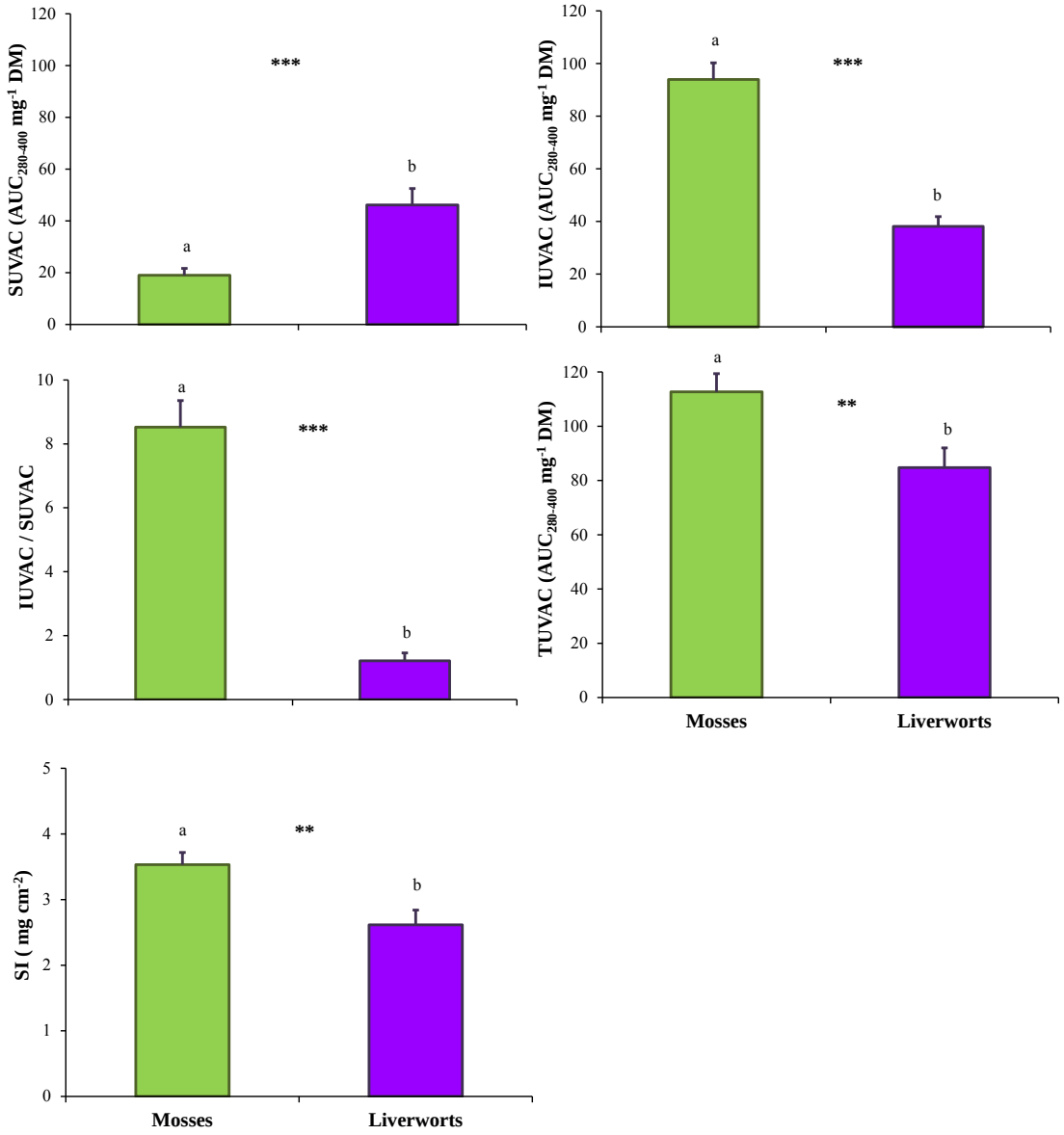


Figure 3.2. Differences in UVACs and sclerophylly index (SI) between liverworts and mosses. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Significance levels for Student's *t* tests are shown, and different letters mean significant differences with a determinate significance level: ***, $p<0.001$; **, $p<0.01$. For liverworts, $n = 22$; for mosses, $n = 64$.

Nevertheless, Buda *et al.* (2013) found large amounts of UVACs, specifically *m*- and *p*-coumaric acid and caffeic acid, in the cuticle of *Physcomitrella patens* leaves. This finding opens new perspectives on the compartmentation of UVACs in bryophytes and their implication in UV protection, given that, presumably, cuticle compounds would be very effective as UV screens.

Apart from the difference in UVACs compartmentation, liverworts and mosses also differed in SI, since mosses were significantly more sclerophyllous than liverworts (Fig. 3.2). In tracheophytes, SI of leaves increase with the development of nonphotosynthetic structures such as hairs, epicuticular waxes, cuticles, epidermal cells, cell walls, lignin, cellulose, mechanical and vascular tissues, together with the content of organic and inorganic solutes (Björkman, 1981; Lambers *et al.*, 1998). In bryophytes, SI, in the way it has been measured in this study, may mainly depend on the proportion of leaves and stems, the leaf architecture, the ratio of typically photosynthetic tissues against typically non-photosynthetic tissues, the leaf thickness, the development of cell walls (in comparison with protoplasts), the presence and proportion of vascular and supporting-mechanical tissues, and the content of organic and inorganic compounds. In the present study, SI discriminated well the sclerophyllous bryophytes (*e.g.* Polytrichaceae) from the soft ones (*e.g. Jungermannia* and other leafy liverworts with simple non-conduplicate leaves, or liverworts with a simple thallus). In addition, sclerophylly itself may be a protecting mechanism against UV through increasing the path which radiation must cross to reach potential targets within the cells. Thus, SI should be a routine measurement in the studies on the effects of UV on bryophytes.

Regarding hornworts, more species should be analysed to make a more solid comparison with liverworts and mosses. Nevertheless, at first sight this group would be more related to liverworts than to mosses, probably due to the fact that hornworts share their thalloid structure with some liverworts.

Phylogenetic aspects: acrocarpous and pleurocarpous mosses

In the ordination performed by PCA (Fig. 3.1), the two main evolutionary lineages within mosses, acrocarpous and pleurocarpous, can also be distinguished across axis I, with acrocarpous more near to liverworts than pleurocarpous.

Acrocarpous mosses produce sporophytes at the tips of the main stems, and grow erect and tufted, whereas pleurocarpous mosses have lateral sporophytes and prostrate growth, forming profusely branched carpets. As occurred between mosses and liverworts, pleurocarpous mosses accumulated higher bulk levels of IUVACs and TUVACs than acrocarps, and less bulk levels of SUVACs, thus showing higher I/S quotients. In this sense, pleurocarpous mosses would be better UV-protected than acrocarpous mosses by the higher amount of IUVACs. This could counteract the greater radiation they receive due to their prostrate growth form and the consequently greater surface they expose to the light. The differences shown by the PCA between acrocarpous and pleurocarpous mosses are also revealed in Fig. 3.3. Pleurocarpous mosses had significantly higher bulk levels of IUVACs and TUVACs, and higher I/S quotients, than acrocarpous mosses, but acrocarpous had significantly higher bulk levels of SUVACs. SI did not show significant differences between these two groups of mosses.

The clear differences in UVACs between acrocarpous and pleurocarpous mosses became more and more diluted as the evolutionary lineages within mosses were analysed in depth (Figs. 3.4 – 3.6). When mosses were divided into Sphagnales, Polytrichales, other acrocarpous mosses and pleurocarpous mosses, significant differences appeared in all the variables considered, but not all the groups were well-defined because of the high variability within each group (Fig. 3.4). Pleurocarpous were the best defined group, on the basis of lower bulk levels of SUVACs and higher bulk levels of IUVACs and I/S quotients. Also, Polytrichales and the category of "other acrocarpous" had higher bulk levels of SUVACs than Sphagnales and pleurocarpous, and Sphagnales showed low values of all the UVAC variables (although not always showing significant differences with the remaining groups). When mosses were divided considering Sphagnales, Polytrichales, Encalyptales, Dicranidae (with 3 Orders), Bryidae (with 3 Orders), and pleurocarpous, all the variables showed significant differences, but the groups were ill-defined, probably due, again, to high within-group variability (Fig. 3.5). Similar ill-defined groups were obtained when 11 Orders were considered (Fig. 3.6). Thus, the differentiation power of UVACs was enough to distinguish the two main evolutionary lineages within mosses (acrocarpous and pleurocarpous), but not to go beyond.

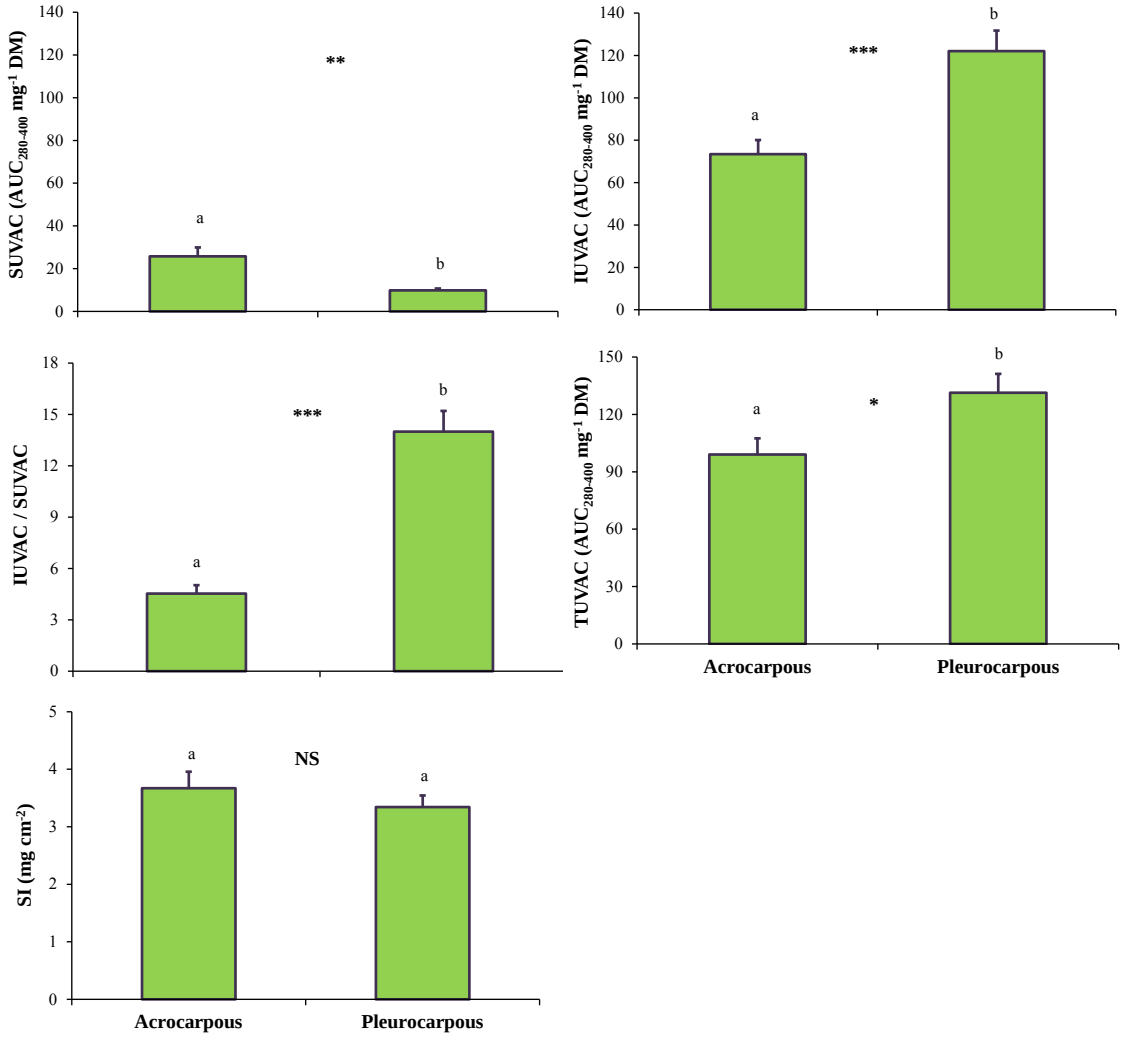


Figure 3.3. Differences in UVACs and sclerophylly index (SI) between acrocarpous and pleurocarpous mosses. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Significance levels for Student's *t* tests are shown, and different letters mean significant differences with a determinate significance level: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; NS, non-significant. For acrocarpous mosses, $n = 37$; for pleurocarpous mosses, $n = 27$.

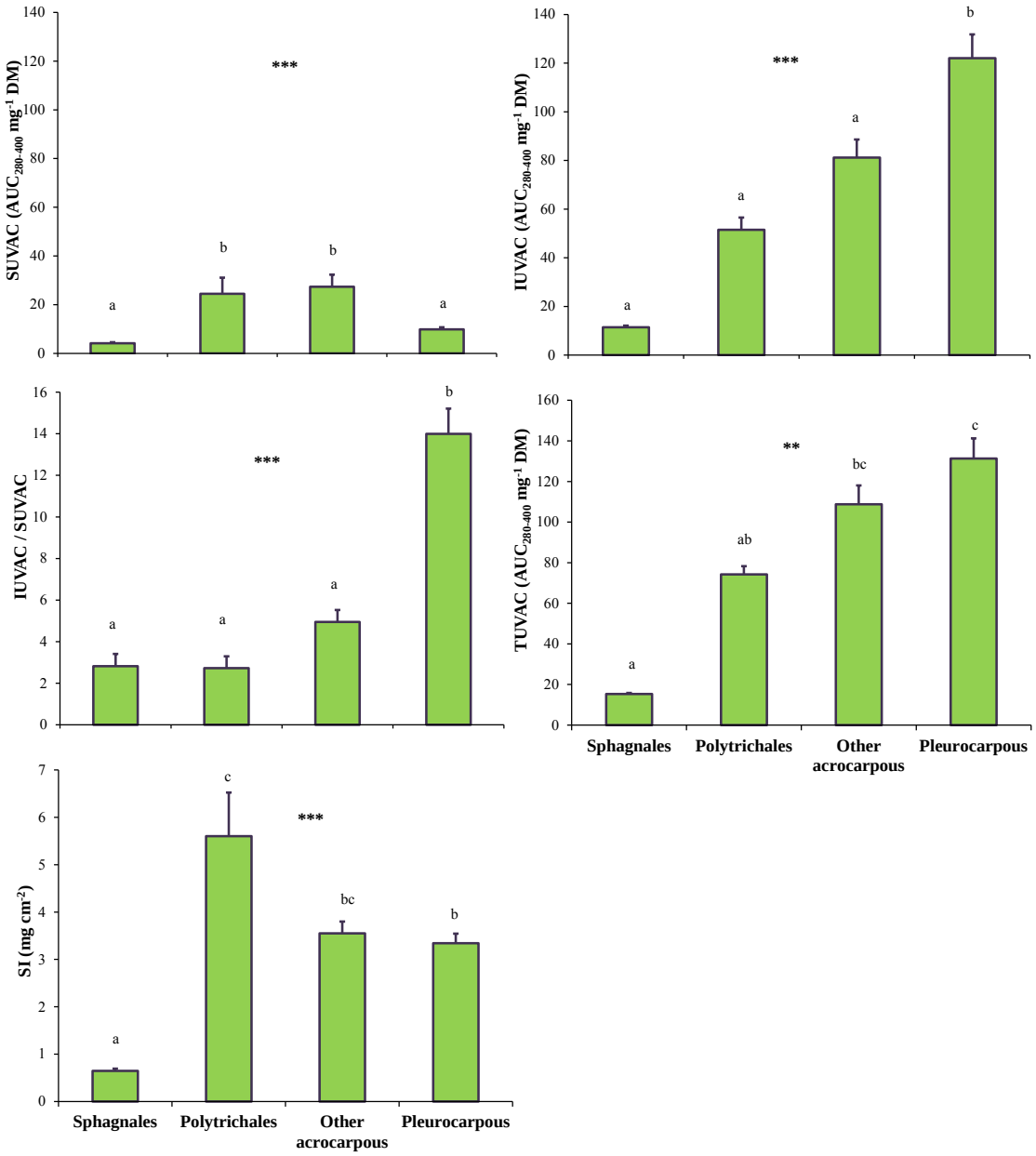


Figure 3.4. Differences in UVACs and sclerophylly index (SI) between different evolutionary lineages of mosses: Order Sphagnales, Order Polytrichales, other acrocarpous mosses, and pleurocarpous mosses. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm (AUC280-400) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): ***, $p<0.001$; **, $p<0.01$. For Sphagnales, n = 2; for Polytrichales, n = 5; for other acrocarpous mosses, n = 30; for pleurocarpous mosses, n = 27.

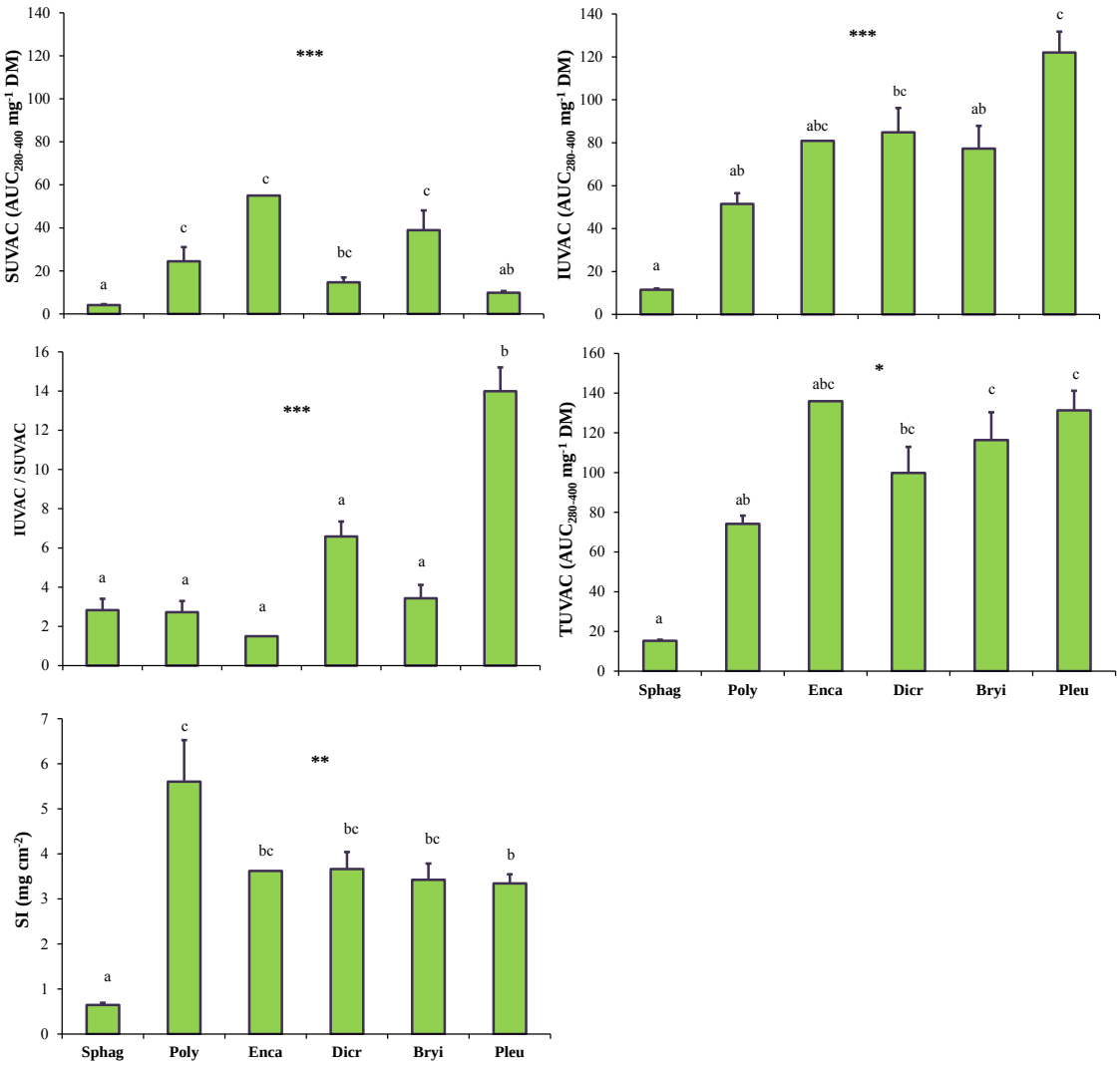


Figure 3.5. Differences in UVACs and sclerophylly index (SI) between different evolutionary lineages of mosses: Orders Sphagnales, Polytrichales, and Encalyptales, Subclass Dicraniidae (comprising the Orders Grimmiales, Dicranales and Pottiales), Subclass Bryidae (comprising the Orders Bryales, Orthotrichales and Hedwigiales), and pleurocarpous mosses (Orders Hookeriales and Hypnales). SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

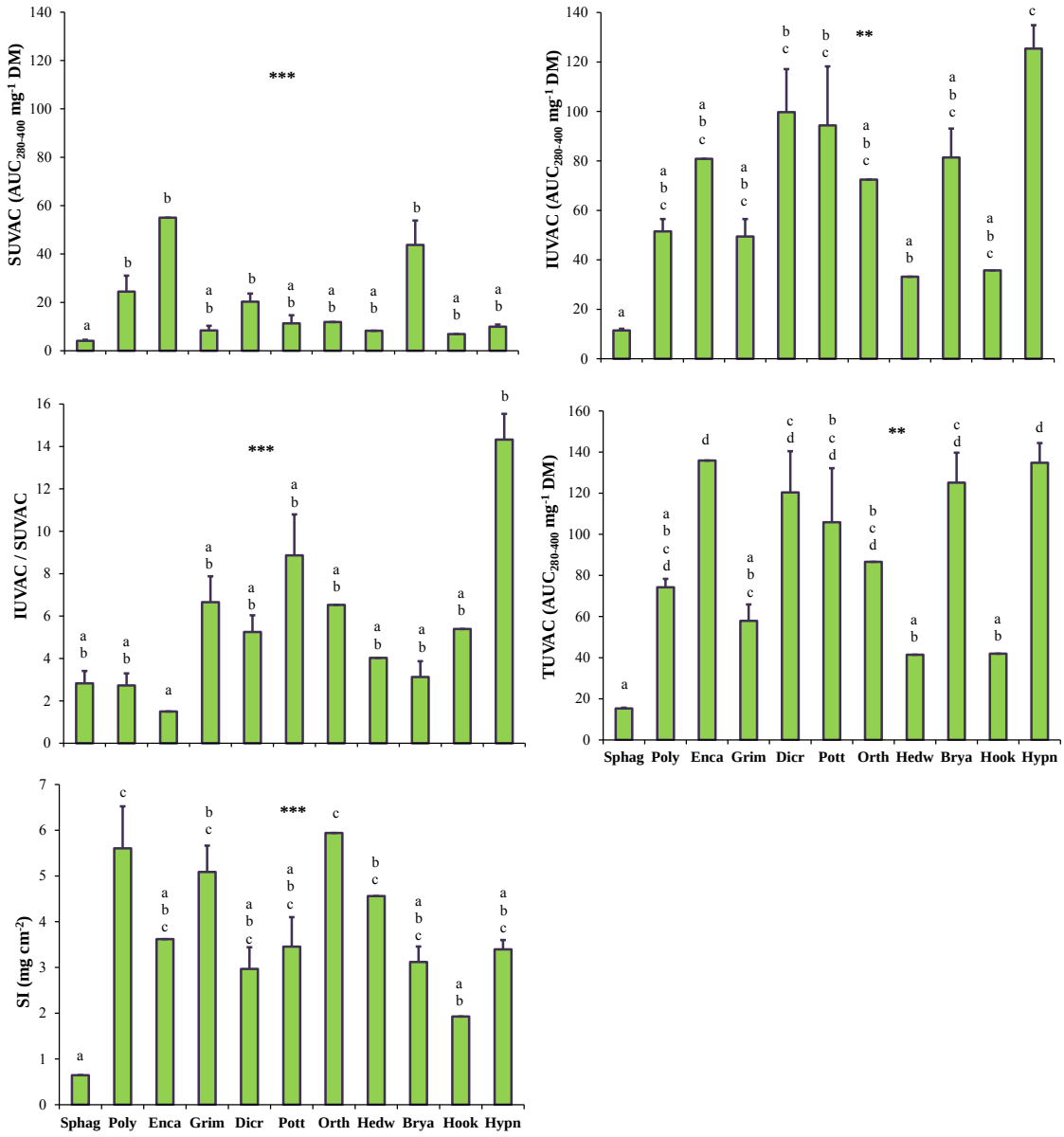


Figure 3.6. Differences in UVACs and sclerophylly index (SI) between different evolutionary lineages of mosses: Orders Sphagnales, Polytrichales, Encalyptales, Grimmiales, Dicranales, Pottiales, Orthotrichales, Hedwigiales, Bryales, Hookeriales and Hypnales. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): ***, $p<0.001$; **, $p<0.01$.

Phylogenetic aspects: Sphagnales

The case of Sphagnales must also be highlighted. The two *Sphagnum* species analyzed were clearly grouped in the PCA plot, because of their extremely low bulk levels of both IUVACs and TUVACs (Fig. 3.1). In addition, some differential characteristics could be observed with respect to other groups of mosses (Figs. 3.4 – 3.6). The segregation of *Sphagnum* species has been confirmed in a different study using 8 additional species from Norwegian peatlands (Soriano *et al.*, 2013). It can be hypothesized that, in *Sphagnum*, UVACs are accumulated only in relation to chlorocysts, dramatically limiting the accumulation capacity. Thus, the photophily and consequent UV tolerance of many *Sphagna* may be based on other mechanisms than UVAC accumulation. This is particularly interesting in plants as *Sphagna*, that are frequently sun-exposed while they are hydrated, and thus cannot tolerate UV through desiccation (Takács *et al.*, 1999).

Phylogenetic aspects: evolutionary lineages within liverworts

The main evolutionary lineages within liverworts (simple thalloids, complex thalloids, and leafy) were not discriminated in the PCA (Fig. 3.1). Simple thalloids have little internal tissue specialization, whereas complex thalloids have the thallus differentiated in photosynthetic and storage parenchyma, and also pores to allow gas interchange. Leafy liverworts have monostratified leaves. The three groups appeared intermixed in the PCA plot because there was no difference between their bulk levels of SUVACs, IUVACs and TUVACs, neither between their I/S quotient nor SI (Fig. 3.7). Thus, liverworts were a much more homogeneous group than mosses regarding the levels and proportions of UVACs. When leafy liverworts were divided into two groups (liverworts with either simple and conduplicate leaves), homogeneity was still conserved because no difference in UVACs between the four groups appeared (Fig. 3.8), as well as it happened when the 7 different Orders were considered (Fig. 3.9). However, liverworts with conduplicate leaves showed significantly higher values of SI than the remaining groups (Fig. 3.8), in accordance with their more complicate structure.

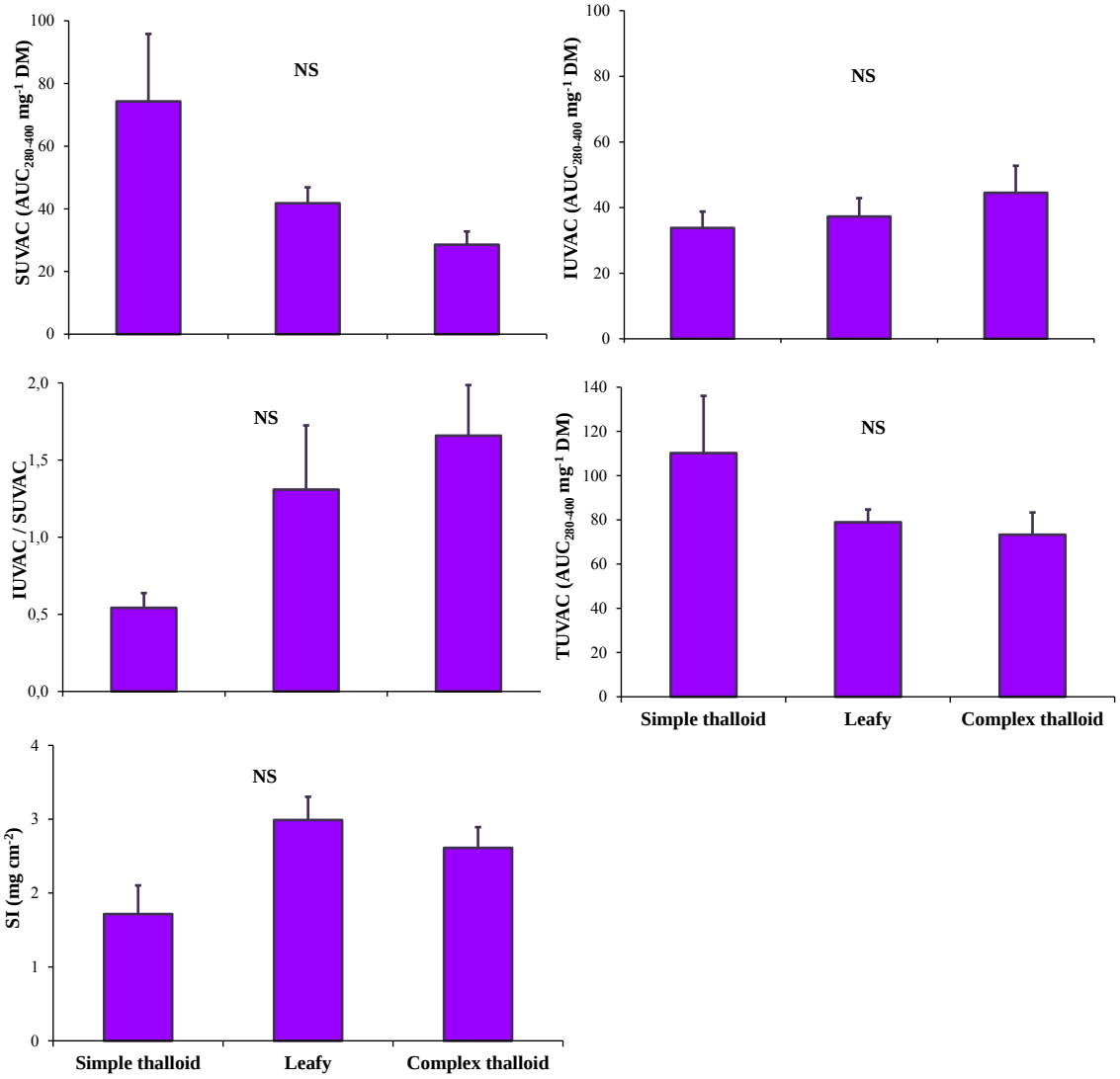


Figure 3.7. Differences in UVACs and sclerophylly index (SI) between different evolutionary lineages of liverworts: simple thalloids, leafy, and complex thalloids. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Significance levels for ANOVAs or Kruskal-Wallis tests are shown. NS, non-significant.

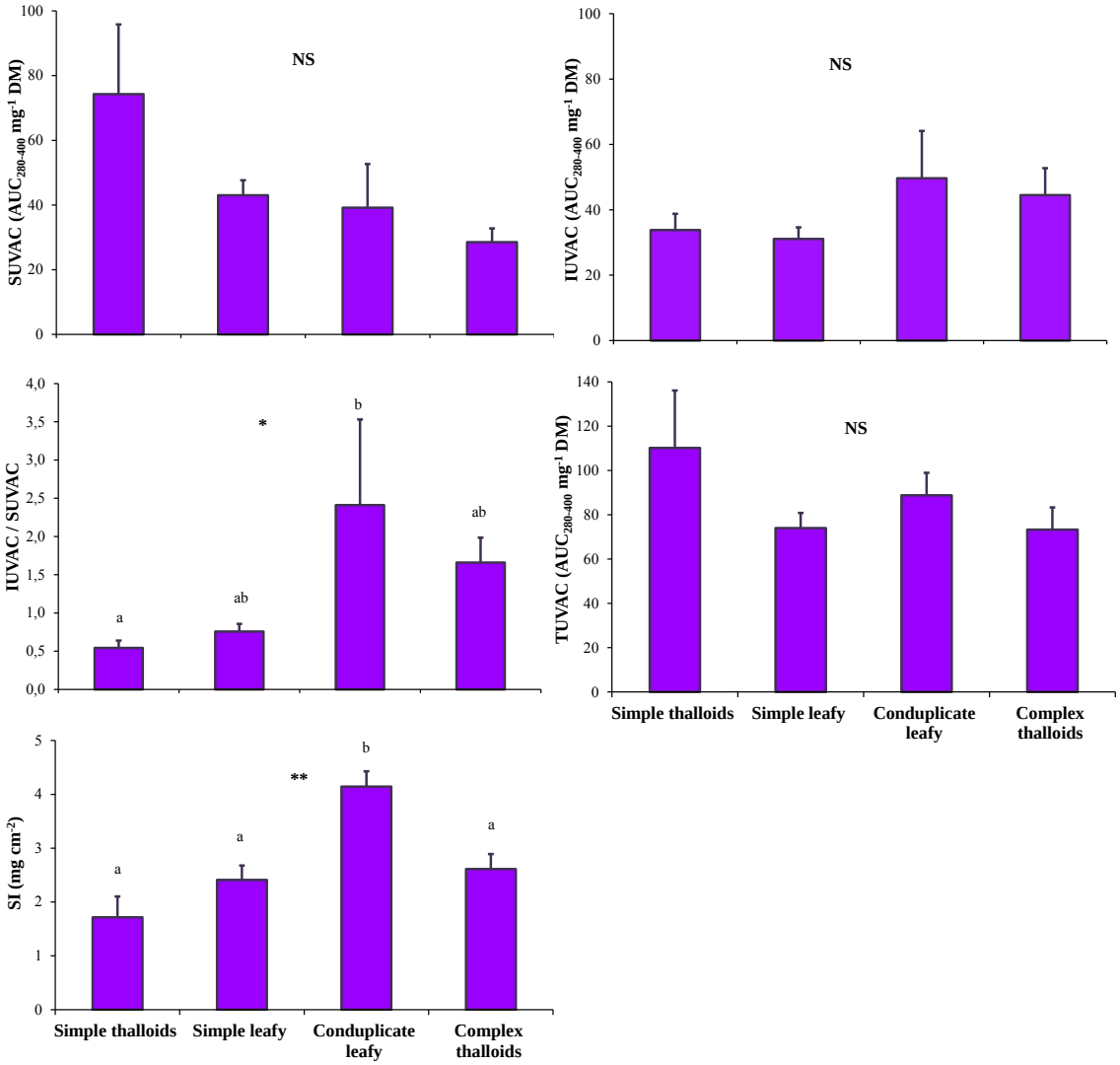


Figure 3.8. Differences in UVACs and sclerophylly index (SI) between different evolutionary lineages of liverworts: simple thalloids, leafy with simple leaves, leafy with conduplicate leaves, and complex thalloids. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): **, $p<0.01$; *, $p<0.05$; NS, non-significant.

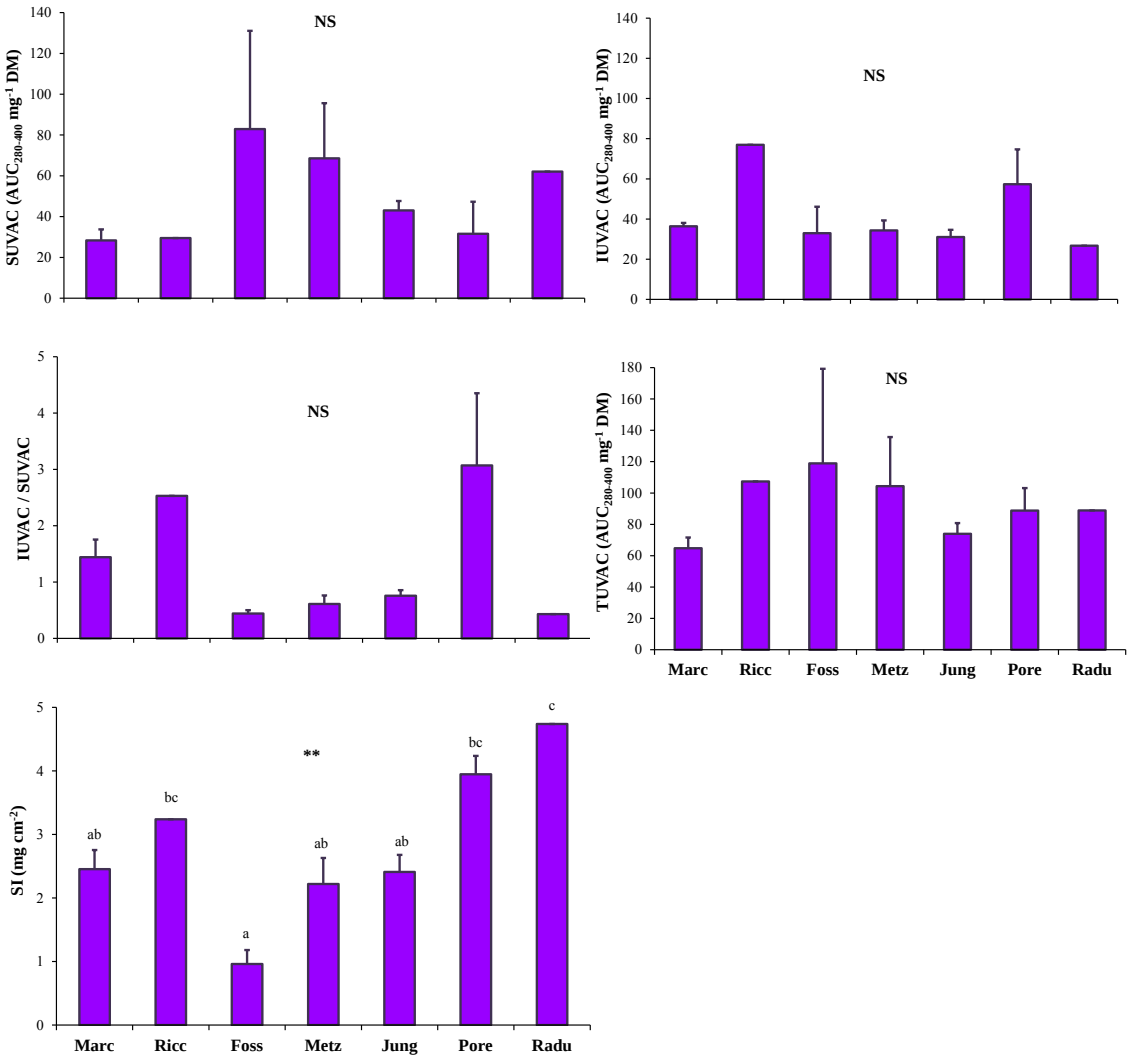


Figure 3.9. Differences in UVACs and sclerophylly index (SI) between different evolutionary lineages of liverworts: Orders Marchantiales, Ricciales, Fossombroniales, Metzgeriales, Jungermanniales, Porellales, and Radulales. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280\text{-}400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): **, $p<0.01$; NS, non-significant.

Phylogenetic aspects: differentiation of the taxonomical Orders within bryophytes

Fig. 3.10 shows the differentation among the 19 Orders in which the bryophytes studied were classified. The effect of the Order was significant in all the variables considered. However, no clear group was differentiated, probably because of, once again, the high variability within each Order. The best characterized Order, as discussed above, was Sphagnales, with very low bulk levels of SUVACs, IUVACs and TUVACs. Also, Sphagnales showed the lowest values of SI.

Phylogenetic aspects: exceptions in the PCA ordination

We have demonstrated that UVAC compartmentation can serve to differentiate liverworts and mosses, and also acrocarpous and pleurocarpous mosses (Fig. 3.1). However, given the distribution of these groups in the PCA plot, there were some exceptions to the general rule.

On one hand, some mosses, such as *Atrichum undulatum* and *Bryum pseudotriquetrum*, were embedded within liverworts, showing higher bulk levels of SUVACs than the generality of mosses. *Atrichum undulatum* possesses coumarin derivatives (Asakawa *et al.*, 2013) which could be located in the soluble fraction (vacuoles), as occurs with other coumarins in, for example, *Jungermannia exsertifolia* subsp. *cordifolia* (Arróniz-Crespo *et al.*, 2006). In the case of *Bryum pseudotriquetrum*, its position in the PCA plot confirms the findings of Clarke & Robinson (2008), who found that UVACs compartmentation in this species was clearly different to that found in other mosses (*Ceratodon purpureus* and *Schistidium antarctici*), probably because its reddish colour in stems and leaves is due to UVACs located in the vacuoles. In this line, Webby *et al.* (1996) found a great number of flavonoids in *Bryum algens* (a synonym of *Bryum pseudotriquetrum*), some of which are located in the vacuolar fraction (Fabón *et al.*, 2012b). These findings would justify the particular position of *Bryum pseudotriquetrum* in the PCA plot.

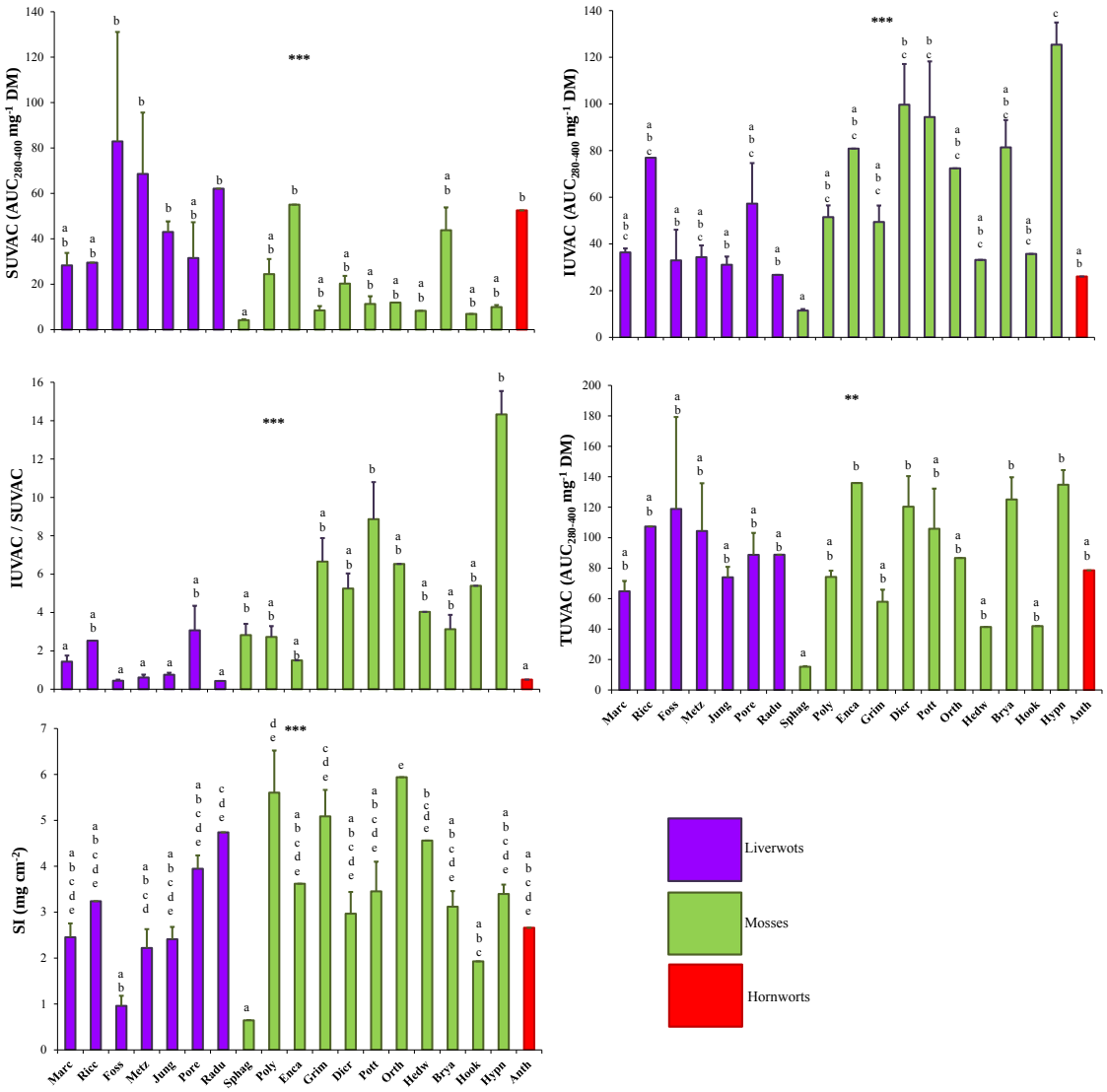


Figure 3.10. Differences in UVACs and sclerophylly index (SI) among the 19 Orders in which the bryophytes studied were classified: 7 Orders of liverworts (Marchantiales, Ricciales, Fossombroniales, Metzgeriales, Jungermanniales, Porellales, and Radulales), 11 of mosses (Sphagnales, Polytrichales, Encalyptales, Grimmiales, Dicranales, Pottiales, Orthotrichales, Hedwigiales, Bryales, Hookeriales and Hypnales), and 1 of hornworts (Anthocerotales). SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): ***, $p<0.001$; **, $p<0.01$.

There were also exceptions in the opposite sense, that is, liverworts that were grouped within mosses. This was the case of *Porella arboris-vitae*, *Frullania tamarisci* and *Riccia beyrichiana*. The two first ones share the possession of peculiar colours and even flavours. *Porella arboris-vitae* has a bronze colour with metallic sheen, and tastes acrid and spicy, probably because of the presence of diverse terpenoids (Asakawa *et al.*, 2013). If these features are related to its situation within mosses is unknown, but another species in the same genus, *Porella platyphylla*, that lacks particular colours or flavours, was clearly placed within the liverworts. *Frullania tamarisci* is reddish brown, as other members of this genus, but again it is unknown if this colour is related with its place in the PCA. Many coloured UVACs can be located in the cell walls (Martensson & Nilsson, 1974), in particular the anthocyanidin riccionidin A in *Cephaloziella varians* (Newsham, 2010). This suggests that the three mentioned liverworts could accumulate UVACs in their cell walls, which would increase their bulk levels of IUVACs and would place them within mosses in the PCA plot.

Bryum weigelii and *Bartramia pomiformis* were rare cases because they appeared isolated towards the positive part of axis II, suggesting they had considerably higher bulk levels of SUVACs than the generality of mosses. The position of *Bryum weigelii* in the PCA plot could be justified because it has luteolinidin glucosides in the cell sap, thus in the soluble fraction (Martensson & Nilsson, 1974). In contrast, other reddish species of *Bryum*, such as *B. alpinum*, have the pigmentation fixed to the cell walls (Martensson & Nilsson, 1974), and thus it appeared near the positive extreme of axis I. Thus, *Bryum* seems to be a diverse genus regarding the modalities of UVACs levels and compartmentation, with *Bryum pseudotriquetrum*, *Bryum weigelii* and *Bryum alpinum* following different modalities. With respect to *Bartramia pomiformis*, Asakawa *et al.* (2013) pointed out the presence of diverse exclusive flavonoids (bartramiaflavone, bartramiatriluteolin, strictatriluteolin) in species of the genus *Bartramia*, such as *B. pomiformis* and *B. stricta*, and it is known that flavonoids can be located in the soluble fraction (Agati & Tattini, 2010; Agati *et al.*, 2012). In general, exceptions to the usual distribution of liverworts and mosses in the PCA plot were related with particular characteristics of the species regarding the presence and location of specific UVACs either in the soluble or insoluble fractions. It should be remarked that all the mosses making exceptions to the usual location of mosses in the PCA plot were acrocarpous, and that they belonged to different families. This supports the idea

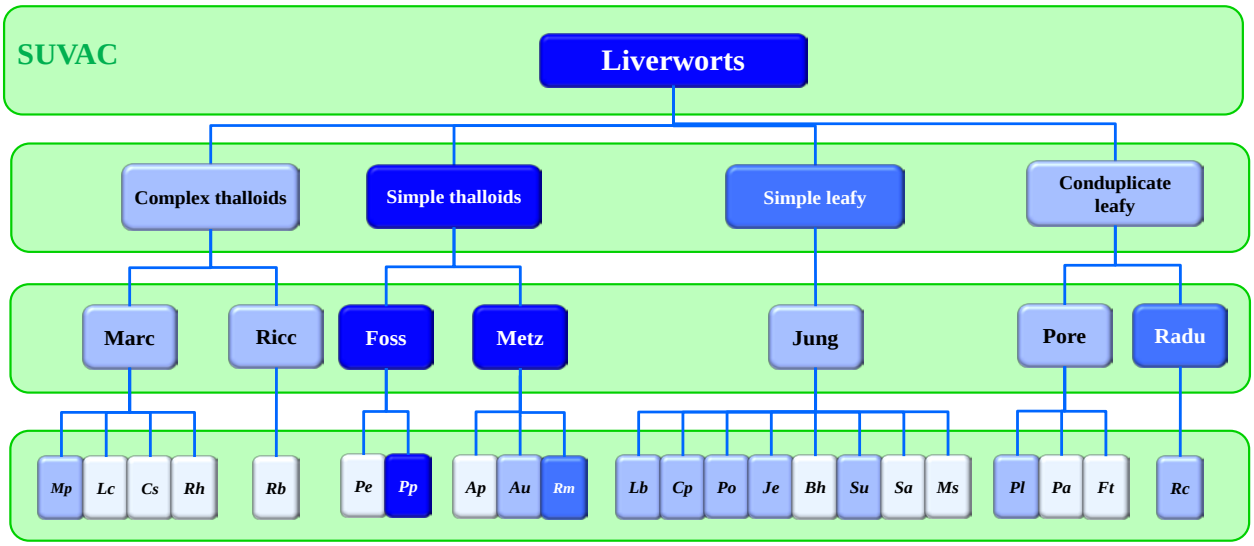
that acrocarpous mosses are more diverse than pleurocarpous mosses (Dicranidae include about 15% of moss genera, but nearly 30% of the phylogenetic diversity, whereas the Hypnanae contain about 45% of moss genera, but a lower percentage of phylogenetic diversity: Cox *et al.*, 2010), also regarding UVACs levels and compartmentation.

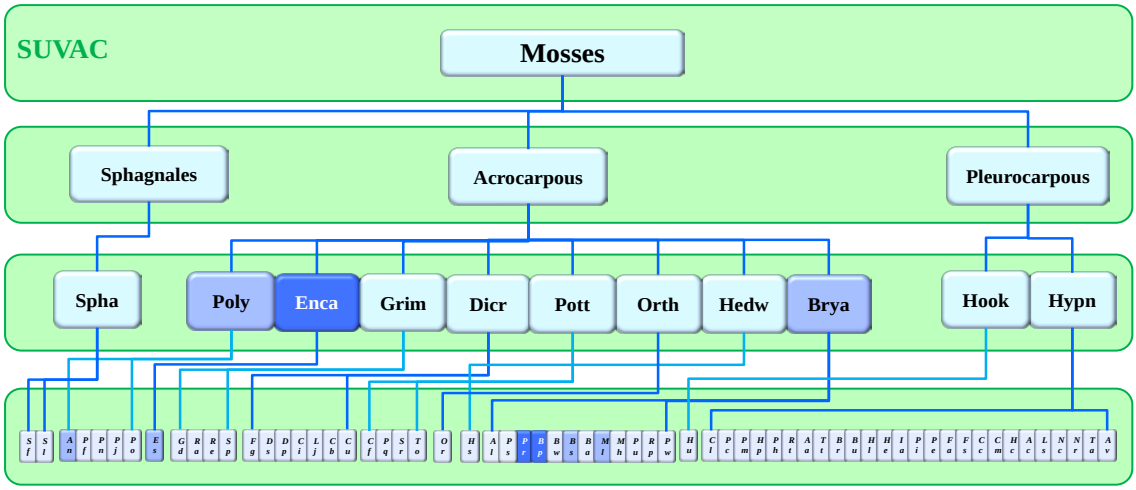
Finally, the relevant case of *Sphagnum* species, which appeared isolated with respect to the rest of mosses in the PCA plot, has been discussed above.

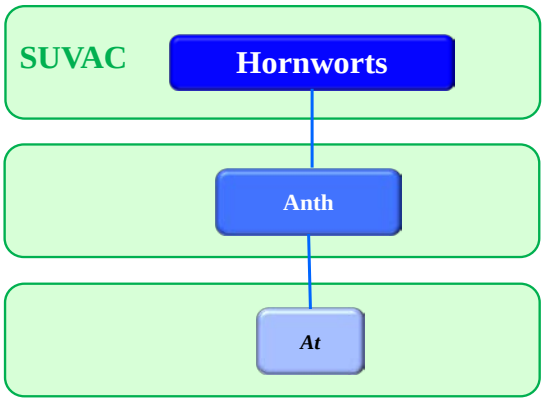
Phylogenetic aspects: a global scheme of UVACs compartmentation in bryophytes

Fig. 3.11 shows a synthetic global scheme representing the diversity of the levels and compartmentation of UVACs in the three evolutionary lineages of bryophytes (liverworts, mosses and hornworts), representing also the main lineages within liverworts and mosses, and then the corresponding Orders and species. The soluble (SUVACs) and insoluble (IUVACs) fractions were differentiated, and their levels were classified in quartiles. Liverworts and hornworts showed higher bulk levels of SUVACs than mosses, especially in simple thalloid liverworts of the Orders Fossombroniales and Metzgeriales (first quartile). These Orders were followed by Radulales (liverworts), Encalyptales (mosses) and Anthocerotales (hornworts), which occupied the second quartile, and then, in the third quartile, by the remaining Orders of liverworts (Marchantiales, Ricciales, Jungermanniales and Porellales), together with two Orders of mosses (Polytrichales and Bryales). The remaining Orders of mosses, including all the Hypnales, showed the lowest bulk levels of SUVACs and occupied the fourth quartile.

With respect to the species, the simple thalloid livertwort *Pellia ephyphilla* showed the highest bulk levels of SUVACs (first quartile), followed in the second quartile by *Riccardia multifida* (another simple thalloid livertwort) and two acrocarpous mosses (*Bryum weigelii* and *Bartramia pomiformis*). The third quartile was occupied by 9 liverworts of different lineages, the hornwort *Anthoceros punctatus* and 4 acrocarpous mosses (*Mnium lycopodioides*, *Encalypta streptocarpa*, *Bryum pseudotriquetrum* and *Atrichum undulatum*). The remaining species of both liverworts and mosses showed the lowest levels (fourth quartile).


SUVAC
Liverworts
Complex thalloids
Simple thalloids
Simple leafy
Conduplicate leafy
Marc
Ricc
Foss
Metz
Jung
Pore
Radu
Mp
Lc
Cs
Rh
Rb
Pe
Pp
Ap
Au
Rm
Lb
Cp
Po
Je
Bh
Su
Sa
Ms
Pl
Pa
Ft
Rc


SUVAC
Mosses
Sphagnales
Acrocarpous
Pleurocarpous
Spha
Poly
Enca
Grim
Dicr
Pott
Orth
Hedw
Brya
Hook
Hypn


SUVAC
Hornworts
Anth
At

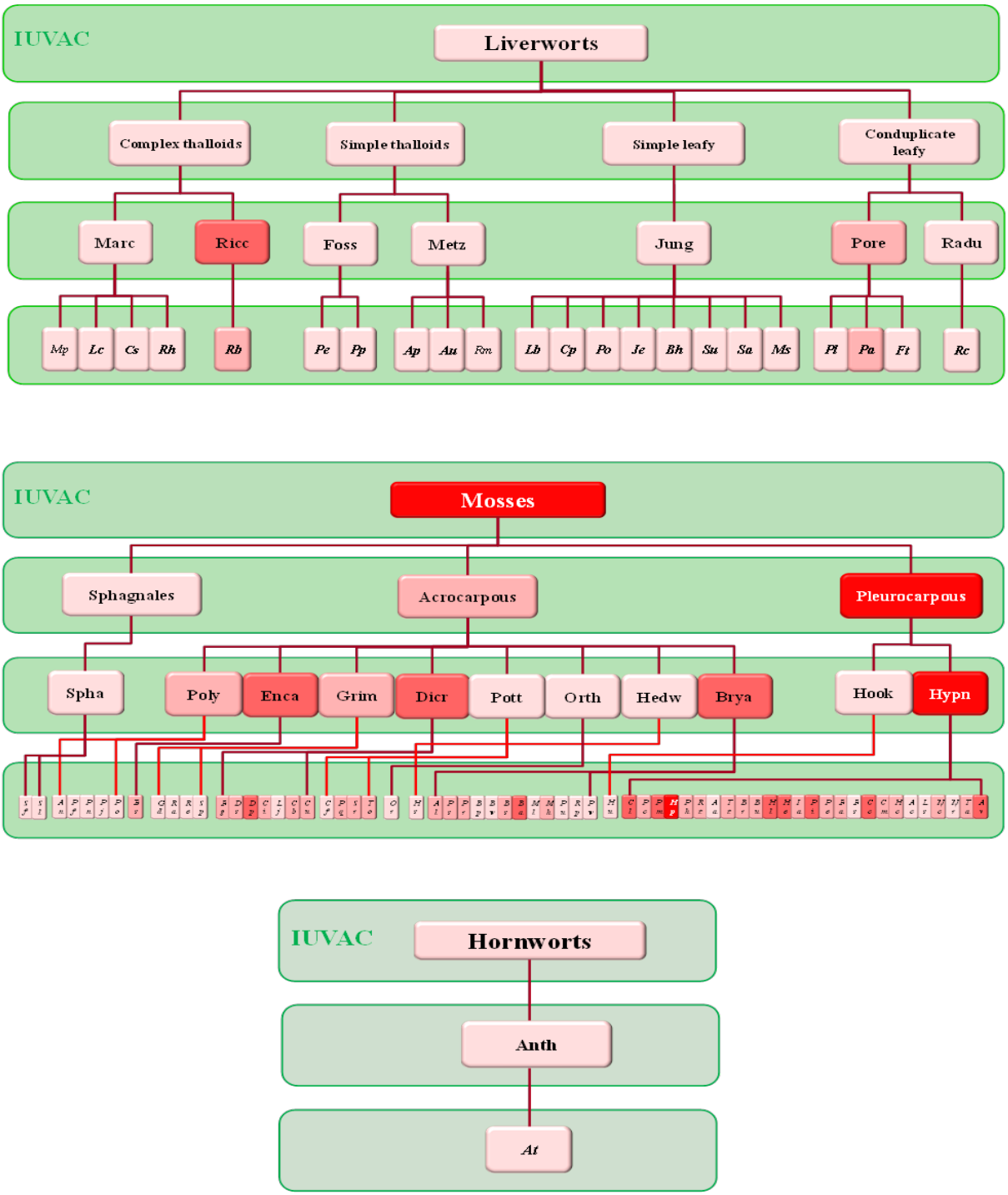


Figure 3.11. A synthetic global scheme representing the diversity of the levels and compartmentation of UVACs in the three evolutionary lineages of bryophytes (liverworts, mosses and hornworts), differentiating the soluble (SUVACs, in blue) and insoluble (IUVACs, in red) fractions. For both SUVACs and IUVACs, the colour intensity has been divided into four levels representing quartiles (more intensity means higher UVACs levels). For liverworts, complex thalloids, simple thalloids, leafy with simple leaves, and leafy with conduplicate leaves, have been distinguished, and within each evolutionary lineage, the corresponding Orders and species (following the codes in Table 3.1) have been placed. For mosses, Sphagnales, acrocarpous and pleurocarpous have been differentiated, as well as the corresponding Orders and species within each lineage.

Regarding the IUVACs, the highest levels were shown by pleurocarpous mosses belonging to the Hypnales. In the second quartile, three Orders of acrocarpous mosses (Encalyptales, Dicranales and Bryales), together with the complex thalloid livertworts of the Order Ricciales. In the third quartile, two Orders of acrocarpous mosses (Polytrichales and Grimmiales) and the liverworts with conduplicate leaves of the Order Porellales. Finally, the lowest bulk levels of IUVACs were shown by the remaining Orders of mosses (Sphagnales, Pottiales, Orthotrichales and Hedwigiales) and liverworts (Marchantiales, Fossombroniales, Metzgeriales, Jungermanniales and Radulales), together with the Order Anthocerotales of hornworts.

The species showing the highest bulk levels of IUVACs (first quartile) was the pleurocarpous moss *Hylocomium splendens*. In the second quartile, 7 other pleurocarpous mosses and 2 acrocarpous mosses were placed. In the third quartile, there were 14 acrocarpous and 13 pleurocarpous mosses, together with the complex thalloid livertwort *Riccia beyrichiana* and the leafy liverwort *Porella arboris-vitae*, with conduplicate leaves. Finally, the lowest levels of bulk IUVACs were shown by the two *Sphagnum* species, the remaining acrocarpous and pleurocarpous mosses, the remaining liverworts and the hornwort.

In general, the groups with higher levels of SUVACs showed lower levels of IUVACs, and vice-versa, except Sphagnales, which showed low levels of both types of compounds.

Ecological aspects: correlations between environmental variables and UVACs

An initial idea (already expressed above) on the relationship between UVAC compartmentation and ecology is that, if it is assumed that the cell wall-bound compounds are more effective UV screens than the vacuolar compounds (Clarke & Robinson, 2008), it could be suggested that mosses, preferentially accumulating cell wall-bound compounds, are better adapted to UV-rich environments than liverworts. This matches well with the general ecology of mosses and liverworts in nature: mosses tend to prevail in sun-exposed habitats, whereas liverworts are more abundant in more shaded and humid environments (Vanderpoorten & Goffinet, 2009).

Table 3.2. Correlation coefficients among the physiological and the numerical environmental variables and bryological attributes shown in Table 3.1 (except altitude, latitude and longitude). For details of the codes of the respective variables, see Table 3.1. Significant correlations are differentiated in different colours depending on the significance level.

Ori	Imm	Len	Exp	L	T	TJan	TJul	F	Pre	K	R	N	SI	SUVAC	IUVAC	I/S	TUVAC	
1	0.10	0.13	0.20	0.22	0.18	0.01	0.08	0.08	-0.09	0.13	0.14	0.19	0.01	0.05	0.10	0.13	0.11	**Ori**
	1	0.33	0.08	0.18	-0.07	-0.17	-0.20	0.70	0.19	0.03	-0.04	0.17	-0.19	0.09	-0.11	-0.12	-0.05	**Imm**
		1	0.02	0.03	-0.07	-0.03	-0.01	0.22	0.01	0.16	-0.09	0.08	-0.03	-0.23	0.12	0.25	-0.01	**Len**
			1	0.39	-0.13	-0.01	-0.10	-0.11	0.14	0.01	-0.33	-0.27	0.27	-0.19	-0.12	-0.05	-0.23	**Exp**
				1	0.15	0.02	-0.07	-0.02	0.07	-0.06	-0.22	-0.33	0.15	-0.08	0.04	0.05	0.00	**L**
					1	0.47	0.47	-0.12	-0.41	-0.29	0.25	0.26	-0.16	-0.10	0.03	0.06	-0.03	**T**
						1	0.91	-0.21	-0.78	-0.40	0.12	0.27	0.02	-0.29	0.11	0.20	-0.03	**Tjan**
							1	-0.25	-0.91	-0.24	0.23	0.45	-0.05	-0.25	0.10	0.20	-0.03	**Tjul**
								1	0.26	0.03	0.01	0.19	-0.28	0.28	-0.24	-0.20	-0.09	**F**
									1	0.12	-0.27	-0.50	0.09	0.23	-0.13	-0.23	-0.01	**Pre**
										1	-0.01	-0.09	-0.10	0.19	0.10	0.04	0.20	**K**
											1	0.35	-0.12	-0.02	0.15	0.22	0.14	**R**
												1	-0.24	-0.05	-0.01	0.07	-0.03	**N**
													1	-0.23	0.13	0.13	0.01	**SI**
														1	-0.27	-0.55	0.25	**SUVAC**
															1	0.67	0.86	**IUVAC**
																1	0.40	**I/S**
																	1	**TUVAC**

$p < 0.05$
$p < 0.01$
$p < 0.001$

Once expressed this general idea, the first approach to evaluate the influence of environmental conditions on UVACs levels and compartmentation was to calculate the correlations between the physiological variables and the quantitative environmental variables and bryological attributes used (Table 3.2). Some environmental variables and attributes were positively correlated because they represented the same ecological gradient. In this sense, the Ellenberg value for light (L) was positively correlated with orientation (the more southern orientation, the more light received) and the exposure index estimated *in situ* for each sample (thus, this index rendered similar information to L). Also, the three variables related with temperature (the Ellenberg value for temperature (T), TJan and TJul), that were positively correlated between them, were also negatively correlated with annual precipitation, and thus a warmer climate would mean a drier climate. Due to the same reason, TJan and TJul (but not T) were negatively correlated with the Ellenberg value for moisture (F). F was positively correlated with

annual precipitation, because both variables represented the same environmental gradient. F was also positively correlated with the immersion index. This solid set of environmental correlations suggested that the environmental variables formed a reliable frame to search for relationships between environment and UVACs.

There were some correlations between the physiological variables. The bulk level of SUVACs was negatively correlated with the bulk level of IUVACs (this is logical because, in general, the bryophyte species and groups with higher levels of SUVACs showed lower levels of IUVACs, and vice-versa). The bulk levels of both SUVACs and IUVACs were positively correlated with the bulk level of TUVACs, because both SUVACs and IUVACs contribute to TUVACs. The I/S quotient was positively correlated with its numerator (IUVACs) and negatively with its denominator (SUVACs).

There were hardly any solid correlations between environmental variables and UVACs. In particular, and surprisingly, none of the factors presumably related with the UV level received by the samples (orientation, exposure index, and L) was significantly correlated with the UVAC variables (SUVACs, IUVACs, TUVACs and I/S), except the unexpected negative correlation found between the bulk level of TUVACs and the exposure index. This general lack of positive correlations highly contrasted with the fact that UVACs increase in response to UV-B radiation under natural conditions, both in bryophytes and angiosperms (Searles *et al.*, 2001b; Newsham & Robinson, 2008; Núñez-Olivera *et al.*, 2009). This increase has also been found under enhanced UV indoors (Martínez-Abaigar & Núñez-Olivera, 2011). In this sense, UVACs are considered the most UV-responsive variable in plants, although this response depends on the species, the UVAC fraction considered (soluble or insoluble), the UV dose received by the samples, etc. Therefore, there is an apparent discrepancy between the frequent response of UVACs to UV and the lack of relationship we have observed in our study between UVACs and the environmental variables, especially those determining the UV levels received by the samples. However, UVAC induction has been reported, both indoors and outdoors, mainly under enhanced UV, either caused naturally by the stratospheric ozone depletion (for example, over Antarctica: Newsham & Robinson, 2008) or by the use of lamps providing higher-than-natural UV doses (Searles *et al.*, 2001a; see a recent review in Martínez-Abaigar & Núñez-Olivera, 2011). When bryophytes have been exposed to natural ambient UV levels, changes in UVACs are more infrequent, although they can sometimes be found (Newsham & Robinson,

2008; Núñez-Olivera *et al.*, 2009). All these considerations suggest that, at least to a certain extent, UVACs are mainly constitutive and privative of each species, and not especially inducible under natural ambient UV doses. Even species belonging to the same genus, as we have seen with *Porella platyphylla* and *P. arboris-vitae*, or with *Bryum* species, can show notoriously different UVAC accumulations and compartmentations, and consequently they can be relatively separated in the PCA ordination (Fig. 3.1).

There was a positive correlation between the bulk level of SUVACs and both F and the annual precipitation. This may reflect the different general ecology of liverworts and mosses, since liverworts occupy more humid environments (Vanderpoorten & Goffinet, 2009) and have higher proportions of the soluble fraction. This would also be in line with the negative correlation between F and the bulk level of IUVACs, which were especially high in mosses, that occupy less humid environments than liverworts. Other correlations between UVACs and environmental variables seemed to be spurious, such as the negative correlations between the bulk level of SUVACs and TJan and TJul.

SI was negatively correlated with F and positively with the exposure index. This would mean that water restrictions and high sun exposures cause morphogenic responses in bryophytes, increasing their sclerophylly.

Ecological aspects: PCA ordination using both environmental and physiological variables

To explore synthetically the relationships between environmental factors and UVACs, the samples were ordinated by PCA on the basis of both environmental and physiological data. The first three axes accumulated 51.1% of the variance (23.1% for axis I, 14.9% for axis II and 13.1% for axis III). The plot generated with the two first axes is shown in Fig. 3.12. The most significant loading factors towards the positive part of axis I were TJul, TJan, T, N, and I/S. Towards the negative part of axis I, the most significant loading factors were annual precipitation, the bulk level of SUVACs,

and F. For axis II, the most significant loading factors towards the positive part were the bulk level of TUVACs, I/S, and SI, whereas towards the negative part, the most significant factors were F and N.

This ordination did not differentiate mosses and liverworts as clearly as the ordination using only UVACs (Fig. 3.1), given that the influence of environmental variables was highly significant for both parts of axis I and the negative part of axis II. In addition, the variance accumulated by the first two axes was notably lower than in the ordination of Fig. 3.1. In general, liverworts are placed around the negative part of axis II and in the third quadrant, because they prefer more moist environments (high F and precipitation values) and accumulate higher levels of SUVACs. Mosses appear notably dispersed, especially the acrocarpous ones, and the only group conserving a certain unity is the group of pleurocarpous mosses, that are mainly placed in the first quadrant and around the positive part of axis I, preferring (or tolerating) higher values of temperature (TJan, TJul, and T), and showing higher levels of both IUVACs and TUVACs, and higher I/S and SI values. The only hornwort appears relatively isolated in the second quadrant, especially influenced by environmental variables, mainly by high values of F and N. Within this general frame, there were some exceptions. Two typically desiccation-tolerant acrocarpous mosses (*Syntrichia ruralis* and *Pleurochaete squarrosa*) appeared in the group of pleurocarpous mosses, towards the extreme positive part of axis I, and three acrocarpous mosses (*Philonotis seriata*, *Bryum weigelii* and *Mnium lycopodioides*) were placed near the liverworts, at the extreme negative part of axis I. This again reflects the great diversity of acrocarpous mosses. In addition, two pleurocarpous mosses (*Antitrichia curtipendula* and *Fontinalis squamosa*) appeared embedded, respectively, within acrocarpous mosses and within liverworts, probably influenced by environmental variables (the immersion index in the case of the aquatic *F. squamosa*). The two species of Sphagnales, again, show an atypical position, mainly surrounded by liverworts. In contrast, two liverworts (*Porella arboris-vitae* and *P. platyphylla*) were placed among mosses.

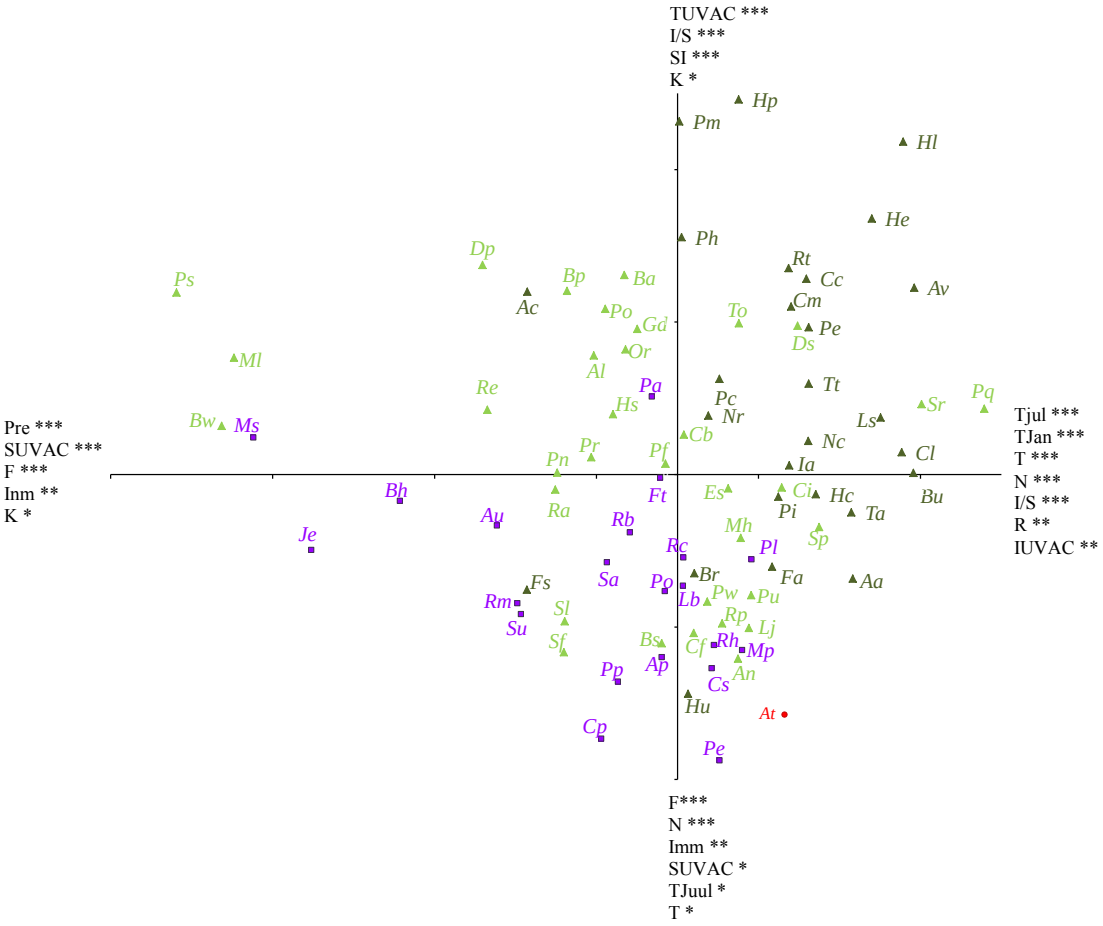


Figure 3.12. Ordination, through Principal Components Analysis (PCA), of the samples of the 87 bryophytes studied, on the basis of both environmental and physiological variables. Liverworts are represented by purple quadrats, mosses by green triangles and the only hornwort by a red circle. Within mosses, acrocarpous mosses are represented by light green triangles and pleurocarpous mosses by deep green triangles. Significant loading factors for the positive and negative parts of each axis, together with their corresponding significance levels, are shown. ***, $p<0.001$; **, $p<0.05$; *, $p<0.01$. For identification of species and environmental and physiological variables, see their codes in Table 3.1. Axis 1 is the horizontal one, and axis 2 is the vertical one. Each tic-mark on the axes represents one unit.

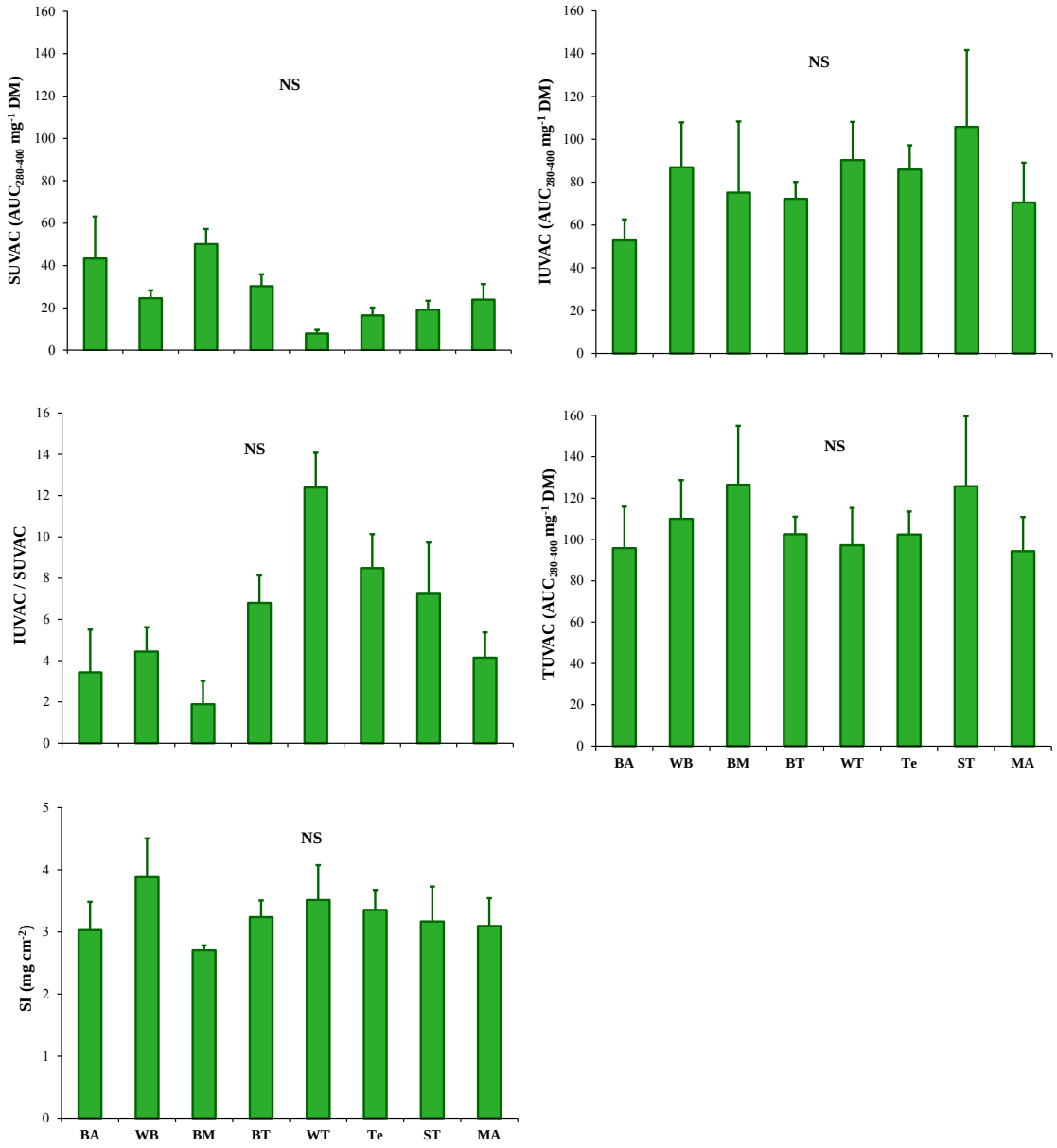


Figure 3.13. Differences in UVACs and sclerophylly index (SI) as influenced by the major biomes considered: BA, boreo-arctic montane; WB, wide-boreal; BM, boreal-montane; BT, boreo-temperate; WT, wide-temperate; Te, temperate; ST, southern-temperate; and MA, mediterranean-atlantic. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Results of ANOVAs or Kruskal-Wallis tests were all non-significant (NS).

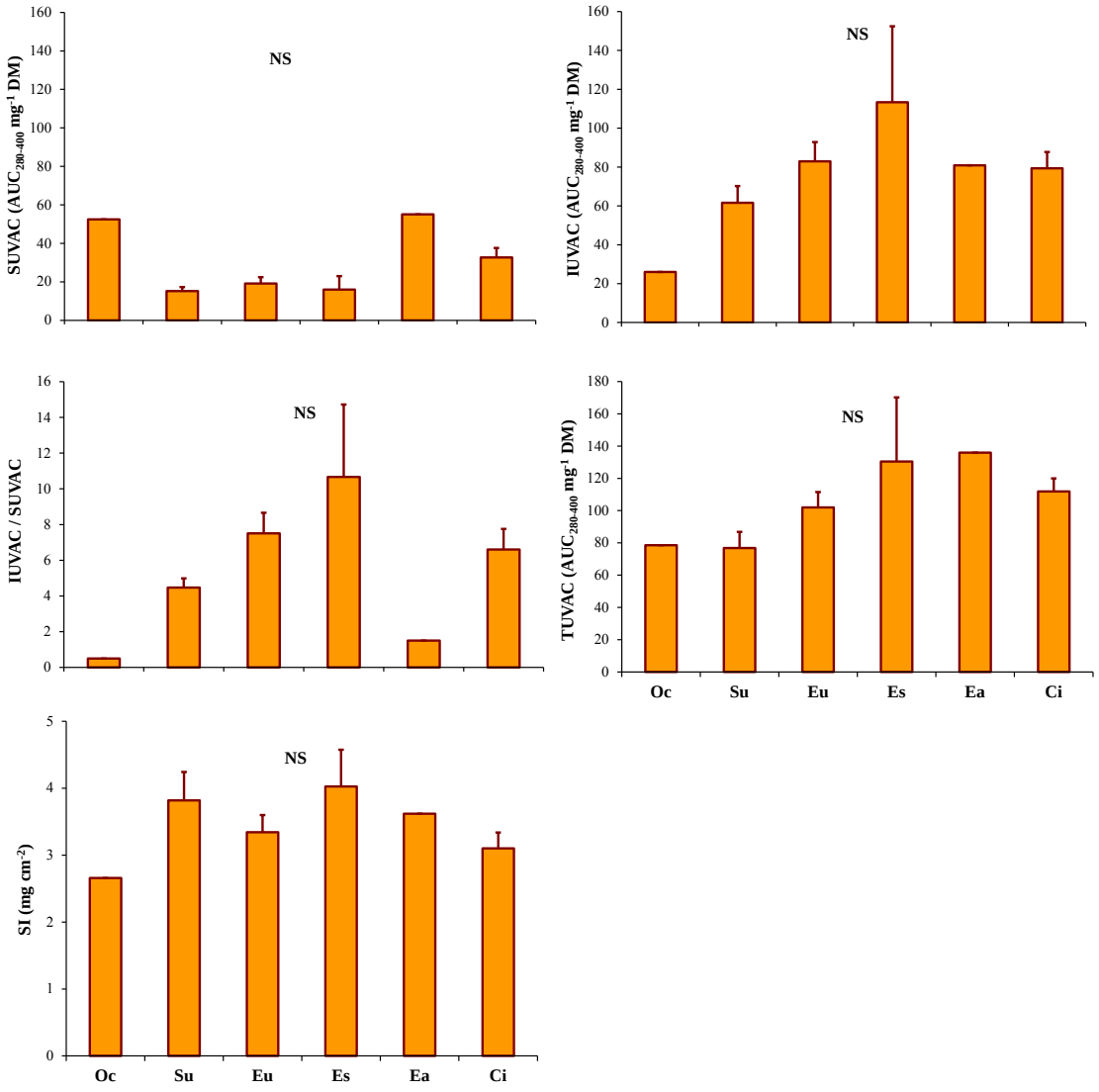


Figure 3.14. Differences in UVACs and sclerophylly index (SI) as influenced by the eastern limits considered: Oc, oceanic; Su, suboceanic; Eu, European; Es, Eurosiberian; Ea, Eurasian; Ci, circumpolar. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Results of ANOVAs or Kruskal-Wallis tests were all non-significant (NS).

The influence of other (qualitative) bryological attributes on UVACs

There was no influence of the biogeographical units (major biome and eastern limit) on the UVACs and the SI (Figs. 3.13 and 3.14). This was expected because this lack of influence was concordant with the general lack of solid correlations existing between the physiological and environmental variables (Table 3.2). In particular, the major biome would be related to environmental variables such as TJan, Tjul and T, whereas the eastern limit would be related to K, and only a few solid correlations were found between these quantitative variables and UVACs and SI. Thus, it is logical that the major biome and the eastern limit had no influence on UVACs and SI. In addition, there was a great variability within each group, and this could obscure a possible influence.

The influence of life form was only significant on the I/S quotient (Fig. 3.15), but probably this influence was spurious because there was, again, a great variability within each category of life form. Similar spurious effects of the sex could be observed on the bulk level of SUVACs and the I/S quotient (Fig. 3.16), because it is difficult to explain that the monoecious species had higher bulk levels of SUVACs and lower I/S quotients than the dioecious species.

Overall, the analysis of the influence of the qualitative bryological attributes (major biome, eastern limit, life form, and sex) on UVACs and SI could have served to have a kind of "negative" controls, because we did not expect any influence of these attributes. In this way, this lack of influence would have contrasted with the great influence of the environmental variables on UVACs and SI that we expected to find. However, this environmental influence was slighter than expected, and the "negative" controls have not been useful to highlight a more relevant influence of environmental variables.

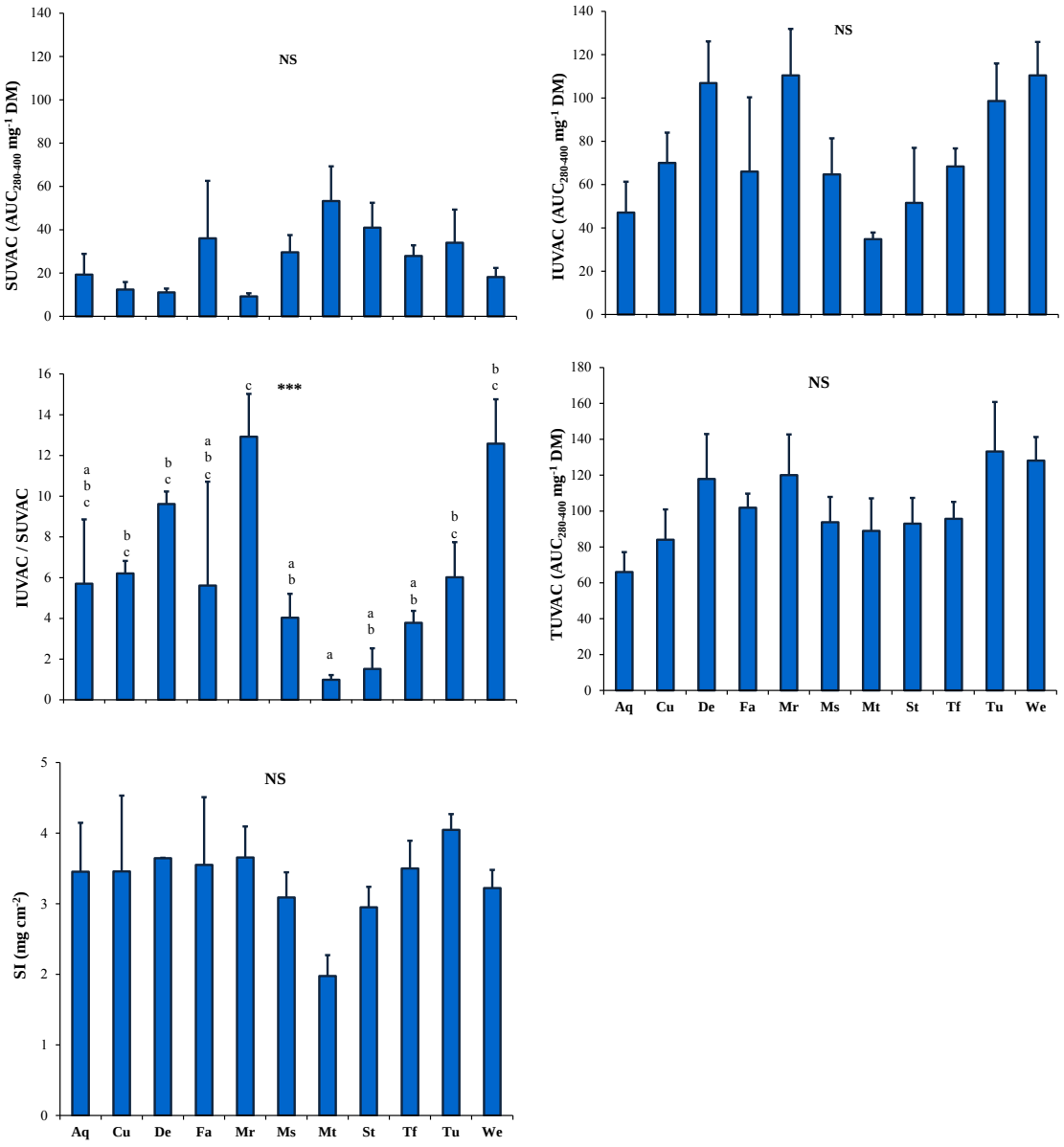


Figure 3.15. Differences in UVACs and sclerophylly index (SI) as influenced by the life forms considered: Aq, aquatic trailing; Cu, cushion; De, dendroid; Fa, fan; Mr, mat-rough; Ms, mat-smooth; Mt, mat-thalloid; St, solitary thalloid; Tf, turf; Tu, tuft; and We, weft. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (ANOVAs or Kruskal-Wallis tests): ***, $p<0.001$; NS, non-significant.

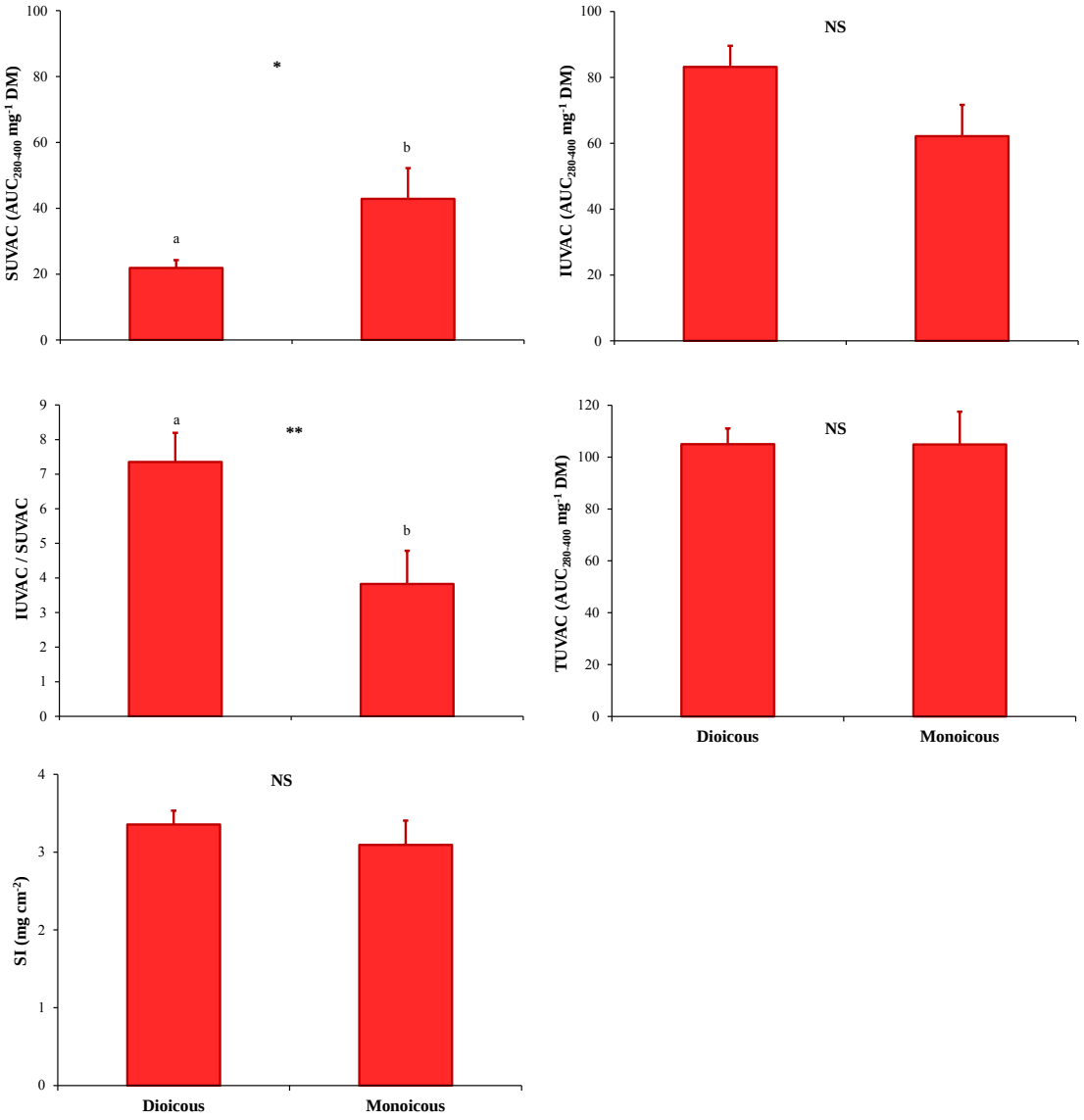


Figure 3.16. Differences in UVACs and sclerophylly index (SI) as influenced by distribution of the sex organs. SUVAC, IUVAC and TUVAC, the bulk levels of (respectively) soluble, insoluble, and total UVACs, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per unit of DM. IUVAC/SUVAC, the quotient between the bulk levels of IUVACs and SUVACs. Different letters mean significant differences with a determinate significance level (Student's *t* tests): **, $p<0.05$; *, $p<0.01$; NS, non-significant.

3.5. CONCLUSIONS

1) Liverworts and mosses differ in the levels and compartmentation of UVACs. In general, liverworts had higher levels of SUVACs and lower levels of IUVACs than mosses, and vice-versa. This difference represents an additional evidence of the phylogenetic distance between both groups, that nowadays is considered to be deeper than previously thought. Thus, UVACs compartmentation represents a new ecophysiological trait that could be evolutionarily important in the colonization of new UV-rich environments after the conquest of land by plants. More data are needed regarding hornworts to place them appropriately in this context, but the first data suggest they are more related to liverworts than to mosses.

2) Exceptions to the general rule described above may appear in cases of species with a peculiar UVAC composition or compartmentation, such as *Atrichum undulatum*, *Bartramia pomiformis*, and species of *Bryum*, *Porella* and *Frullania*.

3) UVAC compartmentation may help differentiate the main evolutionary lineages in mosses (acrocarpous and pleurocarpous), and also peculiar mosses as *Sphagna*, but still not the main evolutionary lineages in liverworts or the different Orders of mosses and liverworts. Plerocarpous mosses constituted the most homogeneous phylogenetic group, regarding UVACs levels and compartmentation, among the groups differentiated in our study.

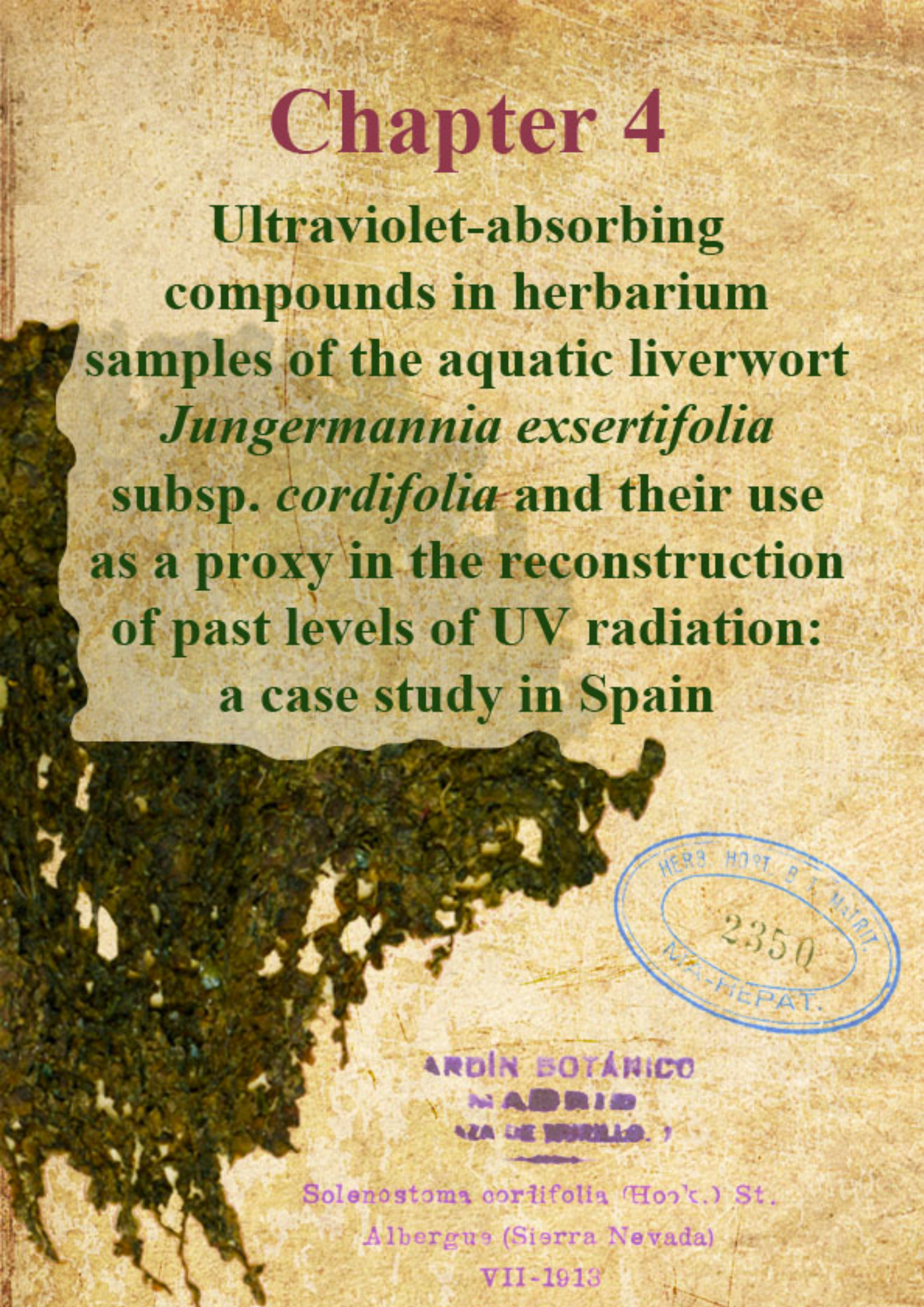
4) Assuming that cell wall-bound compounds are more efficient UV screens than vacuolar compounds, the higher amount of cell wall-bound UVACs in mosses than in liverworts would have allowed mosses to be more competitive in sunny UV-rich environments. Thus, mosses and liverworts may have evolved differently in this regard and thus they have tended to occupy different environments.

5) The lack of clear and solid relationships between UVACs accumulation and compartmentation, and the environmental variables (in particular those determining the UV amount received by the samples), suggests that UVACs are, at least to a certain extent, constitutive and privative of each species, and may not be usually induced by the natural ambient UV levels.

6) Liverworts and mosses also differed in their sclerophylly index, since mosses (in particular, Polytrichaceae) were significantly more sclerophyllous than liverworts (especially species constituted by simple leaves or simple thalli).

Chapter 4

Ultraviolet-absorbing compounds in herbarium samples of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* and their use as a proxy in the reconstruction of past levels of UV radiation: a case study in Spain



4.1.-ABSTRACT

Ultraviolet-absorbing compounds (UVACs) were analysed in 90 herbarium specimens of the aquatic leafy liverwort *Jungermannia exsertifolia* subsp. *cordifolia* collected in Spain in the period 1913-2006, in order to evaluate their usefulness in the reconstruction of past UV levels. We applied novel aspects such as: 1) the use of collection periods within the year longer to those previously used; 2) the use, for the first time in bryophytes, of both the soluble and insoluble fractions of UVACs (respectively, SUVACs and IUVACs) as biological proxies to reconstruct past UV, considering for this aim both the bulk levels of UVACs and the concentrations of individual specific UVACs, in the two fractions. These methodological approaches could contribute to the generation of more reliable models based on variables responding more specifically to UV radiation.

The bulk level of SUVACs was not correlated with that of IUVACs in the samples analysed, suggesting that both types of compounds may play different roles in the cells: UV screening and antioxidants. *p*-Coumaroylmalic acid (C1) was the only UVAC showing significantly higher concentrations after the onset of stratospheric ozone degradation than before it. The Principal Components Analysis (PCA) conducted using the physiological variables considered and the environmental variables under which the samples were growing, clearly separated the samples on the basis of the season when they were collected and their geographical origin. Both of these factors represented gradients of UV radiation. In addition, the PCA suggested that the different storage conditions in the different herbaria did not affect much the ordination of the samples. The bulk level of SUVACs, together with the concentrations of three individual soluble UVACs (C1, C3 and C5), were higher in Spain than in northern Europe, suggesting a better UV protection in the region exposed to higher UV levels. The biologically effective UV-B radiation, weighted using the Plant Damage action spectrum, was reconstructed using a model composed by the collection month and the bulk level of IUVACs, and did not show a clear trend in the period 1913-2006. This

lack of trend might be influenced by several factors (short sampling period within the year, uncertainties about UV exposure of the samples at the moment of sampling, possible breakdown of UVACs during sample storage in herbaria, and limited availability of UV climatic data), but was coincident with other UV reconstructions.

Overall, UVACs of herbarium samples of *J. cordifolia* have demonstrated a wide variety of relationships with UV radiation in the temporal and spatial scales, and particularly the bulk levels of IUVACs can be used as a biomonitor of changes in past UV levels.

4.2.- INTRODUCTION

Measurements of UV radiation at ground level were not geographically generalized until the 1990s (McKenzie *et al.*, 2011). Therefore, the set of UV data available is notably limited, and thus reconstructions of past UV levels on different temporal scales, from decades to millions of years, have been undertaken using climatic models (Lindfors *et al.*, 2003, 2007; Kvalevag *et al.*, 2009; Herman 2010a, 2010b; Kaurola *et al.*, 2010; Watanabe *et al.*, 2012). These reconstructions can improve the evaluation and interpretation of the effects of the increased UV-B levels produced in the biosphere as a consequence of stratospheric ozone degradation. Given that stratospheric ozone is a key factor influencing the UV-B levels reaching the ground, parallel reconstructions of ozone have been done. Nevertheless, it is important to note that the results of UV and ozone reconstructions strongly depend on the variables considered, particularly if aerosols, clouds, and pollutants are included in the models. For example, simulations through 1960-2100 show that UV radiation in the northern mid-latitudes is projected to increase in the 21st century despite the expected recovery of the stratospheric ozone layer, because reductions in aerosols and clouds are expected to overcompensate for the effect of ozone recovery. This strongly contrasts to previous assessments considering only the effect of long-term changes in stratospheric ozone.

In addition to climatic models, different biological variables have been used to reconstruct past UV and ozone levels: phenolic compounds in plant pollen, spores, fossil remains and herbarium specimens; scytonemin and carotenoids in algae from Antarctic lake sediments; species composition of diatoms; damage in DNA recovered from human urine and bird guano; carbon isotope composition of plants, etc. These biological methods have recently been reviewed (Björn & McKenzie, 2007; Rozema *et al.*, 2009; McKenzie *et al.*, 2011). In particular, flavonoids and hydroxycinnamic acid

(HCA) derivatives have been useful in these reconstructions (Rozema *et al.*, 2001, 2009; Blokker *et al.*, 2005, 2006; Huttunen *et al.*, 2005a; Lomax *et al.*, 2008; Otero *et al.*, 2009; Ryan *et al.*, 2009; Willis *et al.*, 2011). This may be logical because the levels of these compounds are determined in a significant manner by the levels of UV radiation, and can act as UV screens and antioxidants in plant cells.

Obviously, biological models for past UV and ozone reconstruction have more limitations than purely climatic models, such as the availability of samples, the environmental conditions (radiation, temperature, etc.) to which samples were exposed at the moment of collection, the preservation of the samples, etc.

Bryophytes are structurally simple plants that have traditionally been used as bioindicators/biomonitors of diverse types of pollution on both terrestrial and aquatic environments (Glime, 1992, 2011; Ah-Peng & Rausch de Traubenberg, 2004; Martínez-Abaigar & Núñez-Olivera, 2011; Harmens *et al.*, 2013; Gecheva & Yurukova, 2014). Bryophytes have also been used to reconstruct past levels of UV and ozone, mainly using herbarium samples of mosses and liverworts, together with moss spores. The most frequently used variable in bryophytes for reconstruction studies has been their phenolic composition, either expressed as the bulk level of UVACs or the concentrations of individual compounds (Markham *et al.*, 1990; Rozema *et al.*, 2001; Huttunen *et al.*, 2005a, 2005b; Blokker *et al.*, 2006; Otero *et al.*, 2009; Ryan *et al.*, 2009). This is congruent with the fact that the increase in phenolic compounds is the most usual response of bryophytes to enhanced UV-B (Newsham & Robinson, 2009). Among the individual compounds, flavonoids (luteolin, apigenin) and HCA derivatives (*p*-coumaroylmalic acid, *p*-coumaric acid, ferulic acid) have mostly been used for this aim (Otero *et al.*, 2009; Ryan *et al.*, 2009). In some cases, the ratios between different compounds (for example between *p*-coumaric and ferulic acids) have also resulted useful in this context (Blokker *et al.*, 2006).

In bryophytes, both the bulk level of UVACs (global UV absorbance) and the individual specific compounds can be measured in two cell compartments (Schnitzler *et al.*, 1996; Fabón *et al.*, 2010): the soluble fraction (mainly located in the vacuoles) and the insoluble fraction (bound to the cell walls). Each fraction may have a different composition, may respond differently to UV, and may represent a different protection

mechanism against UV effects (Fabón *et al.*, 2010). It has been postulated that the cell wall-bound fraction would represent a more efficient UV screen than the vacuolar fraction (Clarke & Robinson, 2008). This analytical differentiation between soluble and insoluble fractions has rarely been considered in bryophytes until recently (Fabón *et al.*, 2010, 2012a, 2012b; Soriano *et al.*, 2013; Hespanhol *et al.*, 2014; Martínez-Abaigar *et al.*, 2014), and has never been applied in bryophytes to the reconstruction of UV or ozone past levels. It would be adequate to test this possibility, especially considering that UVACs located bound to cell walls may be better preserved than those located in vacuoles, and thus they could be more adequate for retrospective studies (Rozema *et al.*, 2001; Willis *et al.*, 2011).

Evidently, biological reconstructions using bryophytes have similar limitations as those described above. However, significant results have already been obtained in UV and ozone reconstructions using bryophytes (Huttunen *et al.*, 2005a, 2005b; Otero *et al.*, 2009; Ryan *et al.*, 2009), and thus it is interesting to progress in this line (Björn & McKenzie, 2007).

Among bryophytes, one of the most frequently used species for UV bioindication/biomonitorization purposes has been the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, on the basis of the following points (Martínez-Abaigar & Núñez-Olivera, 2011): 1) possession of a variety of secondary metabolites, such as many different phenolic constituents; 2) responsiveness of some of its UVACs to UV radiation; 3) relatively large size, which allows for an easy manipulation; 4) growth in extensive and unmixed masses that provide plenty of biomass for experiments; 5) availability of healthy biomass throughout the year, given the long-lived perennial character of this species; 6) prevention of the interference of the typical transitory desiccation of bryophytes, because this is an aquatic species mostly living permanently submerged (thus, its responses to UV may be easier to interpret and model than those of terrestrial species); and 7) wide distribution range over the northern hemisphere, which facilitates studies across wide geographical scales.

The aims of the present study were to analyse UVACs in herbarium samples of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* from all over Spain, and to evaluate their usefulness in the reconstruction of past UV levels, applying novel aspects such as: 1) the use of collection periods within the year presumably longer to those previously used in northern Europe, which were severely limited by bad weather conditions (Otero *et al.*, 2009); and 2) the use, for the first time in bryophytes, of both the soluble and insoluble fractions of UVACs (respectively, SUVACs and IUVACs) as biological proxies to reconstruct past UV, considering for this aim both the bulk levels of UVACs and the concentrations of individual specific UVACs, in the two fractions considered. Given that both fractions may represent different biological meanings (Clarke & Robinson, 2008), and that each individual UVAC may respond differently to UV (Fabón *et al.*, 2010), these methodological approaches could contribute to the generation of more reliable models based on variables responding more specifically to UV radiation.

4.3.-MATERIALS AND METHODS

Plant material

The aquatic leafy liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váňa (hereafter *J. cordifolia*) was used in this study (Fig. 4.1). This dioicous liverwort is large (shoots up to 10 cm long and 5 mm wide) and strongly aromatic, typically growing in extense and swollen masses on rocks in oligotrophic circumneutral mountain streams. The leaves are about 2 mm long and wide, characteristically heart-shaped, with a broad and clasping base narrowing rapidly to the stem and a narrower, rounded tip. Its polygonal cells lack trigones and have thin walls and 2-3 oil bodies per cell. It may have red-brown tones, and even black, to its dominantly deep green colour. The main biological and ecological attributes for this liverwort can be found in Hill *et al.* (2007). Associated species can be *Chiloscyphus polyanthos*, *Scapania undulata*, *Brachythecium rivulare, Platyhypnidium riparioides* and *Bryum pseudotriquetrum*. Following Duell (1983), its geographical distribution is subarctic-subalpine. It can be found in the northern hemisphere, including Europe, Asia and North America.

A total of 90 herbarium specimens of *J. cordifolia* coming from all over Spain were obtained from fourteen herbaria of the Iberian Peninsula: ARAN-VIT, BC (BCB), BCB, FCO-Briófitos, GDAC, LISU, MA-Hepat, MACB, MUB, PAMP, SALA-Bryo, VIT, and the personal Herbarium of Javier Martínez-Abaigar (Table 4.1). The geographic location of the 90 samples used in this study is shown in Fig. 4.2 and detailed in Table 4.1.

Figure 4.1. An extense mass of *Jungermannia exsertifolia* subsp. *cordifolia* growing in a mountain stream in La Rioja (Spain), together with details of leafy shoots (photograph courtesy of Des Callaghan), heart-shaped leaves, and polygonal cells lacking trigones and showing peripheral chloroplasts and hyaline oil bodies. A typical herbarium sample is also shown.

Analysis of UV-absorbing compounds (UVACs)

UV-absorbing compounds of the liverwort apices were analyzed in the soluble fraction (SUVAC), mainly located in the vacuole, and in the insoluble fraction (IUVAC), bound to the cell walls. In both fractions, the bulk level of UVACs was analysed globally by spectrophotometry, and several individual UVACs were analysed individually by HPLC, following Schnitzler *et al.* (1996), Arróniz-Crespo *et al.* (2006) and Fabón *et al.* (2010).

To begin with, dry shoot apices were frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany), and then 5 ml of methanol:water:7 M HCl (70:29:1, v/v/v) was added for extraction (24 h at 4 ºC in the dark). The extract was centrifuged at 6000 *g* for 15 min at 10ºC to differentiate two fractions of UVACs. In the supernatant of the centrifugation, we measured the soluble compounds (SUVAC), and in the pellet, after alkaline digestion, the insoluble compounds (IUVAC) (Clarke & Robinson, 2008).

In the supernatant of the methanol extraction described above, the bulk level of SUVAC was measured as the area under the absorbance curve in the intervals 280-400 nm ($AUC_{280-400}$) and 280-315 nm ($AUC_{280-315}$) per unit of DM), using a Perkin-Elmer λ35 spectrophotometer (Perkin-Elmer, Wilton, CT, USA). These intervals correspond either to the whole of UV-A and UV-B radiations or only to UV-B radiation.

The pellet of the methanol extraction was hydrolysed with 2 ml 1 M NaOH for 3 h at 80ºC, acidified to pH 1.0 with HCl and extracted three times with ethyl acetate. After evaporation, the material was dissolved in 100% methanol and the bulk level of IUVAC was spectrophotometrically measured in the same units previously described for the bulk level of SUVAC.

Table 4.1. Data of the 90 samples of *Jungermannia exsertifolia* subsp. *cordifolia* used in this study: herbaria where the samples were deposited and corresponding label number, locality and province where the samples were collected, geographical data of each sample (longitude, latitude and altitude), and collection date.

Sample number	Herbarium / Label number	Locality and province	Longitude (ºE)	Latitude (ºN)	Altitude (m)	Collection date
1	MACB / 13679	Cuevas del Valle (Ávila)	-5.01	40.32	1260	07-09-1984
2	MACB / 62157	Viella (Lérida)	0.76	42.63	1630	23-07-1988
3	MACB / 79067	P.N. Aigüestortes (Lérida)	1.00	42.59	2100	08-07-2001
4	MUB / 4598	Viella (Lérida)	0.76	42.63	1630	23-07-1988
5	MUB / 21924	Benasque (Huesca)	0.63	42.68	1800	22-08-2006
6	SALA-BRYO / 212	Candelario (Salamanca)	-5.77	40.34	1230	18-10-1984
7	SALA-BRYO / 702	Sierra Béjar (Salamanca)	-5.76	40.31	1856	19-07-1985
8	SALA-BRYO / 703	Navacarros (Salamanca)	-5.69	40.38	1820	15-07-1983
9	SALA-BRYO / 704	Navacarros (Salamanca)	-5.69	40.38	1820	15-07-1983
10	SALA-MUSCI / 2045	Viella (Lérida)	0.76	42.63	1630	23-07-1988
11	GDAC / 12102	Sierra Nevada (Granada)	-3.42	37.02	2700	13-08-1976
12	GDAC / 24658	Pajares (La Rioja)	-2.59	42.03	1200	16-11-1986
13	MA-HEPAT / 1873	Sierra Nevada (Granada)	-3.38	37.09	2500	07-1913
14	FCO-BRIOFITOS / 4561	Vegadeo (Asturias)	-7.03	43.40	320	16-03-1974
15	FCO-BRIOFITOS / 1160	Viella (Lérida)	0.76	42.63	1630	23-07-1988
16	FCO-BRIOFITOS / 209	Vegadeo (Asturias)	-7.03	43.40	320	16-03-1974
17	LISU / 175285	Sierra Nevada (Granada)	-3.33	37.05	2955	11-07-1997
18	LISU / 055963	Salardú (Lérida)	0.97	42.64	1850	05-09-1988
19	BCB / 46913	Setcases (Gerona)	2.26	42.42	2200	15-07-1985

Sample number	Herbarium / Label number	Locality and province	Longitude (ºE)	Latitude (ºN)	Altitude (m)	Collection date
20	BCB / 46914	Pajares (La Rioja)	-2.59	42.03	1300	08-03-1987
21	BCB / 21912	Villasimpliz (León)	-5.66	42.91	1140	1974
22	BCB / 20636	Candelario (Salamanca)	-5.77	40.34	1230	18-10-1984
23	BCB / 20637	Estany Xic (Lérida)	0.88	42.61	2200	24-08-1980
24	BCB / 16432	Nuria (Gerona)	2.16	42.42	2500	01-09-1956
25	BCB / 16434	Sant Maurici (Lérida)	1.00	42.58	2000	13-06-1956
26	BCB / 32330	Villalonga del Ter (Gerona)	2.26	42.42	2200	27-07-1984
27	BCB / 16433	Vall San Nicolau (Lérida)	0.95	42.58	2000	03-08-1959
28	BCB / 16435	Vall San Nicolau (Lérida)	0.96	42.58	2100	03-08-1959
29	BCB / 16428	Valle de Ordesa (Huesca)	-0.04	42.65	1450	17-08-1965
30	BCB / 16429	Panticosa (Huesca)	-0.23	42.78	2000	14-07-1965
31	BCB / 25964	Salardú (Lérida)	0.97	42.64	2250	05-09-1988
32	BCB / 25963	Setcases (Gerona)	2.26	42.41	1900	17-10-1983
33	BCB / 25962	Panticosa (Huesca)	-0.23	42.78	2000	1965
34	BCB / 32934	Benasque (Huesca)	0.64	42.67	2250	07-1966
35	BCB / 41365	Estany Llonc (Lérida)	0.95	42.58	2000	19-07-1944
36	BCB / 25965	Vega Liébana (Cantabria)	-4.73	43.03	2200	14-08-1987
37	BCB / 45753	Boquerón de Bobias (León)	-4.76	43.03	1900	14-08-1987
38	BCB / 45756	Caldevilla Valdeón (León)	-4.95	43.10	1750	17-08-1989
39	BCB / 45752	Pico Curavacas (Palencia)	-4.68	42.98	1900	12-07-1988
40	BCB / 52485	Sierra Nevada (Granada)	-3.33	37.05	2955	11-07-1997
41	BCB / 52724	Sierra Nevada (Granada)	-3.34	37.03	2500	14-07-1997

Sample number	Herbarium / Label number	Locality and province	Longitude (ºE)	Latitude (ºN)	Altitude (m)	Collection date
42	BCB / 52615	Sierra Nevada (Granada)	-3.34	37.05	2920	14-07-1997
43	BCB / 54032	Estanys de la Pera (Gerona)	1.60	42.46	2350	06-07-1999
44	BCB / 54031	Clots del Port (Gerona)	1.60	42.46	2450	06-07-1999
45	BCB / 54343	Cassibrós (Lérida)	1.26	42.70	1550	29-06-1998
46	BCB / 54232	Pardines - Tragurà (Gerona)	2.22	42.33	1850	05-08-1999
47	BCB / 54710	Vall de la Llosa (Gerona)	1.70	42.44	1650	07-07-1999
48	BCB / 54102	Tredòs (Lérida)	0.93	42.65	1770	13-07-1998
49	BCB / 54034	Vall de la Llosa (Gerona)	1.70	42.44	1650	07-07-1999
50	BCB / 54342	Vallibierna (Huesca)	0.65	42.61	2300	02-08-1997
51	BCB / 54037	Estanys de la Pera (Gerona)	1.60	42.46	2350	06-07-1999
52	BCB / 54036	Estanys de la Pera (Gerona)	1.60	42.46	2350	06-07-1999
53	BCB / 54033	Vall de la Llosa (Gerona)	1.70	42.45	1720	07-07-1999
54	BCB / 55838	Alt Aneu (Lérida)	1.08	42.61	1500	28-08-2006
55	VIT / 649/87	Ceanuri (Vizcaya)	-2.77	43.04	1150	04-07-1987
56	VIT / 541/86	Tredòs (Lérida)	0.96	42.66	1800	21-07-1986
57	VIT / 1104/86	Tredòs (Lérida)	0.96	42.66	1800	21-07-1986
58	VIT / 1084/86	Areu (Lérida)	1.41	42.63	2400	20-07-1986
59	VIT / 134/94	Boca de Huérgano (León)	-4.77	43.06	1600	07-06-1994
60	VIT / 32821 (602/04)	Panticosa (Huesca)	-0.24	42.79	2350	22-07-2004
61	ARAN - VIT / 1397	Panticosa (Huesca)	-0.23	42.77	1800	23-07-1984
62	VIT / 30068 (1143/02)	Montanuy (Huesca)	0.71	42.59	2250	12-09-2002
63	VIT / 22370 (852/98)	Jaca (Huesca)	-0.50	42.82	2100	26-08-1998

Sample number	Herbarium / Label number	Locality and province	Longitude (ºE)	Latitude (ºN)	Altitude (m)	Collection date
64	VIT / 817/86	Sarría (Álava)	-2.83	43.03	790	30-09-1986
65	VIT 7 657/86	Murúa (Álava)	-2.74	43.00	700	24-09-1986
66	GDAC	Sierra Nevada (Granada)	-3.37	37.08	2850	10-1970
67	GDAC	Sierra Nevada (Granada)	-3.37	37.08	2600	04-11-1971
68	BC (BCB)	Jaizquibel (Guipúzcoa)	-1.84	43.36	50	28-09-1930
69	PAMP / 6624	Viella (Lérida)	0.76	42.63	1630	23-07-1988
70	BCB / 4002	Viella (Lérida)	0.77	42.62	1620	15-09-1984
71	PAMP / 323	Sierra Nevada (Granada)	-3.38	37.09	2600	07-1970
72	J. Martínez Abaigar	Lumbreras (La Rioja)	-2.62	42.03	1450	30-07-1987
73	J. Martínez Abaigar	Lumbreras (La Rioja)	-2.63	42.01	1800	12-11-1986
74	J. Martínez Abaigar	Lumbreras (La Rioja)	-2.59	42.03	1350	27-10-1985
75	J. Martínez Abaigar	Lumbreras (La Rioja)	-2.59	42.03	1350	22-11-1986
76	J. Martínez Abaigar	Villoslada Cam. (La Rioja)	-2.67	42.07	1200	28-08-1986
77	J. Martínez Abaigar	Villoslada Cam. (La Rioja)	-2.66	42.60	1400	28-08-1986
78	J. Martínez Abaigar	Villoslada Cam. (La Rioja)	-2.68	42.07	1140	28-08-1986
79	J. Martínez Abaigar	Villoslada Cam. (La Rioja)	-2.69	42.04	1400	26-08-1986
80	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.92	42.18	1000	03-09-1996
81	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.95	42.16	1000	25-06-1994
82	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.98	42.22	1750	03-09-1996
83	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.90	42.02	1850	10-08-1995
84	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.95	42.16	1000	25-06-1994
85	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.90	42.02	1850	17-10-1996

Sample number	Herbarium / Label number	Locality and province	Longitude (ºE)	Latitude (ºN)	Altitude (m)	Collection date
86	J. Martínez Abaigar	Mansilla Sierra (La Rioja)	-2.95	42.16	1000	25-06-1994
87	BCB / 346	Vall de Mulleres (Lérida)	0.75	42.63	1700	24-07-1983
88	BCB / 1397	Viella (Lérida)	0.76	42.63	1630	23-07-1988
89	BCB / 733	Pajares (La Rioja)	-2.59	42.03	1200	16-11-1986
90	BCB / 1530	Bono (Huesca)	0.72	42.61	1950	21-08-1986

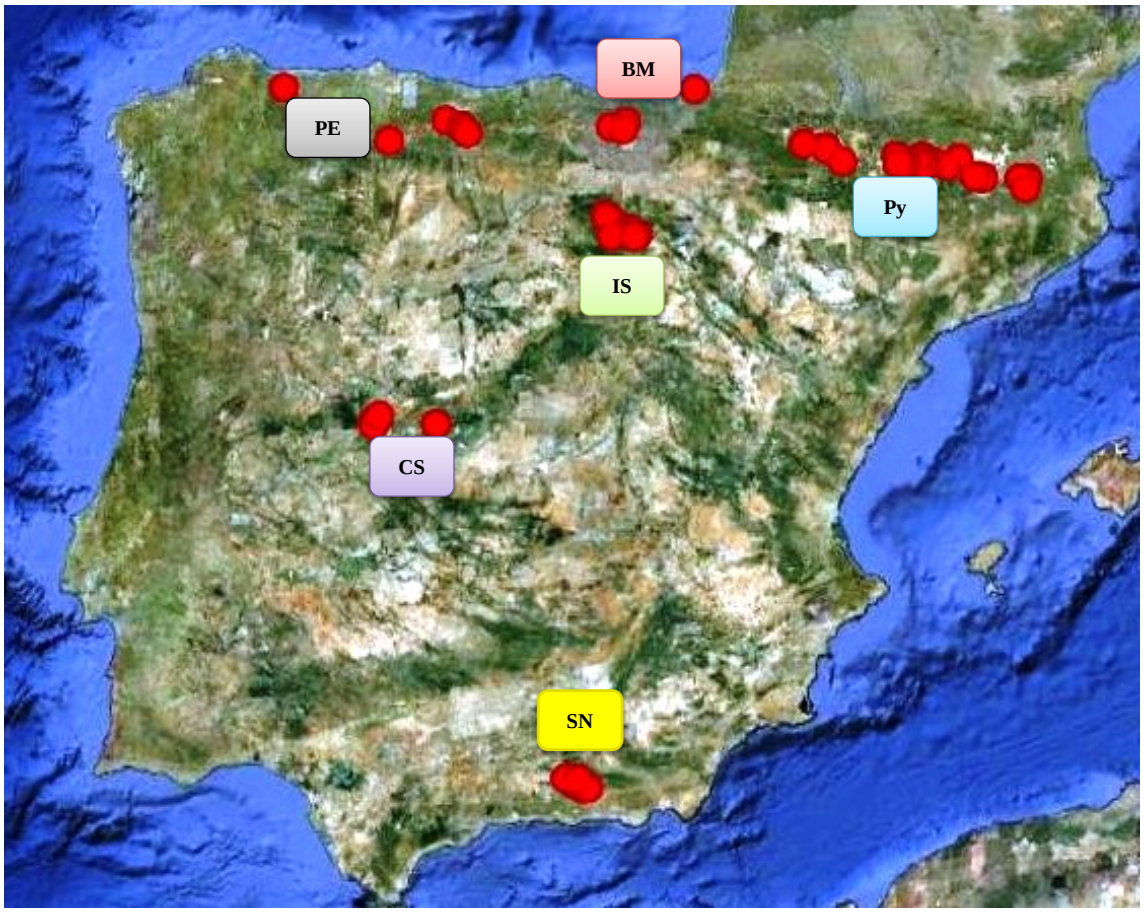


Figure 4.2. Geographic location of the 90 herbarium specimens of the aquatic leafy liverwort *Jungermannia exsertifolia* subsp. *cordifolia* used in this study. Abbreviations of the mountain chains where samples were collected are as follows: BM, Basque Mountains; CS, Central System; IS, Iberian System; PE, Picos de Europa; Py, Pyrenees; SN, Sierra Nevada.

Individual UVACs were measured by high-performance liquid chromatography (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA). 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 of two components: solvent A, acetic acid pH 2.4 and 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 absorption spectra were recorded in the wavelength range between 200 and 500 nm.

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. In the insoluble fraction (IUVAC), and after alkaline 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 previously been identified using Nuclear Magnetic Resonance and Mass Spectrometry (Arróniz-Crespo *et al.*, 2006). 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*-coumaric and *p*-coumaroylmalic acids, ferulic acid (Fluka) for ferulic and feruloylmalic acids, and 7,8-dihydroxy-4-phenyl-coumarin (Aldrich) for C4 and C5. The relationship between *p*-coumaric and ferulic acids was useful in the assessment of long-term changes in UV-B radiation (Lomax et al., 2008). Thus, the C6/C7 ratio was calculated in the insoluble fraction, and by analogy, the C1/C3 ratio in the soluble fraction. All the measurements were obtained in triplicate (three samples for each herbarium specimen, using around 12 mg DM for each sample).

Environmental data

Stratospheric ozone data were obtained from the Total Ozone Mapping Spectrometer (TOMS) website (http://toms.gsfc.nasa.gov/ozone) for each collection site in which samples had been collected from 1979 to present, since this is the period available for ozone in this webpage. Ozone data were obtained for the collection day and also for the two days before, because a 3-day period seems to be enough to find responses in the levels of UVACs (Jansen *et al.*, 1998). Other studies use similar periods of 1-3 days (Newsham *et al.*, 2002; Newsham, 2003; Sullivan *et al.*, 2007).

Since ozone levels for 1 and 3 days were highly correlated (r = 0.74 p<0.001), 1-day data were further used for data treatment.

Radiation data for the collection day were obtained using Engelsen's model Version 2.3 (Engelsen & Kylling, 2005), available at the website of the Norwegian Institute for Air Research (NILU) (http://zardoz.nilu.no/~olaeng/fastrt/fastrt.html). This model estimates both UV radiation doses and Ultraviolet Index on the Earth's surface, starting from different data, such as daily stratospheric ozone, longitude, latitude, altitude and date. Thus, radiation data could only be obtained for the days in which ozone data were available. This model was applied to obtain daily doses of UV-A, UV-B, and weighted UV-B (using both CIE Erythema –skin redness- and Plant Damage action spectra). Weighted UV-B corresponds to the biologically effective UV-B (UV-B_{BE}). As in the case of ozone, radiation data were also obtained for two days prior to collection of each sample, but only 1-day data were processed because of both data sets were highly correlated.

Longitude, latitude and altitude were obtained from the labels of herbarium specimens where available, and the data for the remaining specimens were generated with geographic information systems (GIS) starting from the data provided on the labels.

Data analysis

We tested the influence of several independent variables on the global and individual levels of SUVACs and IUVACs, as well as on the ratios C1/C3 and C6/C7. The independent variables were: the age of the samples (collection year); the month of collection; the latitude, longitude and altitude where the samples were collected; the total ozone; and the different formulations of radiation variables. All these variables will be hereafter referred as a whole as "environmental variables".

Data analysis included, firstly, bivariate correlations between all of the variables used (Pearson's coefficients). Correlations were obtained using all samples, and also using only the samples collected in July, the month with the highest number of

collections. In this last way we tried to eliminate the noise caused by the seasonal changes in UV radiation on the UVACs. Regression analyses were also conducted to model the relationships between selected variables, taking the environmental variables as independent variables and the physiological variables (UVACs) as dependent variables. In the regression analyses, coefficients of determination (r^2) were calculated.

UVACs of the samples collected before and after the onset of the ozone depletion process were compared using a Student's *t* test. This method was also used to compare the UVAC values between the samples from Spain (this study) and those collected in northern Europe (Otero *et al.*, 2009).

The samples were ordered by Principal Component Analysis (PCA) taking into account both the environmental and physiological data.

Finally, a stepwise multiple linear regression analysis was applied to try to explain the UV-B_{BE} radiation (weighted using the Plant Damage action spectrum), as a function of environmental and physiological variables in the period for which UV radiation values were available (1979-2006). The data generated by this analysis were then compared, using linear regression, with data of the same variable obtained by Engelsen's model. Given that both sets of data were closely correlated, and thus stepwise regression produced a good estimation of the data generated by Engelsen's model, UV-B_{BE} (Plant Damage) daily doses were reconstructed for the whole period in which herbarium samples were available (1913-2006). This allowed to analyse the trend of temporal changes in UV-B_{BE}. In the stepwise regression analysis, the probability values referred to the significance of the slope of the regression lines were calculated, as determined by the two-sided *t*-test (*t* is the *t*-statistics). All statistical procedures were performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

4.4.- RESULTS AND DISCUSSION

Geographic distribution of the herbarium samples

The geographic distribution of the herbarium samples of *J. cordifolia* used in this study (Table 4.1, Fig. 4.2) matched well with the real distribution of this species in Spain, where it had been found in the main chains of mountains: Pyrenees, Picos de Europa, Basque Mountains, Iberian System, Central System and Sierra Nevada (Martínez-Abaigar & Ederra, 1992). Thus, the herbarium survey carried out for this study was a good approach to this real distribution. It has to be noted that, within the Iberian Peninsula, *J. cordifolia* has recently been found in northern Portugal (Garcia *et al.*, 2012).

Summary of physiological and environmental variables

Table 4.2 shows a summary of numerical values of the different physiological and environmental variables used in this study. The collection period covered 93 years, from 1913 to 2006, which is a narrower period in comparison to that used in a similar study conducted in northern Europe (1850-2006: Otero *et al.*, 2009). This seems logical due to the greater bryological tradition in this last territory. Collections were made between March and November, mostly in July (38 collections, 42% of the total). The remaining collections were made in August (19), September (11), June and October (6 each), November (5) and March (3). The month of collection was unknown in two samples. The seasonal distribution of the collections was 72% in summer, 22% in autumn, and only 3% in spring. No collection was carried out in winter. In northern Europe (Otero *et al.*, 2009), the highest number of collections was also made in July, but the collection period within the year was shorter (June-October), probably due to climatic restrictions. Nevertheless, in Spain only 9% of the samples were collected out

from the period June-October, and thus we could get only a limited representation of spring samples (and no representation, again, of winter samples). Overall, in Spain, the sampling period within the year did not result as wide as expected on the basis of the milder climate with respect to that in northern Europe. This fact obviously limited the capability to reconstruct past UV using samples of *J. cordifolia*, since, for example, winter samples representing the baseline of UVACs contents (Núñez-Olivera *et al.*, 2009) were lacking.

Latitude and longitude gradients were, respectively, 6º and 9º. The latitudinal gradient represented approximately 700 km distance. In northern Europe, this gradient was wider (10º: Otero *et al.*, 2009). The altitude interval in Spain was 50-2995 m, and most samples (77%) were collected between 1000 and 2200 m. This altitude gradient was much wider than that in northern Europe (20-800 m: Otero *et al.*, 2009), in accordance with the higher altitudes occurring in Spain.

Daily values of ozone varied between 289 and 378 DU. They were slightly higher than those recorded in northern Europe (268-355: Otero *et al.*, 2009), which is logical due to the stronger stratospheric ozone degradation towards Arctic zones than in mid-latitudes (McKenzie *et al.*, 2007), although ozone values naturally increase with increasing latitude from the equator to the poles. Finally, and logically, both intervals and mean values of all the UV variables (daily doses of UV-A, unweighted UV-B, and UV-B_{BE} weighted with Erythemal and Plant Damage spectra) were higher in Spain tan in northern Europe (Otero *et al.*, 2009), due to the lower latitudes in Spain and the consequent more perpendicular reception of solar radiation.

The bulk levels of SUVAC were clearly higher than those of IUVAC, as occurs usually in liverworts and contrary to mosses (see Chapter 3). This matches well with the results obtained is fresh samples of *J. cordifolia* (Fabón *et al.*, 2010; Chapter 5). Among the individual UVACs, C2 was clearly the most abundant one, followed by C1, C4, C5, C7 and C3. C6 was the less abundant UVAC. This ordination coincides with that found in herbarium samples of *J. cordifolia* from northern Europe (Otero *et al.*, 2009).

Table 4.2. Summary of numerical values (minimum, maximum, and mean) of the different physiological and environmental variables used in this study, together with the number of data for each case. SUVAC and IUVAC, bulk levels of, respectively, soluble and insoluble UV-absorbing compounds (as the AUC, area under the absorbance curve, in the interval 280-400 nm). C1 to C7, individual UV-absorbing compounds already named in the text. DU, Dobson units.

Variable	n	Min	Max	Mean
SUVAC ($AUC_{280-400}$ mg^{-1} DM)	89	6.54	52.45	26.50
IUVAC ($AUC_{280-400}$ mg^{-1} DM)	90	8.76	24.30	13.60
C1 (µmol g^{-1} DM)	90	0.00	17.45	5.16
C2 (µmol g^{-1} DM)	90	0.00	47.26	13.79
C3 (µmol g^{-1} DM)	90	0.00	11.87	2.63
C4 (µmol g^{-1} DM)	90	0.00	21.62	5.17
C5 (µmol g^{-1} DM)	90	0.00	21.41	4.75
C6 (µmol g^{-1} DM)	90	0.40	2.89	0.98
C7 (µmol g^{-1} DM)	90	0.96	10.17	3.99
C1/C3	73	0.38	5.39	2.02
C6/C7	90	0.08	0.89	0.27
Collection year	90	1913	2006	
Collection month	88	3	11	7.7
Latitude (ºN)	90	37.03	43.40	42.55
Longitude (ºE)	90	-7.03	2.26	-3.15
Altitude (m)	90	50	2955	1775
Daily ozone (DU)	70	289	378	319
UV-A Dose (kJ m^{-2} d^{-1})	70	451	1768	1432
UV-B Dose (kJ m^{-2} d^{-1})	70	5.3	53.5	35.8
Weighted UV (CIE Erythemal) (kJ m^{-2} d^{-1})	70	0.69	6.73	4.21
Weighted UV (Plant Damage) (kJ m^{-2} d^{-1})	70	0.44	8.92	5.27

Correlations between the variables used

The two expressions calculated for the bulk level of SUVAC ($AUC_{280-315}$ and $AUC_{280-400}$) were significantly and positively correlated ($r = 0.99$ $p<0.001$), as well as the same two expressions referred to IUVAC ($r = 0.97$ $p<0.001$). Thus, both wavelength intervals gave the same type of information, and only $AUC_{280-400}$ will be used to describe results. For the bulk level of SUVAC, the correlation between both expressions is concordant with previous results obtained in a number of liverworts and mosses (Otero *et al.*, 2008), whereas that correlation had not been previously analysed for the bulk level of IUVAC.

The remaining correlations between the used variables, both environmental and physiological, are shown in Table 4.3. The bulk level of SUVAC was not correlated with that of IUVAC. This may indicate a different function for both types of compounds. IUVACs, that are bound to the cell walls, could play a more efficient UV screening role (Clarke & Robinson, 2008), whereas SUVACs could also be antioxidants. The bulk level of SUVAC was significantly and positively correlated with all the individual soluble UVACs (C1 to C5), as in Otero *et al*. (2009), but the bulk level of IUVAC was not correlated with the individual insoluble UVACs (C6 and C7). On the other hand, the bulk level of IUVAC was negatively correlated with some soluble compounds (C1, C2 and C4). This would suggest a metabolic connection between vacuolar and cell wall-bound compounds, and the decrease in certain vacuolar compounds would lead to a global increase in cell wall-bound compounds. All the individual soluble UVACs were positively correlated (as in Otero *et al*., 2009), as occurred with the two insoluble UVACs, but soluble UVACs were also positively correlated with an insoluble UVAC (C7). Thus, the relationships between the different individual UVACs were complex and remain to be completely elucidated.

All the variables indicative of UV radiation were strongly and positively correlated. This is logical because all of them were derived from the same model (Engelsen & Kylling, 2005). All the UV variables were also positively correlated with the collection year for the period in which UV levels could be calculated (1979-2006). This could suggest that UV-B in particular, and UV in general, increased along that period due to both ozone degradation and global warming (that may cause less

cloudiness and a consequent increase in solar radiation at mid-latitudes: McKenzie *et al.*, 2011). Nevertheless, determination coefficients (r^2) in the linear regressions between UV variables and the collection year of the samples were low (around 0.10), and thus the collection year did not explain much variability of UV levels. In addition, many uncertainties still remain regarding the influence of global warming on UV levels (McKenzie *et al.*, 2011).

The collection year was also positively correlated with all the soluble UVACs, but not with the insoluble UVACs. Correlations between the year and the soluble UVACs had previously been found (Otero *et al.*, 2009), which could be interpreted in two different ways: 1) UVACs would be broken down as storage period of the samples increased, which would limit the use of these compounds in retrospective studies; and 2) at least some UVACs have really increased in response to the increase in the UV-B levels derived from ozone degradation. Both hypotheses may be partially true. On one hand, phenolic derivatives can be broken down with time in stored plant samples, although the breakdown velocity may depend on the type of compound (Björn & McKenzie, 2007; Huttunen *et al.*, 2005a, 2005b). For example, Huttunen *et al.* (2005a) did not find any influence of storage or age on the oxidation of some moss flavonoids, and *p*-coumaric acid and other HCA derivatives seem to be chemically stable because they have been found in fossil pollen, mostly in cell walls (Rozema *et al.*, 2001, 2009; Blokker *et al.*, 2005, 2006; Lomax *et al.*, 2008; Willis *et al.*, 2011). This did not fit well with the fact that in our case the UVACs that were positively correlated with the collection year were the soluble compounds and not the insoluble ones. On the other hand, some UVACs from *J. cordifolia*, in particular C1 and C5, increased with increasing UV, under both laboratory and field conditions (Martínez-Abaigar *et al.*, 2003; Arróniz-Crespo *et al.*, 2006, 2008a, 2008b; Núñez-Olivera *et al.*, 2009). It is challenging that changes in C1 (the most UV-responsive UVAC of *J. cordifolia*, that in addition results specifically induced by UV-B: Martínez-Abaigar & Núñez-Olivera, 2011) seemed to show a relationship rather exponential than linear (graph not shown). This would support the hypothesis that C1 has been accumulated more strongly in the liverwort after the development of stratospheric ozone degradation (around 1980). In fact, C1 was the only physiological variable whose values prior to 1980 were significantly lower than those found after 1980: 3.18 ± 0.67 (n = 19) *vs.* 5.69 ± 0.58 (n = 71) (means ± SE).

Table 4.3. Correlation coefficients among physiological and environmental variables. CIE and PD, weighted UV-B using either CIE Erythema (skin redness) or Plant Damage action spectra. SUVAC and IUVAC, bulk levels of, respectively, soluble and insoluble UV-absorbing compounds. C1 to C7, concentrations of individual UV-absorbing compounds already named in the text. Significant correlations are differentiated in different colours depending on the significance level. Note that similar coefficients may show different significance levels depending on the value of *n*, which may be different for each pair of variables.

Alt	Lat	Long	UV-A Dose	UV-B Dose	CIE	PD	Ozone	Year	Month	SUVAC	IUVAC	C1	C2	C3	C4	C5	C6	C7	C1/C3	C6/C7	
1	-0.51	0.34	0.46	0.52	0.39	0.54	-0.11	0.05	0.08	0.04	0.34	-0.02	0.01	0.19	-0.09	0.26	0.16	0.08	-0.28	0.08	**Altitude**
	1	0.33	-0.09	-0.17	-0.05	-0.21	0.13	0.13	-0.14	0.08	-0.23	0.16	-0.12	0.05	0.05	0.04	0.03	0.13	0.27	0.07	**Latitude**
		1	0.26	0.25	0.25	0.24	-0.02	0.09	0.00	0.19	-0.12	0.33	0.03	0.31	0.10	0.30	0.31	0.35	0.20	0.10	**Longitude**
			1	0.98	0.79	0.95	0.25	0.37	-0.78	0.21	0.18	0.13	0.10	0.13	0.11	0.12	0.06	0.13	0.11	0.06	**UV-A Dose**
				1	0.83	0.99	0.09	0.32	-0.73	0.17	0.23	0.10	0.02	0.12	0.05	0.09	0.09	0.14	0.09	0.04	**UV-B Dose**
					1	0.84	0.04	0.25	-0.57	0.11	0.23	0.10	-0.01	0.12	0.00	0.04	0.15	0.13	0.12	-0.06	**CIE**
						1	0.00	0.30	-0.69	0.16	0.26	0.10	0.00	0.12	0.03	0.07	0.10	0.15	0.08	0.02	**PD**
							1	0.23	-0.35	0.14	-0.05	0.10	0.28	0.07	0.24	0.07	-0.12	-0.06	0.09	0.01	**Ozone**
								1	-0.03	0.15	0.00	0.26	0.28	0.25	0.23	0.24	0.05	0.14	-0.01	-0.02	**Year**
									1	-0.12	0.04	-0.09	-0.17	-0.03	-0.08	0.06	0.03	-0.18	-0.17	-0.08	**Month**
										1	-0.13	0.70	0.58	0.56	0.71	0.55	-0.09	0.40	0.36	-0.04	**SUVAC**
											1	-0.28	-0.29	-0.15	-0.32	-0.19	0.11	-0.12	-0.26	-0.02	**IUVAC**
												1	0.62	0.67	0.60	0.53	0.06	0.46	0.66	0.00	**C1**
													1	0.60	0.67	0.54	-0.01	0.34	0.15	-0.02	**C2**
														1	0.54	0.75	0.14	0.52	-0.10	0.08	**C3**
															1	0.46	-0.04	0.31	0.19	-0.06	**C4**
																1	0.11	0.36	-0.08	0.04	**C5**
																	1	0.47	-0.07	0.28	**C6**
																		1	0.07	0.00	**C7**
																			1	-0.03	**C1/C3**
																				1	**C6/C7**

$p<0.05$
$p<0.01$
$p<0.001$

The collection month was negatively correlated with ozone (as in Otero *et al.*, 2009), probably because collections were carried out mainly in the period June-November, and in the studied area ozone maxima are reached in spring (March-May) whereas the minima are recorded in autumn (October-November) (Häder *et al.*, 2007). The collection month was also negatively correlated with all the expressions of UV radiation, again because collections were mainly made in June-November, a period of the year in which UV levels progressively decrease after the summer solstice (see for example Núñez-Olivera *et al.*, 2011).

The positive correlation between altitude and UV variables was logical, because UV levels, especially UV-B, increase with altitude (Björn *et al.*, 1998; Arróniz-Crespo *et al.*, 2006). It may also be interesting the positive correlation between altitude and both C5 concentration and the bulk level of IUVAC, which could be due to a higher protection in samples coming from higher altitudes. In fresh samples of *J. cordifolia*, altitude was also positively correlated to C5 (Arróniz-Crespo *et al.*, 2006).

Latitude, another key factor influencing UV levels received at the ground level (Björn, 1999), was negatively correlated with the bulk level of IUVACs. This may be logical because UV increases with decreasing latitude, and thus the plant would be better protected at lower latitudes. However, no correlation between latitude and SUVACs was found.

Weighted UV-B using the Plant Damage spectrum was positively correlated with the bulk level of IUVACs, but not of SUVACs. Given this correlation, and the fact that these cell wall-bound UVACs would be more efficient UV screens than vacuolar ones (SUVACs) (Clarke & Robinson, 2008), the bulk level of IUVACs would be a good response variable to UV levels in herbarium samples of *J. cordifolia*.

Other significant correlations, such as those found between longitude and UV variables, as well as those found between longitude and the concentrations of C1, C3, C5, C6 y C7, seemed to be a consequence of the positive relationship between longitude and altitude, since the highest mountains were situated towards the east. The ratios C1/C3 and C6/C7, which have been useful to assess long-term changes in UV-B (Blokker *et al.*, 2006; Lomax *et al.*, 2008), were not correlated with any UV variable.

Given that seasonal changes in UV could mask the correlations between the different variables, correlations were recalculated using only the collections made in July, the month with the highest number of collections. This new set of correlations (data not shown) was hardly different to the previous one. Nevertheless, some new

correlations appeared. For example, latitude was negatively correlated (p<0.001) with all the UV variables (except with the UV-erythemal), something logical because UV levels increase with decreasing latitude (Björn, 1999). Latitude was also negatively correlated (p<0.05) with C2 concentration, which would suggest that C2 is induced by an increase in UV related to a decrease in latitude. However, C2 did not respond to enhanced UV under both laboratory and field experiments (Arróniz-Crespo *et al.*, 2006, 2008 a, 2008 b; Otero *et al.*, 2006; Fabón *et al.*, 2010). Finally, a new correlation was found between the unweighted UV-B and the bulk level of IUVACs (p<0.01). This would be added to the already found correlation between this physiological variable and the weighted UV-B using the Plant Damage spectrum. This fact promoted the relationship between UV-B and the bulk level of IUVACs, supporting the hypothesis that IUVACs (located in cell walls) would be more efficient UV screens than vacuolar SUVACs (Clarke & Robinson, 2008). Thus, the bulk level of IUVACs was selected to model the reconstruction of past UV (see below).

Ordination of samples by Principal Components Analysis (PCA)

The samples were ordinated by Principal Components Analysis (PCA) on the basis of both environmental and physiological data. The first three axes accumulated 55% of the variance (29% for axis I, 16% for axis II and 10% for axis III). The plot generated with the two first axes is shown in Fig. 4.3. The most significant loading factors (p<0.001) towards the positive part of axis I were all the UV radiation variables, together with the bulk level of SUVACs, all the individual soluble UVACs (C1 to C5), one insoluble UVAC (C7) and the collection year. Towards the negative part of axis I, the only significant loading factor was the collection month (p<0.001). For axis II, the most significant loading factors (p<0.001) towards the positive part were the bulk level of SUVACs and the concentrations of C1, C2 y C4, whereas towards the negative part the most significant factors were all the UV radiation variables, the bulk level of IUVACs and altitude.

This ordination clearly differentiated the samples collected in summer from those collected in autumn, on the basis of the loading factors described above. Summer samples would be associated to higher UV levels, higher bulk levels of SUVACs and IUVACs, higher concentrations of all the individual UVACs (except C6), more recent

collections and higher altitudes. In contrast, autumn samples were obviously collected in more advanced months of the year, and showed high bulk levels of SUVACs and high concentrations of the individual SUVACs, but not especially high bulk levels of IUVACs. The complete lack of samples collected in winter, and the scarcity of samples collected in spring, may limit the discrimination power of the PCA, since the bulk level of SUVAC and the concentrations of several individual soluble UVACs show seasonal changes in *J. cordifolia*, with higher values in summer and autumn which were coincident with higher UV levels (Núñez-Olivera *et al.*, 2009). The lack or scarcity of winter and spring samples was probably responsible for the fact that some loading factors, such as the bulk level of SUVACs or the concentrations of individual soluble UVACs appeared on both axes. This also limited the discrimination power of the PCA. In spite of the limited number of winter-spring samples, the PCA was notably robust because it was importantly determined by environmental factors controlling UV levels, such as altitude and latitude, as well as by different expressions of the accumulation of UVACs, which is a recognized physiological mechanism induced by UV radiation (Martínez-Abaigar *et al.*, 2003).

In the PCA, samples were also ordinated as a function of their geographic origin, because samples from the northern and central mountains were notably separated from most of those collected in southern mountains (Sierra Nevada). This differentiation was mainly determined by axis II and seems logical because, in accordance with the loading factors for the positive part of axis II, samples from northern mountains were collected at higher latitudes and had higher levels of SUVACs, whereas samples from Sierra Nevada were collected at lower latitudes and had lower levels of SUVACs. Lower latitudes imply higher temperatures, but aquatic bryophytes can hardly survive when they are exposed to temperatures higher than 15ºC (Glime, 1987). Thus, the lower latitudes of Sierra Nevada were compensated by the higher altitudes (and concomitant lower temperatures) of this mountain chain, and thus aquatic bryophytes could survive in Sierra Nevada streams. Lower latitudes and higher altitudes are associated with higher UV levels (Björn *et al.*, 1998), and thus samples from Sierra Nevada should be well protected against these UV levels. This can be seen in the PCA because IUVACs appear as a loading factor for the negative part of axis II, which means that samples from Sierra Nevada had higher bulk levels of IUVACs, which are presumably better UV screens than SUVACs (as previously discussed: Clarke & Robinson, 2008).

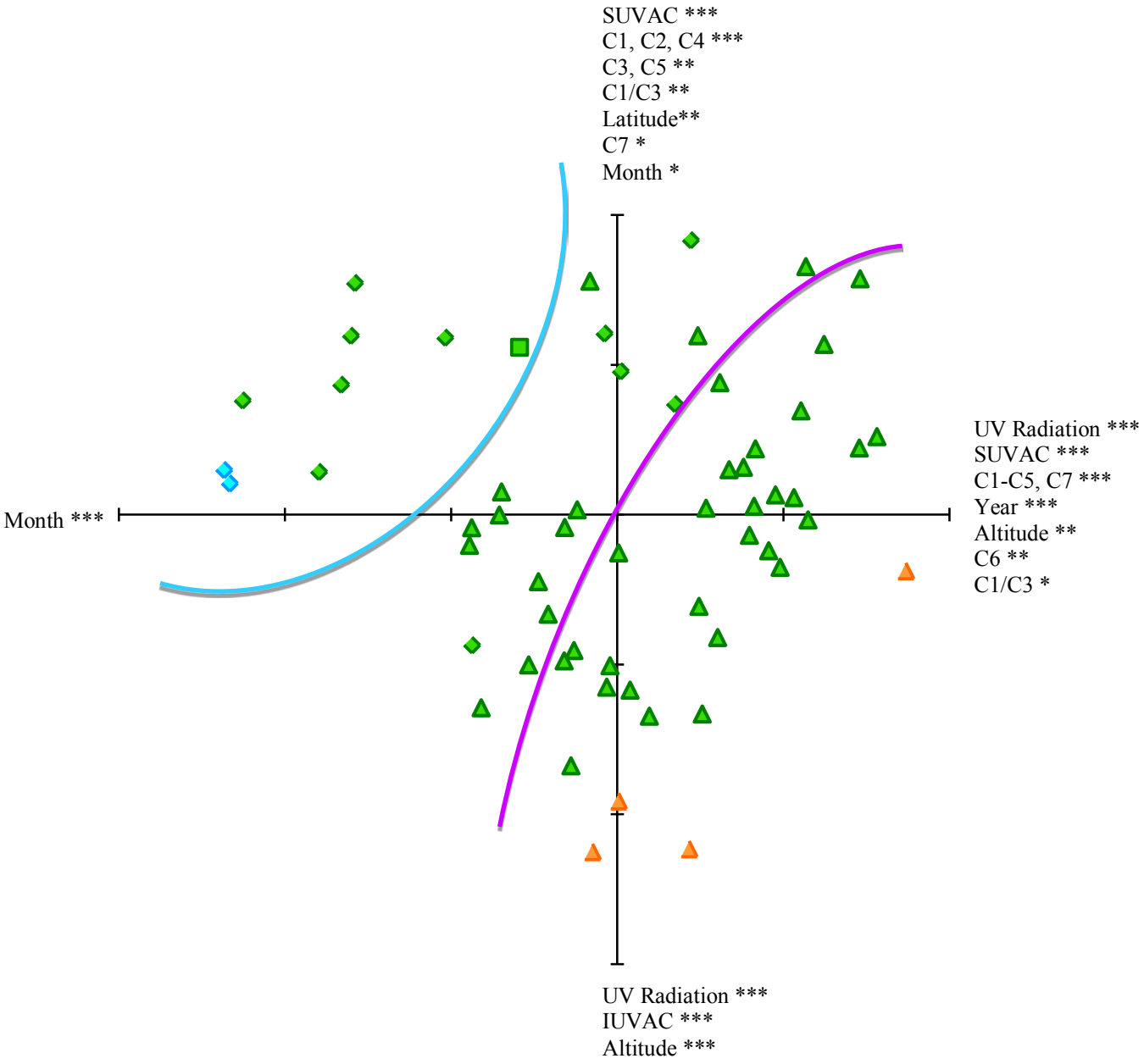


Figure 4.3. Ordination of the sampling localities used in this study through Principal Components Analysis (PCA), using both environmental and physiological variables. Significant loading factors for the positive and negative parts of each axis, together with their corresponding significance levels, are shown. ***, $p<0.001$; **, $p< 0.01$; *, $p<0.05$. SUVAC and IUVAC, bulk levels of, respectively, soluble and insoluble UV-absorbing compounds. C1 to C7, individual UV-absorbing compounds already named in the text. Axis 1 is the horizontal one, and axis 2 is the vertical one. Each tic-mark on the axes represents one unit. Samples collected in the different seasons are differentiated: spring (quadrat), summer (triangle), and autumn (diamond). Samples coming from northern (green), centre (blue) and southern (orange) Spain are also distinguished.

Another interesting fact is that, on axis II, the bulk levels of SUVACs and IUVACs were situated on opposite parts of the axis. This would support, as discussed previously, that both fractions would play different physiological roles: SUVACs would be more related to antioxidant systems (this property has been demonstrated in similar phenolic compounds to those analysed in the present study: Sroka, 2005), whereas IUVACs would be specialized in UV screening, benefiting from their location in cell walls.

A second PCA was conducted using only physiological variables (Fig. 4.4). Axis I accumulated 38% of the variance and axis II 18%. The plot generated with these two axes is shown in Fig. 4.4. This PCA had less differentiation power of the samples than the previous one. Apparently, the samples were not ordinated by the main factors influencing UV levels, such as collection date (samples collected in each season were not grouped separately), altitude or latitude. However, as occurred in the previous PCA, the bulk levels of SUVACs and IUVACs appeared as loading factors in different axes, supporting the hypothesis that their physiological meaning is different. In addition, in this PCA, the influence of the homogeneity of some samples and their storage conditions in the different herbaria on the levels of UVACs can be analysed.

Following the interchange program Brioteca Hispánica (Casas, 1993), some samples were divided into subsamples which were distributed among the main Spanish bryological herbaria. This affected, for example, to the sample 1339, collected in Viella (Pyrenees); it was divided into 6 subsamples (numbers 2, 4, 10, 15, 69 and 88 in Table 4.1) which are presently stored in 6 different herbaria. Other samples affected by this interchange program were those from Candelario in the Central System (numbers 6 and 22) and Sierra Nevada (17 and 40). The subsamples of a same sample are very close on the PCA plot, which reveals a notable homogeneity in their UVACs characteristics (except one of the subsamples from Viella). Thus, the growing form in extense masses, typical of *J. cordifolia*, allows to divide the mass into several samples to be distributed among several herbaria, and the storage conditions of each herbarium do not seem to have an important influence on the UVACs characteristics. This is the first time that the effects of these aspects on UVACs analysis are evaluated.

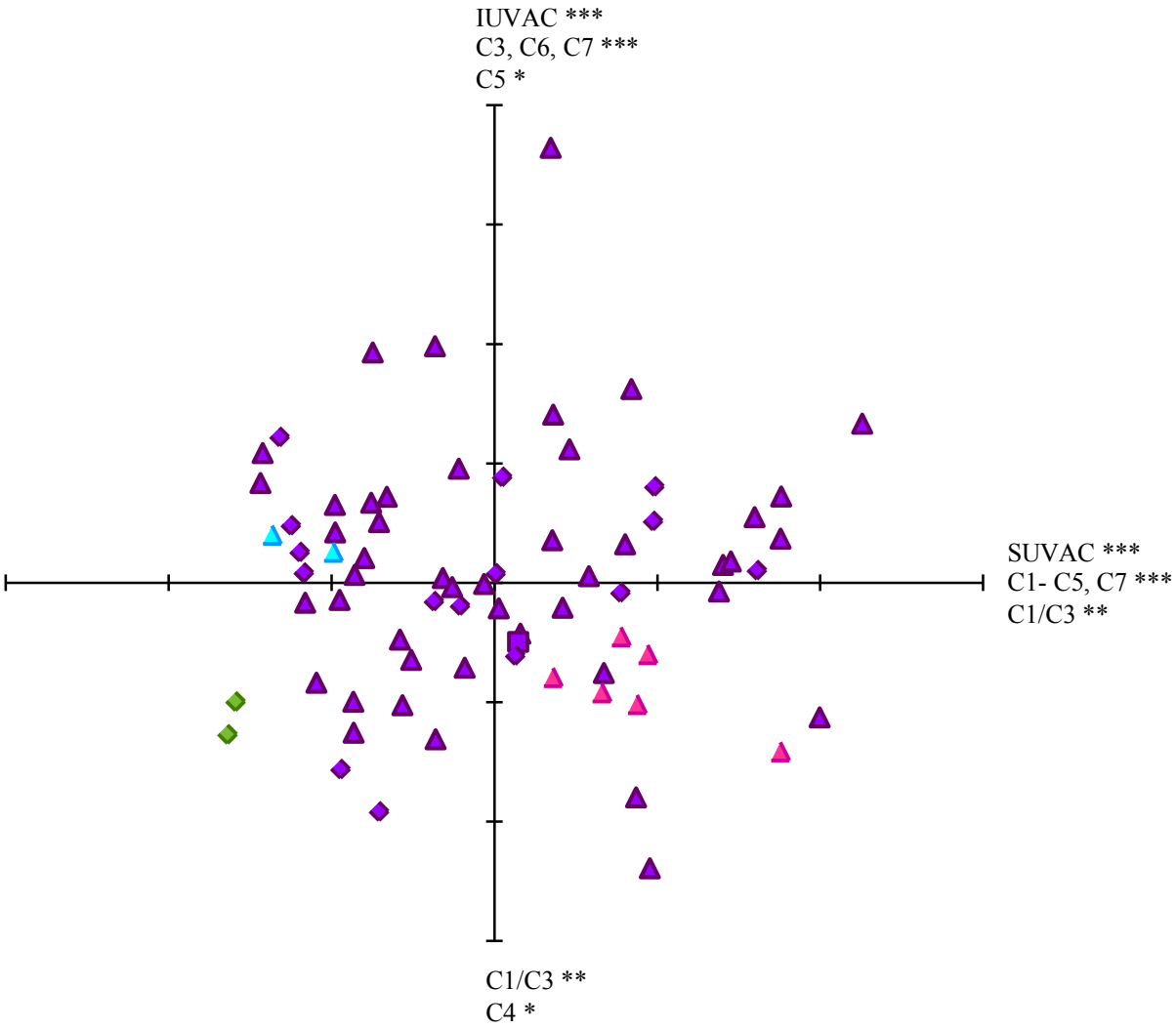


Figure 4.4. Ordination of the sampling localities used in this study through Principal Components Analysis (PCA), using only physiological variables. Significant loading factors for the positive and negative parts of each axis, together with their corresponding significance levels, are shown. ***, $p<0.001$; **, $p< 0.01$; *, $p<0.05$. SUVAC and IUVAC, bulk levels of, respectively, soluble and insoluble UV-absorbing compounds. C1 to C7, individual UV-absorbing compounds already named in the text. Axis 1 is the horizontal one, and axis 2 is the vertical one. Each tic-mark on the axes represents one unit. Samples collected in the different seasons are differentiated: spring (quadrat), summer (triangle), and autumn (diamond). Subsamples corresponding to a same sample are highlighted using different colours: Viella (numbers 2, 4, 10, 15, 69 and 88 in Table 4.1) in pink; Candelario (numbers 6 and 22) in green, and Sierra Nevada (17 and 40) in blue.

Comparison between Spain and northern Europe

The values of the physiological variables in Spain (this study) and northern Europe (Otero *et al.*, 2009) were compared (Fig. 4.5). It must be taken into account that IUVACs and C4 were not analysed in the study concerning northern Europe, and that the periods in which samples had been collected are different (1913-2006 for Spain and 1850-2006 for northern Europe). Nevertheless, the Spanish samples had significantly higher bulk levels of SUVACs and higher concentrations of C1, C3, and C5 than samples from northern Europe. These differences were probably due to the fact that UV levels received by *J. cordifolia* samples in Spain are higher than those received in northern Europe (Table 4.2; Otero *et al.*, 2009; Häder *et al.*, 2007). In our study, the higher UV levels found in Spain were not only due to the lower latitudes (37.03-43.40º in Spain *vs.* 61.06-70.67º in northern Europe), but also to the higher altitudes (mean values of 1775 and 465 m, respectively) at which our samples were collected in comparison with the northern Europe samples. Seasonal variations in UV had probably less influence in the differences found, because in both Spain and northern Europe most samples were collected in summer.

Most of these results could be expected because the bulk levels of SUVACs and C1 have been found to be UV-B-responsive variables in *J. cordifolia* under both laboratory and field conditions (Martínez-Abaigar *et al.*, 2003; Otero *et al.*, 2006; Arróniz-Crespo *et al.*, 2008a, 2008b; Núñez-Olivera *et al.*, 2009; Fabón *et al.*, 2010). In particular, C1 is the most inducible compound in *J. cordifolia* in response to enhanced UV-B (Martínez-Abaigar & Núñez-Olivera, 2011). C5 has also been induced by enhanced UV-B in the laboratory (Arróniz-Crespo *et al.*, 2008b) and in an altitude gradient in the field (Arróniz-Crespo *et al.*, 2006). In contrast, C3 had never been induced by enhanced UV-B and thus its higher values in comparison with those of north European samples are somewhat surprising. Overall, *J. cordifolia* samples were better UV-protected than samples from northern Europe, in accordance with the higher UV levels they experience.

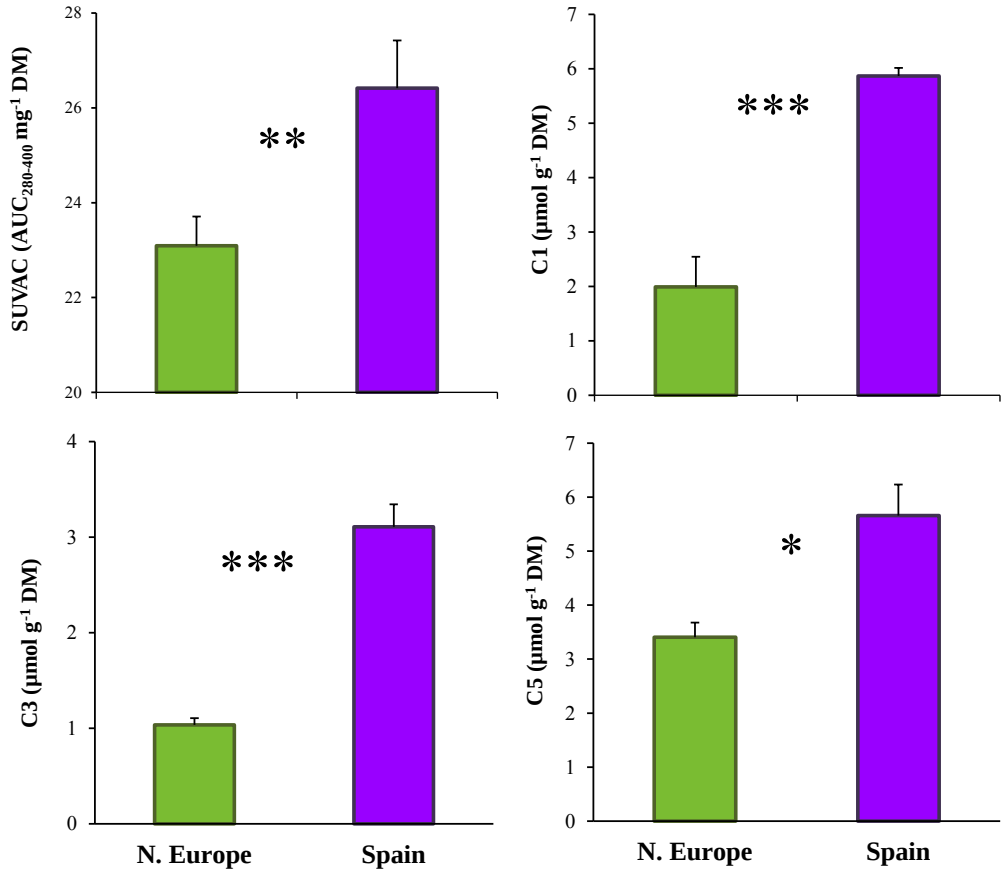


Figure 4.5. Comparison of mean (±SE) values of the bulk levels of soluble UV-absorbing compounds (SUVAC) and the concentrations of the individual UVACs C1, C3 and C5, between the herbarium samples of northern Europe (Otero *et al.*, 2009) and the samples of Spain (this study). Significance levels for Student's *t* tests are shown: ***, $p<0.001$; **, $p< 0.01$; *, $p<0.05$. The names of the compounds are given in the text.

Reconstruction of past levels of UV radiation

The stepwise multiple linear regression analysis applied showed that the best model to explain the UV-B_{BE} radiation (weighted using the Plant Damage action spectrum), as a function of environmental and physiological variables in the period for which UV radiation values were available (1979-2006) was as follows:

$$\text{UV-B}_{BE}\text{ (Plant Damage)} = k_0 + k_1 M + k_2 IUVAC$$

where k_0 is a constant, M is the collection month, $IUVAC$ is the bulk level of IUVACs, and k_1 and k_2 are specific coefficients (Table 4.4).

It was not surprising that the bulk level of IUVACs resulted useful for this model, because it was the physiological variable best correlated with the environmental variables: positively with the UV-B_{BE} (Plant Damage), the unweighted UV-B referred only to the month of July, and altitude, and negatively with latitude. The influence of the collection month was neither surprising because of the progressively decreasing levels of UV radiation in the period June-November (Núñez-Olivera *et al.*, 2011), when most collections were carried out. Moreover, the collection month was strongly and negatively correlated with all the UV variables calculated using the model by Engelsen & Kylling (2005), and thus the k_1 coefficient was negative.

Using the model above, UV-B_{BE} (Plant Damage) data were calculated for the period 1979-2006 and compared with data of the same variable obtained from the model by Engelsen & Kylling (2005). Both sets of data were strongly and positively correlated (Fig. 4.6), and thus our model was a good estimation of the data generated by Engelsen & Kylling's model. This allowed to reconstruct the UV-B_{BE} (Plant Damage) daily doses for the whole period in which herbarium samples were available (1913-2006). UV-B_{BE} (Plant Damage) did not show a clear trend in this period (Fig. 4.6).

Table 4.4. Coefficients for the stepwise multiple linear regression model obtained to calculate the daily dose of biologically effective UV-B ($UV\text{-}B_{BE}$) radiation, weighted using the Plant Damage action spectrum, as a function of the collection month and the bulk level of insoluble UV-absorbing compounds (IUVAC). Values of the associated two-sided *t*-tests and corresponding *p* values are given.

Coefficient	Units	Value	*t*	*p*
k_0	kJ m^{-2} d^{-1}	10.74	9.10	<0.001
k_1	kJ m^{-2} d^{-1} $month^{-1}$	-1.05	-9.63	<0.001
k_2	kJ m^{-2} d^{-1} $(AUC_{280\text{-}400}$ mg^{-1} $DM)^{-1}$	0.20	3.33	<0.001

Real measurements of UV at ground level are scarce, since they were not generalized until the 1990s (McKenzie *et al.*, 2011), and thus past UV reconstruction may be interesting because it can help in the interpretation of the effects of stratospheric ozone degradation on the UV-B levels received in the biosphere. However, UV reconstruction is not an easy task. Reconstructions based on pure climatic models have frequently shown contradictory temporal trends. For example, in northern Europe, models have suggested that UV has increased, decreased, or has not shown a trend since 1980 to present, depending on the locality considered (Lindfors *et al.*, 2007). Herman (2010a), using estimations from satellite data, pointed out that erythemal UV had significantly increased by 4% in mid-latitudes (38.9ºN) in the period 1979-2008, but this increase has not been homogeneous because it has mainly occurred from 1979 to 1998 (due to the decrease in ozone); after 1998, both ozone and UV levels have remained approximately constant. In addition, the UV increase depended on the month. Both factors, multiphasic trends and the influence of month, further complicate the interpretation of the trends generated by the models. Herman (2010b) modelled the $UV\text{-}B_{BE}$ (Plant Damage) trend in the period 1979-2008 and concluded that had increased 8% in the 40º latitude zone in the Northern Hemisphere. In the same latitude region, Kaurola *et al.* (2010) indicated that erythemal UV increased 0-3% per decade in the period 1978-2002.

Other models have covered longer periods. Kvalevag *et al.* (2009) suggested an extensive decrease of surface erythemally-weighted UV as a consequence of pollution, with reductions up to 20% since 1750, particularly in industrialized and highly populated regions. In this line, Watanabe *et al.* (2012) suggested that the surface UV-B radiation decreased between 1850 and 2000 in the extratropics of the Northern Hemisphere (25-60ºN latitude), because the period of stratospheric ozone degradation is compensated by the increase in tropospheric ozone, and thus the troposphere has become less transparent for UV-B.

Kvalevag *et al.* (2009) summarized the recent available studies reporting long term trends in surface UV based on both long-term UV observations (15 cases, overall from 1968 to 2006, showing positive and negative trends, frequently non-significant) and reconstructed data (12 cases, overall from 1893 to 2006, with positive trends in 6 cases). Among the reconstructed data, the most similar latitude to ours was that of Davos (Switzerland, 46.8ºN), which showed a positive trend in the period 1926-2003.

In Spain, no specific reconstruction of past UV has been carried out using climatic models in the period studied in the present work, although real measurements in shorter periods are already available (2000-2009 in Martínez-Lozano *et al.*, 2012; 2002-2011 in Bilbao & Miguel, 2013). This lack of climatic reconstructions in Spain further complicates the interpretation of our biological reconstruction.

Overall, simulated long-term changes in the surface UV radiation using climatic models may show many uncertainties (Watanabe *et al.*, 2012). They are derived from physical and chemical processes associated with ozone, aerosols, and clouds; the anthropogenic influence; and the lack of surface UV observations before the 1990s, which was mentioned above and prevents the validation of the historical simulation results. Furthermore, local real measurements may sometimes contradict model estimations (Krzyscin *et al.*, 2011).

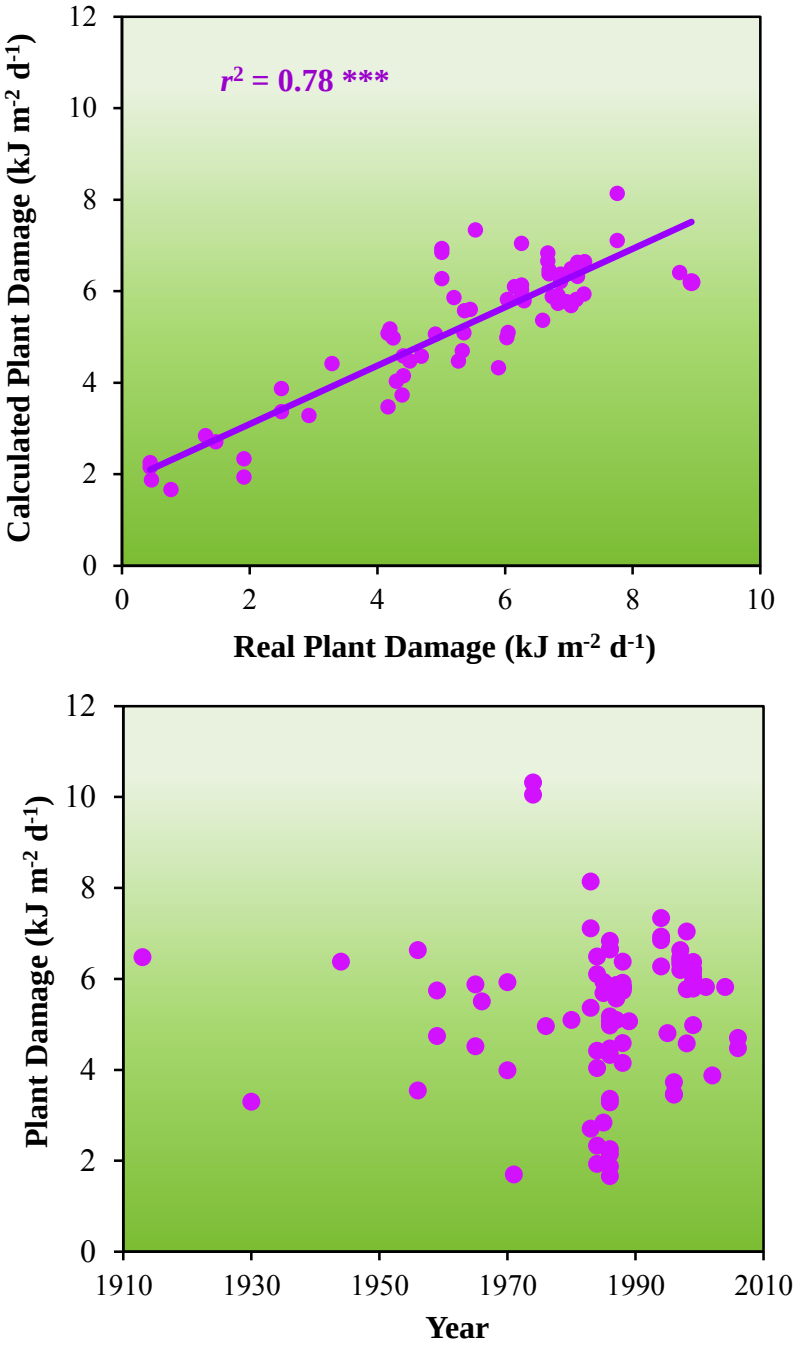


Figure 4.6. Top, linear regression between the daily dose of biologically effective UV-B (UV-B_{BE}) radiation, weighted using the Plant Damage action spectrum, calculated on one hand using the stepwise multiple linear regression model as a function of the collection month and the bulk level of insoluble UV-absorbing compounds (IUVAC) (Calculated Plant Damage), and, on the other hand, using the model by Engelsen & Kylling (2005) (Real Plant Damage). The coefficient of determination and the respective significance level are shown (***, p<0.001). Bottom, temporal changes in the daily dose of biologically effective UV-B (UV-B_{BE}) radiation, weighted using the Plant Damage action spectrum, calculated using the stepwise multiple linear regression model mentioned above for the period 1913-2006.

Regarding biological models, that have been reviewed in Björn & McKenzie (2007), Rozema *et al.* (2009) and McKenzie *et al.* (2011), their reconstruction power may be even more limited than that of climatic models, since the former are based on surrogate biological variables. In fact, most biological models did not find clear UV trends over time. Huttunen *et al.* (2005a) found a lack of a significant temporal trend in the bulk UV-B absorbance of herbarium samples of 9 out of 10 Subarctic mosses in the period 1926–1996, which agreed with climatic UV reconstruction. Lomax *et al.* (2008) reported that the ratio between *p*-coumaric and ferulic acids in spore walls from herbarium samples of *Lycopodium* from Greenland reproduced changes in UV-B in the period 1907–1993, but again these levels were fluctuating and did not show a clear temporal trend. Otero *et al.* (2009), using the concentration of *p*-coumaroylmalic acid in herbarium samples of *J. cordifolia* from northern Europe, did not find significant temporal trends neither in the total stratospheric ozone in the period 1850–2006 nor in UV levels in 1918–2006. Once again, this agreed with the climatic data available. Willis *et al.* (2011) found that the abundance of *p*-coumaric acid in *Pinus spp*. pollen followed a similar fluctuating pattern (no trend, therefore) to that obtained for modelling UV-B flux at a site in Norway over the past 9500 years. Only Ryan *et al.* (2009) reported clear temporal trends in the regression analysis between total flavone concentration and the level of ozone (negative), and between the ratio of luteolin to apigenin and the modelled midday UV-B radiation (positive), using herbarium samples of the moss *Bryum argenteum* for the period 1956-2005. These significant temporal trends were demonstrated in Antarctica, where severe ozone degradation obviously makes easier to find significant relationships between ozone, UV and biological variables. Nevertheless, low r^2 coefficients (0.14-0.26) were obtained in the regressions, which illustrates the difficulties existing to find good relationships even in Antarctica. Overall, the interpretation of historical data is difficult, because specific local environmental conditions in the moment of sampling (clouds, temperature, shade, etc.) are unknown (Ryan *et al.*, 2009).

Taking into account all the above discussion, in particular all the contradictory results and uncertainties associated with the reconstruction of past UV using both climatic models and biological proxies, together with the relatively modest increases or decreases in UV that have been pointed out when found, and noting that the expected

increase in UV radiation attributable to ozone depletion has not been well established by direct measurements of surface UV radiation (McKenzie *et al.*, 2011), our results showing a lack of trend in UV-B_{BE} (Plant Damage) in 1913-2006 may be reasonable because they are in the line of previous results found using different variables, species and periods in different geographical locations.

In addition, there exist some limitations associated to the use of biological proxies for past UV reconstruction that may have affected our study:

1) the short sampling period within the year, because collections in winter (and even in spring) are scarce or unavailable, presumably due to bad weather; this makes impossible to trace the theoretical baseline of the UVACs concentrations, which are the lowest in winter (Núñez-Olivera *et al.*, 2009); regarding this point, our attempt to increase the sampling period within the year with respect to that used in a previous study carried out in northern Europe (Otero *et al.*, 2009) has not been successful because, again, we could get only a limited representation of spring samples and no representation of winter samples, in spite of the milder climate in Spain with respect to that in northern Europe;

2) different factors, most of them unknown, may affect the UV exposure of the samples in the moment of collection, as it has been mentioned above;

3) UV and ozone real data are available only for relatively short periods (approximately, from 1990 for UV and 1979 for ozone), and this makes more difficult to find correlations between environmental and biological variables;

4) it is difficult to evaluate if UVACs are broken down along time in herbarium samples.

Regarding the last point, cell wall-bound phenolic compounds, in particular *p*-coumaric acid and similar compounds, would be good candidates to be used as UV proxies, on the basis of previous studies in which they have proven useful in reconstructions of past UV (Rozema *et al.*, 2001, 2009; Blokker *et al.*, 2005, 2006; Lomax *et al.*, 2008; Willis *et al.*, 2011) or in the assessment of present UV, in both the spatial and temporal scales (Arróniz-Crespo *et al.*, 2006; Núñez-Olivera *et al.*, 2009). In addition, they are chemically stable and can be unaltered for millions of years, for example in pollen, spores or fossil materials (Rozema *et al.*, 2001, 2009; Blokker *et al.*, 2005, 2006; Lomax *et al.*, 2008; Willis *et al.*, 2011). If this is the case, these compounds would also be well preserved in herbarium samples of at most one or two centuries of age. However, in our study, neither *p*-coumaric acid nor the remaining HCA derivatives that were present in the soluble and insoluble fractions of *J. cordifolia* could serve as UV proxies, because none of them was correlated to any of the expressions used to represent UV radiation levels. In contrast, in Otero *et al.* (2009), the HCA derivative *p*-coumaroylmalic acid, analysed in the soluble fraction of *J. cordifolia*, was useful to reconstruct past ozone levels in northern Europe, and this variable was subsequently used to reconstruct past UV levels. In our study, although no individual UVAC was useful as UV proxy, the bulk level of IUVACs was. This seems to be more logical in comparison with the results obtained in Otero *et al.* (2009), considering that cell wall-bound compounds may be less easily broken down than soluble compounds.

4.5.-CONCLUSIONS

1) The bulk level of SUVACs was not correlated with that of IUVACs in the 90 herbarium samples of *J. cordifolia* that were analysed for this study. This suggests that both types of compounds may play different roles in the cells: efficient UV screening for IUVACs, due to their location in the cell walls, and an additional antioxidant role for SUVACs.

2) *p*-Coumaroylmalic acid (C1) was the only UVAC of *J. cordifolia* showing significantly higher concentrations in the years after the onset of stratospheric ozone degradation with respect to the previous years. This indicated its usefulness in the biomonitorization of the UV-B increase associated to ozone degradation.

3) The PCA conducted using the environmental and physiological variables considered, clearly separated the samples collected in summer from those collected in autumn, mainly due to the different UV levels experienced in both seasons and the higher concentrations of protecting UVACs in summer than in autumn. In addition, the PCA ordinated the samples on the basis of their geographical origin, and demonstrated that the different storage conditions in the different herbaria did not affect much that ordination.

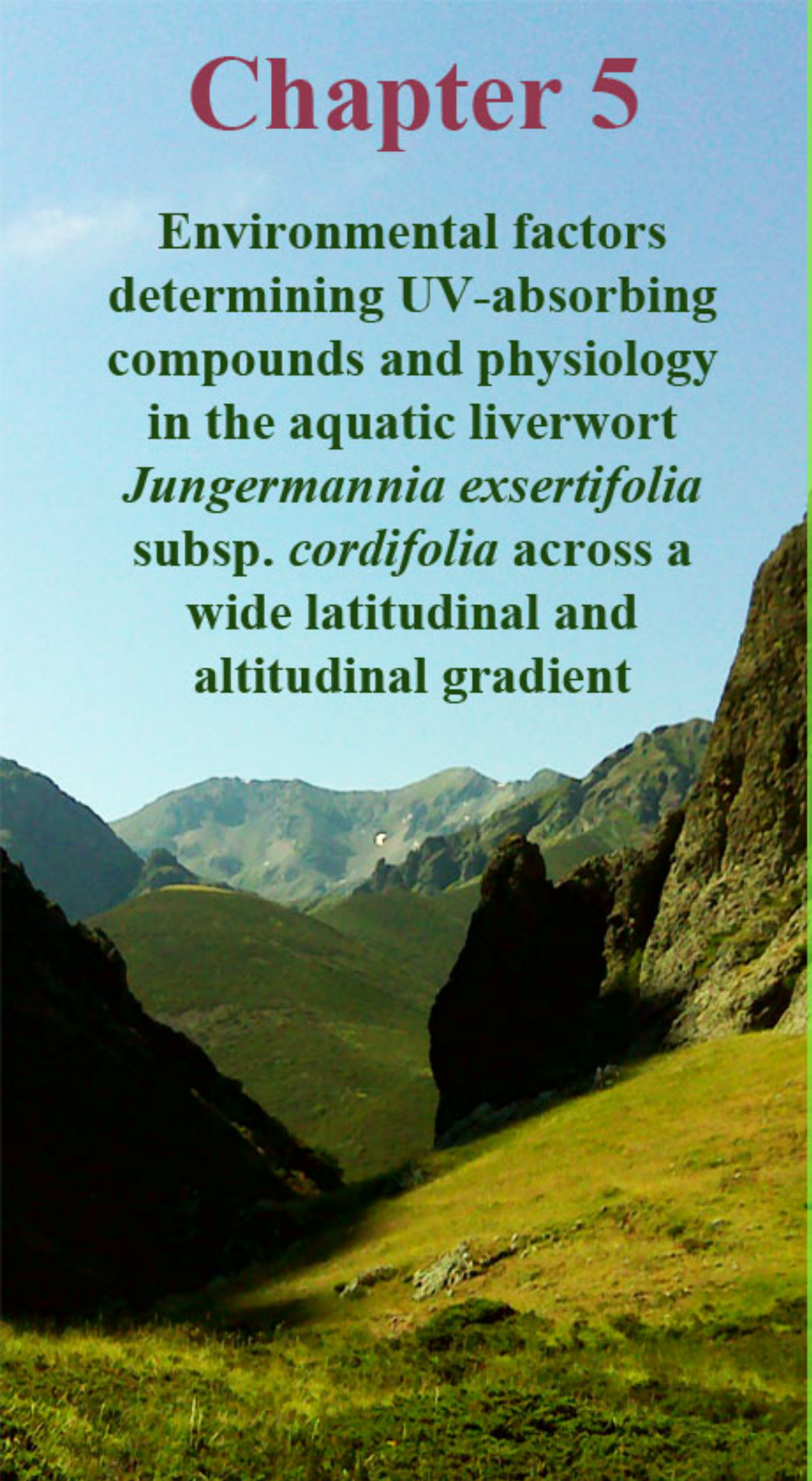
4) The bulk level of SUVACs in *J. cordifolia*, together with the concentrations of three individual soluble UVACs (C1, C3 and C5), were higher in Spain than in northern Europe, suggesting a better UV protection which is in line with the higher UV levels received in Spain.

5) The UV-B_{BE} (Plant Damage) was reconstructed in Spain using a model composed by the collection month and the bulk level of IUVACs of *J. cordifolia*, and did not show a clear trend in the period 1913-2006. This lack of trend might be influenced by several factors (short sampling period within the year, uncertainties about UV exposure of the samples at the moment of sampling, possible breakdown of UVACs during sample storage in herbaria, and limited availability of UV climatic data), but was coincident with other UV reconstructions that have been carried out using other biological variables, species, sampling periods and geographical regions.

6) Overall, UVACs of herbarium samples of *J. cordifolia* have demonstrated a wide variety of relationships with UV radiation in the temporal and spatial scales. This is in line with the fact that UVACs of fresh samples of *J. cordifolia* also respond consistently to UV radiation both temporally and spatially. Thus, they are good biomonitors of changes in past and present UV levels.

Chapter 5

Environmental factors determining UV-absorbing compounds and physiology in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* across a wide latitudinal and altitudinal gradient



5.1.-ABSTRACT

With the aim of evaluating the environmental factors determining the physiology of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* across a wide latitudinal and altitudinal gradient, we analysed UV-absorbing compounds (UVACs), the maximum quantum yield of PSII (F_v/F_m), sclerophylly index and DNA damage in 17 samples of this species, collected in streams of the main mountain chains of Spain: Picos de Europa, Basque Mountains, Pyrenees, Iberian System, Central System, and Sierra Nevada. Sampling was completed in 18 days near the summer solstice. With respect to UVACs, we differentiated methanol-soluble (SUVACs) and insoluble (IUVACs) compounds. In both fractions, the bulk level of UVACs and the concentrations of 7 (5 soluble and 2 insoluble) individual UVACs were measured by, respectively, spectrophotometry and HPLC. In addition, different geographical and environmental variables were obtained for each sampling locality: altitude, latitude, longitude, water temperature, ozone, and radiation data (PAR, unweighted maximum irradiances and daily doses of UV-A and UV-B, and biologically effective UV using two different spectral weighting functions).

The ranges of UV radiation and ozone along the spatial gradient were relatively narrow because of the almost simultaneous sampling, which allowed to strengthen the influence of other remarkable gradients, such as those of latitude (more than 6º), and, especially, of altitude (almost 2500 m, the widest altitudinal gradient to date using one only bryophyte) and water temperature (11ºC). The physiological variables showed a high variability among the localities within each mountain chain, which could obscure the differences among mountain chains. In addition, the differences among and within mountain chains could be due not only to the macroenvironmental factors assessed in our study but also to microenvironmental factors inherent to the strongly dynamic nature of mountain streams. Nevertheless, the ranges of the physiological variables analysed were concordant with the values found in previous studies using *J. exsertifolia* subsp. *cordifolia*. In particular, the homogeneous and relatively high values of F_v/F_m, and the lack of DNA damage in any of the samples analysed, suggested that this

liverwort was well adapted to the environmental conditions across the gradient. Regarding the relationship between UV radiation and the physiological variables, the bulk level of SUVACs, together with the concentrations of two coumarins, were the most UV-responsive variables in the spatial gradient, and thus they could be used for the biomonitorization of ambient UV levels.

The PCA conducted using both the environmental and physiological variables showed that macroenvironmental variables and UVACs were able to ordinate, in a reasonably congruent manner, both the different mountain chains and the different localities within each mountain chain, although microenvironmental factors would be responsible for some differences. The ordination shown by the PCA plot reproduced the location of the mountain chains considered within the Iberian Peninsula.

Finally, UVACs of fresh and herbarium (stored for 12-98 years) samples of *J. exsertifolia* subsp. *cordifolia* were compared, showing that soluble UVACs were significantly higher in fresh than in herbarium samples, whereas insoluble UVACs showed the contrary. These results, which were summarized by PCA, could be due to the degradation of soluble UVACs during storage and/or to their increase in response to the increased UV-B levels caused by stratospheric ozone degradation in the last decades. Anyway, it is clear that IUVACs were much more stable than SUVACs, and thus the former would be more adequate than the latter for retrospective UV biomonitorization.

5.2.- INTRODUCTION

The research about the effects of UV radiation on plants is mainly based on three methodological approaches: UV supplementation using lamps to address the effect of stratospheric ozone degradation, UV exclusion using filters to assess the effects of current UV levels, and exploitation of natural gradients of UV in the temporal or spatial scales (Martínez-Abaigar & Núñez-Olivera, 2011). The advantage of this last kind of studies is that no environmental circumstance of the plants is modified, something greatly needed in the context of UV research. However, these experiments require an accurate measurement of the UV gradient and a critical assessment of other interacting factors which can influence the effects of UV itself. Studies of this type are, for example, temporal variations in the Antarctic during the occurrence of "ozone hole" (Newsham *et al.*, 2005; Robinson *et al.*, 2005), seasonal and interannual variations of UV levels (Núñez-Olivera *et al.*, 2009), or spatial variations with water depth (Häder *et al.*, 1996; Rae *et al.*, 2001; Laurion *et al.*, 2002), latitude (Karsten *et al.*, 1998; Nybakken *et al.*, 2004; Comont *et al.*, 2012) and altitude (Rozema *et al.*, 1997; Filella & Peñuelas, 1999; Neitzke & Therburg, 2000; Arróniz-Crespo *et al.*, 2006; Bernal *et al.*, 2013). In particular, latitude and altitude are important factors influencing UV levels reaching the ground (McKenzie *et al.*, 2011), and the increase in UV radiation with increasing altitude or decreasing latitude provides natural spatial gradients which can be used for research because they can affect the responses of plants to UV.

UV levels decrease with increasing latitude because solar rays are received less perpendicularly. However, there is only limited information on plant responses to UV across latitudinal gradients. It would be expected, for example, that UVACs of a particular species would be lower at higher latitudes. This has been found in marine macroalgae across a latitudinal gradient from polar to temperate regions, where the concentration of mycosporine-like aminoacids (a type of UVACs) increased with increasing natural solar irradiance (Karsten *et al.*, 1998). Also, in an European latitude gradient using *Lolium perenne*, the bulk level of UV-B absorbing compounds showed a

positive trend with solar UV across the gradient, although this relationship was not totally consistent (Comont *et al.*, 2012). However, contradictory responses to latitude, depending on the species and interacting environmental factors, have also been obtained (Nybakken *et al.*, 2004), and even sometimes positive relationships between UVACs and increasing latitude have been found (Jaakola & Hohtola, 2010).

In bryophytes, the effects of latitude on the responses to UV radiation have mainly been studied in herbarium samples. In latitudinal gradients in Finland, the specific UV absorbance of several moss extracts showed a direct (and counterintuitive) relationship with latitude (Huttunen *et al.*, 2005a, 2005b). In contrast, also in northern Europe, the concentration of *p*-coumaroylmalic acid (a derivative of hydroxycinnamic acid) in the liverwort *Jungermannia exsertifolia* subsp. *cordifolia* was negatively correlated with latitude, thus showing higher values at latitudes receiving higher UV levels (Otero *et al.*, 2009). This was supposed to increase UV protection, but studies in greater latitudinal gradients were recommended because the latitude range in that study was relatively narrow and the regression of that compound *vs.* latitude was relatively little significant. Using samples of the same liverwort along a latitudinal gradient in Spain, latitude was negatively correlated with the bulk level of IUVACs, but no correlation between latitude and SUVACs was found (see Chapter 4). In the only study to our knowledge dealing with the effects of latitude on UV responses in fresh samples of bryophytes, there was no significant correlation between flavonoid content (total phenolic content, number of flavonoids, and percentage of luteolin derivatives) and latitude in the moss genus *Plagiomnium*, and thus, presumably, flavonoid variation was more influenced by fine-scale ecological features (Harris, 2009).

The levels of UV radiation increase with increasing altitude because the pathlength of solar rays is shorter and the atmosphere more clear, and both factors reduce UV attenuation. In particular, biologically effective UV-B radiation increases between 5% and 20% per 1000 m altitudinal increase (Björn *et al.* 1998). This increase of UV (especially UV-B) levels with altitude must be taken with caution, because this is true for UV irradiance peaks, but not necessarily for temporally integrated (for example, annual) UV doses, because doses can strongly be influenced by the higher cloudiness associated with high altitudes (Núñez-Olivera *et al.*, 2011). The influence of altitude on the plant responses to UV radiation has been more studied than the influence of latitude.

Particularly, the most studied variable has been the bulk level of SUVACs, which has been found to increase with altitude in leaves or flowering heads (in the case of Asteraceae) of diverse species: *Quercus ilex, Rhododendron ferrugineum*, *Fagus sylvatica*, *Crepis capillaris*, *Hieracium pilosella*, *Hypochaeris radicata* and *Plantago asiatica* (Rozema *et al.*, 1997; Filella & Peñuelas, 1999; Neitzke & Therburg, 2000; Zidorn *et al.*, 2005; Murai *et al.*, 2009). Other variables that have been found to increase with altitude are leaf thickness (Rozema *et al.*, 1997), trichome density (Ruhland *et al.*, 2013), the contents of flavonoids and/or phenolic acids (Zidorn *et al.*, 2005; Spitaler *et al.*, 2006; Bernal *et al.*, 2013), the proportion of flavonoids with a higher antioxidant capacity (Spitaler *et al.*, 2006; Murai *et al.*, 2009) and the carotenoids content (González *et al.*, 2007). All these responses are triggered by UV radiation and can improve the UV protection of the plant. In some cases, negative or neutral responses to altitude have been found. In *Batrachium trichophyllum* and *Potamogeton alpinus*, the contents of UV-B absorbing compounds showed no significant differences between high and low altitude populations (Germ *et al.*, 2002). In the shrub *Artemisia tridentata*, the bulk levels of SUVAC decreased with elevation (Ruhland *et al.*, 2013), and a similar decrease was found in the total amount of flavonoids in leaf cuticles of *Buxus sempervirens* (Bernal *et al.*, 2013). These negative responses suggest that, sometimes, the observed variations along the altitudinal gradients would respond to other factors rather than to UV.

In bryophytes, the influence of altitude on UV responses have been studied in herbarium samples of the liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, in which the bulk level of SUVACs (Otero *et al.*, 2009), the bulk level of IUVACs and the concentration of a coumarin (see chapter 4), were correlated positively with altitude, which could lead to a higher protection in samples from higher altitudes. In fresh samples of the same liverwort, the bulk level of SUVACs, the concentrations of two coumarins, the maximal apparent electron transport rate through PSII and the maximal non-photochemical quenching increased with altitude, whereas photoinhibition percentage decreased (Arróniz-Crespo *et al.*, 2006). Negative responses were found by Harris (2009) in *Plagiomnium* species, since there was no significant correlation between flavonoid content and altitude, which again suggests the influence of microenvironmental factors or, probably, the species, since he studied several different species within the genus *Plagiomnium*.

Overall, more studies are needed in photosynthetic organisms in general, and in bryophytes in particular, to disentangle the effects of latitude and altitude on the physiological responses of the plant (including UVACs) to UV radiation. In this sense, liverworts may have a particular interest because they were the first true plants colonizing land (Zobell *et al.*, 2010), and thus they had to cope with higher UV levels that in the primitive aquatic habitat.

DNA is one of the key molecular targets of UV-B radiation, given that DNA bases directly absorb incident UV-B photons (Jansen et *al.*, 1998). This may lead to the formation of different photoproducts: cyclobutane pyrimidine dimers (CPDs, the major photoproducts) and pyrimidine (6-4) pyrimidone photoproducts. These DNA alterations disrupt cellular metabolism because gene transcription and DNA replication are blocked, and this may decrease growth. 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 (Häder & Sinha, 2005). DNA damage has been measured only in 10 bryophytes, 9 mosses and 1 liverwort (Fabón *et al.*, 2011). Both laboratory and field studies were conducted, using artificially enhanced UV radiation, natural ambient UV levels, or solar radiation deprived of UV-B. In general, DNA damage has been found in bryophytes exposed to artificially enhanced UV-B radiation under laboratory and field conditions, whereas exposure to natural ambient UV-B levels did not result in DNA damage (Fabón *et al.*, 2011). 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 (as it has been said above) liverworts are now considered the earliest diverging land plants. Thus, more experiments measuring DNA damage in liverworts are needed to corroborate or reject previous results. In particular, the effects of latitude or altitude on UV-B-induced DNA damage have not been previously studied in bryophytes or other photosynthetic organisms.

Within the context described above, the aim of the present study was to evaluate the environmental factors determining the physiology of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia* across a wide latitudinal and altitudinal gradient in streams of the main mountain chains of Spain: Picos de Europa, Basque Mountains, Pyrenees, Iberian System, Central System, and Sierra Nevada. Our gradients of latitude (more than 6º) and altitude (almost 2500 m) were remarkable, because, for example, the altitudinal gradient was the widest used, to our knowledge, in the assessment of the influence of altitude on UV effects on photosynthetic organisms using one only species. Moreover, the combination of both latitudinal and altitudinal gradients had not been explored before in this context. Different physiological variables were measured: UVACs, the maximum quantum yield of PSII (F_v/F_m), sclerophylly index and DNA damage. With respect to UVACs, we differentiated methanol-soluble (SUVACs) and insoluble (IUVACs) compounds, which are mainly located in different cell compartments (vacuoles and cell walls, respectively), and, in both fractions, we analysed the bulk level of UVACs and the concentrations of individual (5 soluble and 2 insoluble) UVACs. This analytical approach has rarely been carried out in bryophytes and even in tracheophytes or algae, but it can be interesting because, on one hand, vacuolar and cell wall-bound UVACs may represent different modalities of UV protection, and, on the other hand, each individual compound contributing to the bulk UV absorbance may respond to UV gradients in a different way. Thus, the analyses carried out may provide a wider perspective on the influence of environmental factors on UVACs. Another additional aim of our study was to compare the physiological results obtained in fresh samples of *Jungermannia exsertifolia* subsp. *cordifolia* with those derived from the herbarium samples of the same species studied in Chapter 4, which were collected in the same mountain chains of Spain considering similar latitudinal and altitudinal gradients. This comparison may help assess the influence of sample storage on the integrity of UVACs, as well as the influence of stratospheric ozone degradation on the UVACs of bryophytes.

5.3.- MATERIALS AND METHODS

Plant material and collection sites

We used in this work the liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váňa (hereafter *J. cordifolia*), a characteristic species of oligotrophic circumneutral mountain streams (see a characterization of the species in Chapter 4.3). Samples were collected in the summer of 2011, from 24 June to 11 July, in 17 localities placed in streams of the main mountain systems of Spain (Fig. 5.1; Table 5.1). This sampling roughly corresponded to the distribution of this liverwort in Spain (see Chapter 4.2). Table 5.1 shows the location of the 17 sampling points in the corresponding mountain chains, together with their longitude, latitude, altitude, and environmental data. Two sampling points were selected in Picos de Europa, two in the Basque Mountains, three in the Pyrenees, four in the Iberian System, two in the Central System, and four in Sierra Nevada. The selection of these sites was based on a previous survey through the mountains of Spain, starting from the bibliographic records and herbarium specimens of this liverwort (see Chapter 4.2). This previous survey took place in the summer of 2010. All the localities where the liverwort was found were georreferenced using GPS. The definitive localities were selected taking into account that the samples were fully sun-exposed and submerged in a narrow range from 0 to 5-cm depth, so that depth was not a relevant ecological factor to be considered. In addition, the sampling site should have a relatively easy access to facilitate the transport of material and measurement instruments.

The definitive sampling was carried out in the midday hours of clear days around the summer solstice, to ensure that the samples were receiving the maximum solar irradiances. To increase homogeneity of the irradiances received among the different samples, sampling was completed in the shortest possible period. In this way, samples were more influenced by their "historical" radiation climate than by the specific irradiances to which they had been exposed during the last days prior to sampling.

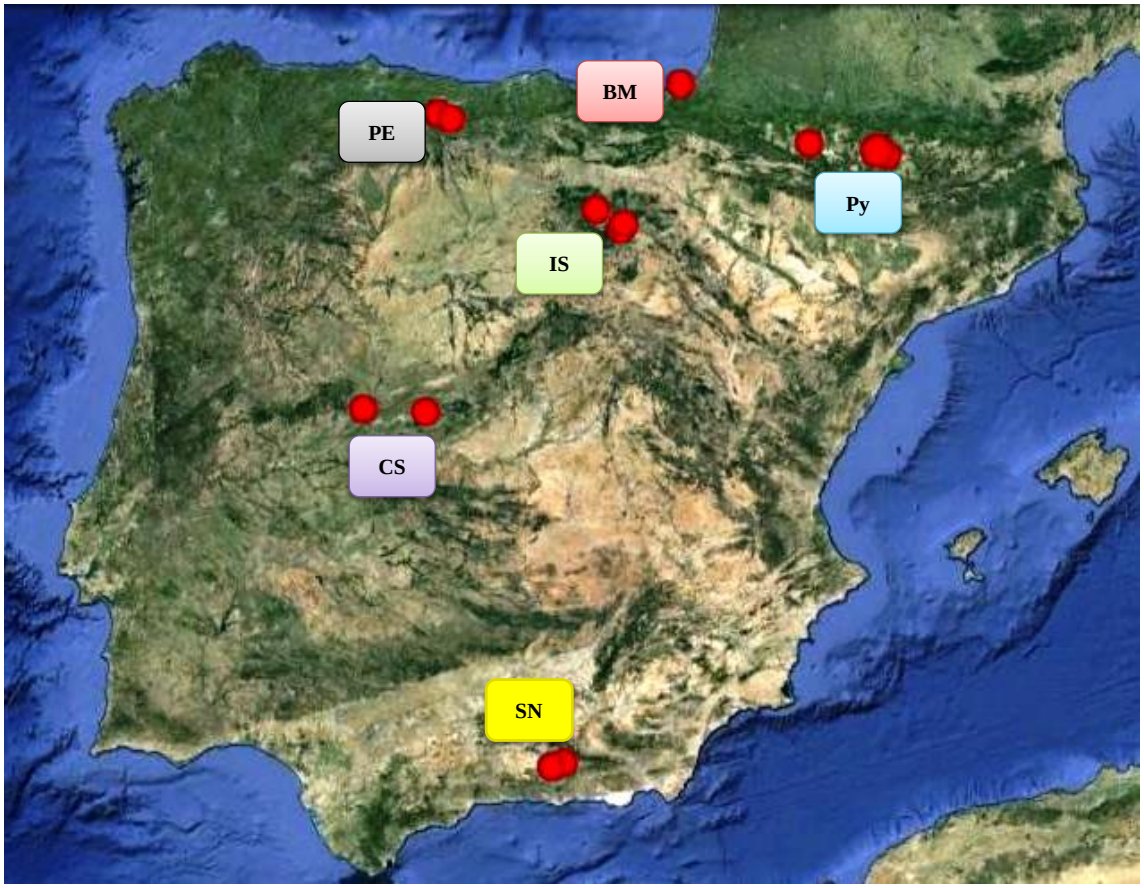


Figure 5.1. Geographic location of the 17 samples of the aquatic leafy liverwort *Jungermannia exsertifolia* subsp. *cordifolia* used in this study. Abbreviations of the mountain chains where samples were collected are as follows: BM, Basque Mountains; CS, Central System; IS, Iberian System; PE, Picos de Europa; Py, Pyrenees; SN, Sierra Nevada.

Environmental variables

Different geographical and environmental variables were obtained for each sampling locality: altitude, latitude and longitude (Oregon 550 GPS, Garmin, Olthe, Kansas, USA); water temperature in the moment of collection (infrared-thermometer with laser pointer, Qualitäts-Erzeugnis, by TFA / Germany); and ozone and radiation data (Table 5.1).

Stratospheric ozone on the sampling day was obtained from *Total Ozone Mapping Spectrometer* (TOMS) webpage (http://toms.gsfc.nasa.gov/ozone). UV-A and UV-B irradiance data at midday on the sampling day were derived from a model (Engelsen & Kylling, 2005) (http://zardoz.nilu.no/~olaeng/fastrt/fastrt.html). In addition, starting from the spectral irradiance data provided by the model for the

sampling day, the biologically effective UV irradiance (UV_{BE}) at midday was calculated using the biological spectral weighting function by Flint & Caldwell (2003). Also, using the same model, we calculated the UV-A and UV-B daily doses for the collection day, and, applying the Plant Damage spectral weighting function by Caldwell (1971), the biologically effective UV-B irradiance ($UV\text{-}B_{BE}$) at midday and the $UV\text{-}B_{BE}$ daily dose for the collection day. We applied two different biological spectral weighting functions because they are conceptually different: the model by Caldwell (1971) is focused on UV-B radiation whereas the model by Flint & Caldwell (2003) takes also into consideration certain UV-A wavelengths. In this way, we could compare the relationships of both models with other environmental and physiological variables, and thus evaluate the influence of UV-A radiation. Photosynthetically active radiation (PAR) data at the midday for the collection day was calculated with the SBDART model ("A research and teaching software tool for plane-parallel radiative transfer in the Earth's atmosphere": Ricchiazzi *et al.*, 1998).

Physiological variables

In vivo chlorophyll fluorescence of PSII was measured *in situ* with a portable pulse amplitude modulation fluorometer (AquaPen-P AP-P 100, Photon System Instruments, Drasov, Czech Republic) using the saturation pulse method (Schreiber *et al.*, 1995; Núñez-Olivera *et al.*, 2004a). Minimal and maximal fluorescence (F_0 and F_m) were measured in samples dark-adapted for 20 min, and the maximum quantum yield of PSII was given by the ratio F_v/F_m, where $F_v = F_m - F_0$.

Diverse physiological variables were going to be expressed on an area basis. For that aim, the area of green and healthy 1.5-cm long apices of the liverwort was measured *in situ* (LI-COR LI-3000 area meter, Lincoln, NE, USA), after removing excess water with filter paper, and several apices (6-8) were grouped to constitute each sample. Samples were stored independently in Eppendorf tubes, which were placed in liquid nitrogen. All these procedures were conducted under natural radiation conditions. Liquid nitrogen vessels were transported to the laboratory, and then the samples in the tubes were stored at -80ºC until analysis. For each destructive analysis, five replicates were prepared *in situ* for each sampling point.

Table 5.1. Data of the 17 samples of *Jungermannia exsertifolia* subsp. *cordifolia* used in this study: stream name and code, mountain chain where it is located, collection date, geographical data (altitude, latitude, and longitude), levels of ultraviolet (UV) and photosynthetic (photosynthetically active radiation, PAR) radiation, stratospheric ozone level, and water temperature. Details on how the different environmental data were obtained or calculated are described in the text. UV_{BE} and $UV\text{-}B_{BE}$, biologically effective UV and UV-B radiation, respectively. DU, Dobson units.

Locality	Mountain Chain	Collection date	Altitude (m)	Latitude (ºN)	Longitude (ºE)	UV-A (W m^{-2})	UV-B (W m^{-2})	UV_{BE} (W m^{-2})	PAR (W m^{-2})	UV-A Dose (kJ m^{-2} d^{-1})	UV-B Dose (kJ m^{-2} d^{-1})	$UV\text{-}B_{BE}$ Dose (kJ m^{-2} d^{-1})	Ozone (DU)	Water temp (ºC)
Lumbreras (Lu)	Iberian System (IS)	24-6-2011	1800	42.01	-2.63	58.4	1.99	1.29	447	1738	48.5	7.67	307	7.3
Senestillos (Se)		24-6-2011	1353	42.03	-2.59	57.7	1.95	1.27	446	1714	47.4	7.47	307	8.0
Lavieja (Lv)		24-6-2011	1277	42.05	-2.59	57.6	1.94	1.27	446	1710	47.2	7.44	307	11.0
Calamantio (Ca)		24-6-2011	990	42.19	-2.93	57.0	1.91	1.26	445	1688	46.4	7.23	307	9.1
Pico (Pi)	Central System (CS)	6-7-2011	1273	40.32	-5.01	57.7	1.88	1.26	447	1694	45.3	6.93	321	13.0
Candelario (Cn)		6-7-2011	1202	40.34	-5.77	57.5	1.86	1.25	446	1690	45.1	6.90	321	15.0
Panticosa (Pa)	Pyrenees (Py)	9-7-2011	1851	42.77	-0.23	57.6	1.87	1.25	442	1708	45.3	6.87	321	7.8
Viella (Vi)		11-7-2011	1635	42.63	0.75	57.3	1.88	1.25	441	1691	45.3	6.94	316	6.4
Benasque (Be)		11-7-2011	1810	42.68	0.63	56.3	1.89	1.26	442	1700	45.7	7.00	316	6.3
Jaizquibel-1 (J1)	Basque Mountains (BM)	27-6-2011	174	43.37	-1.84	55.1	1.83	1.21	439	1642	44.9	7.11	299	12.5
Jaizquibel-2 (J2)		27-6-2011	174	43.37	-1.84	55.1	1.83	1.21	439	1642	44.9	7.11	299	12.5
San Juan-1 (S1)	Sierra Nevada (SN)	4-7-2011	2597	37.08	-3.37	61.2	2.06	1.35	460	1774	48.8	7.54	326	4.9
San Juan- 2 (S2)		4-7-2011	2597	37.08	-3.37	61.2	2.06	1.35	460	1774	48.8	7.54	326	4.9
Dilar-1 (D1)		4-7-2011	2635	37.06	-3.39	61.2	2.06	1.35	460	1775	48.9	7.55	326	3.9
Dilar-2 (D2)		4-7-2011	2635	37.06	-3.39	61.2	2.06	1.35	460	1775	48.9	7.55	326	3.9
Caldevilla (Cl)	Picos de Europa (PE)	1-7-2011	1514	43.11	-4.94	57.0	1.89	1.25	441	1704	46.5	7.21	311	8.3
San Glorio (SG)		1-7-2011	1580	43.06	-4.77	57.2	1.90	1.25	441	1708	46.7	7.25	311	6.5

In parallel to the samples described in the paragraph above, apices of the liverwort were collected and stored in a portable icebox to obtain the sclerophylly index (SI) in the laboratory. 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 liverwort (LI-COR LI-3000 area meter). Previously, fresh mass was measured.

UV-absorbing compounds (UVACs) were analysed differentiating methanol-soluble and insoluble compounds (respectively, SUVAC and IUVAC), that are mainly located, respectively, in the vacuoles or bound to the cell walls (for details, see Chapter 4.2). In both cases, the bulk level of UVACs and the concentrations of several individual UVACs (C1-C7: see Chapter 4.2) were measured by, respectively, spectrophotometry and HPLC, starting from the samples which had been frozen in liquid nitrogen in the field. The ratios C6/C7 and C1/C3 were also obtained.

DNA damage was evaluated by detection of thymine dimers (Sinha *et al.*, 2001; Fabón *et al.*, 2011). 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).

Statistical analysis

Bivariate correlations (Spearman's coefficients) between all of the variables used, both environmental and physiological, were obtained.

Once proved the data met the assumptions of normality (Shapiro–Wilks's test) and homoscedasticity (Levene's test), the effect of the locality on the physiological variables within each mountain chain was tested using one-way analysis of variance (ANOVA), or Student's *t* tests when only two localities were to be compared. In the case of significant differences in ANOVAs, means were then 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.

Student's *t* tests were applied to compare herbarium (Chapter 4) and fresh (this Chapter) samples of *J. cordifolia*. For this aim, we only used the herbarium samples collected in the same period of the year when fresh samples had been collected (June and July), in the same mountain chains, and before 1999. Thus, the herbarium samples had been stored for 12-98 years at the moment of analysis. A total of 43 herbarium samples *vs.* 17 fresh samples were analysed.

The samples were ordinated by Principal Component Analysis (PCA), taking into account both the environmental and physiological data. In addition, another PCA was conducted to ordinate herbarium and fresh samples on the basis of UVACs.

The statistical procedures were performed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

5.4. RESULTS AND DISCUSSION

Geographic distribution of the samples

The geographic distribution of the samples of *J. cordifolia* used in this study (Table 5.1, Fig. 5.1) reproduced the general distribution of this species in Spain, where it has been found in the main chains of mountains: Pyrenees, Picos de Europa, Basque Mountains, Iberian System, Central System and Sierra Nevada (see Chapter 4.3).

Summary of values of environmental variables

Table 5.1 shows the numerical values of the geographical and environmental variables in the sampling points surveyed for this study. The altitude range was 174 (Jaizquíbel) - 2635 m (Dílar). Latitude range was 37.06º N (Dílar) - 43.37º N (Jaizquíbel), and longitude range was from -5.77º E (Candelario) to 0.75º E (Viella). Water temperature was between 3.9ºC (Dílar) and 15.0ºC (Candelario). Ozono levels varied from 299 DU (Jaizquíbel) to 326 DU (Sierra Nevada). With respect to radiation variables, maximum irradiances and doses of PAR, UV-A, UV-B and UV_{BE}, were the highest in Sierra Nevada and the lowest in Jaizquíbel (Basque Mountains), except the $UV\text{-}B_{BE}$ (Plant Damage), which was the highest in Lumbreras (Iberian System) and the lowest in Panticosa (Pyrenees).

For each variable, the minimum value found was between 85% and 95% of the maximum value, except for altitude (7%) and water temperature (26%). This would mean that most gradients were relatively narrow, which was especially true for ozone and radiation variables. The fact that most gradients were narrow was in line with the short period of time (only 18 days) in which sampling was completed. Consequently, other ozone ranges found in the literature were wider than ours (Núñez-Olivera *et al.*, 2009; Otero *et al.*, 2009; Ryan *et al.*, 2009; Turnbull *et al.*, 2009). In our study, the ranges of the radiation variables were also considerably narrow, mostly because of the sampling design, which was thought to prevent the influence of temporal variations. On one hand, the sampling took place around the summer solstice, which favoured that irradiances and doses were near the maximum

values that can be measured (Núñez-Olivera *et al.*, 2011), thus restricting the differences between localities. On the other hand, these differences were further attenuated because the sampling was completed in only 18 days. In the case of radiation variables, the slight differences existing between the localities allowed that the samples in each locality were more influenced by their "historical" radiation climate (mostly dependent on altitude and latitude) than by the specific irradiances to which they had been exposed during the last days prior to sampling. Consequently, altitude and latitude would be the key factors determining the UV doses received by the samples. Nevertheless, even slight differences in UV-B doses (between 29.2 and 30.9 kJ m^{-2} d^{-1}) can cause changes in UVACs in *J. cordifolia* (Arróniz-Crespo *et al.*, 2006).

It may sound strange that the same localities (Jaizquíbel and Sierra Nevada) showed the extreme values of all the radiation variables except the UV-B_{BE} (Plant Damage) doses. However, it must be taken into account that the Plant Damage spectral weighting function is especially sensitive to short UV-B wavelengths, and ozone particularly eliminates these wavelengths. Thus, UV-B_{BE} (Plant Damage) doses were more influenced by ozone values than the remaining radiation variables, and the interaction between ozone, latitude and altitude in the determination of UV-B_{BE} (Plant Damage) doses was different and more complex than in the remaining variables. This caused that the highest and the lowest UV-B_{BE} (Plant Damage) doses were found in different localities to Jaizquíbel and Sierra Nevada.

In contrast to the narrow ozone and radiation gradients used in the present study, the gradients of latitude (more than 6º), and, especially, of altitude (almost 2500 m) and water temperature (11ºC), were remarkable. In fact, this is the study showing the widest altitudinal gradient using one only bryophyte, because the gradients in Arróniz-Crespo *et al.* (2006) and Otero *et al.* (2009) were much narrower (676 and 780 m, respectively). To our knowledge, our altitude gradient was even wider than those used in similar studies using tracheophytes (Rozema *et al.*, 1997; Filella & Peñuelas, 1999; Neitzke & Therburg, 2000; Zidorn *et al.*, 2005; Murai *et al.*, 2009; Bernal *et al.*, 2013). In contrast, our latitudinal gradient was narrower than those used in other studies, either conducted on bryophytes (Harris, 2009; Otero *et al.*, 2009) or tracheophytes (Nybakken *et al.*, 2004; Comont *et al.*, 2012). However, the strong climatic and ecological gradients occurring in Spain from north to south may make our latitudinal gradient sufficiently meaningful to influence the physiology of the plants.

Summary of values of physiological variables

Table 5.2 shows the numerical values of the physiological variables measured in samples of *J. cordifolia* coming from the 17 localities used in this study. F_v/F_m varied between 0.56 (Dílar-1) and 0.70 (Senestillos), and SI between 1.86 mg cm^{-2} (San Glorio) and 3.24 (Jaizquíbel-1). These ranges matched well with those found in previous field studies using *J. cordifolia*, in particular the one in which seasonal and interannual changes were studied (Núñez-Olivera *et al.*, 2009). Thus, the temporal variation summarized the spatial variation and vice-versa.

The bulk level of SUVACs (as $AUC_{280\text{-}400}$ mg^{-1} DM) varied between 20.83 and 43.14, whereas the bulk level of IUVACs between 6.75 and 14.94. In both cases, the lowest values were found in Jaizquíbel-1 and the highest values in Panticosa. In all the localities, the bulk level of SUVACs was higher than that of IUVACs, and thus the ratios I/S (IUVAC / SUVAC) were lower than 1 (between 0.23 in Dílar-2 and 0.43 in Candelario). This is the first study in which both SUVACs and IUVACs were analysed in fresh samples of a liverwort under field conditions, because in previous studies only mosses were used (Clarke & Robinson, 2008; Lappalainen *et al.*, 2008) or IUVACs were not analysed (Newsham et al., 2005; Arróniz-Crespo *et al.*, 2006; Snell *et al.*, 2007, 2009; Núñez-Olivera *et al.*, 2009). The bulk levels of SUVACs found in our study were concordant with those found in previous field studies using *J. cordifolia* (Núñez-Olivera *et al.*, 2009), and the ratios I/S were in line with those found in liverworts in general (Chapter 3) and in herbarium samples of *J. cordifolia* in particular (Chapter 4). No values of IUVACs of fresh samples in the field exist for comparison.

Among the individual compounds, C2 was the most abundant in all the localities, whereas C6 was the less abundant. The highest values of C1, C5, C6 and C7 were found in Panticosa, the highest values of C2 and C3 were found in Jaizquíbel-2, and the highest values of C4 were found in San Juan-1. C4 and C5 were not detected in Jazquíbel-1.

Table 5.2. Numerical values (mean ± SE) of the different physiological variables measured in samples of *J. cordifolia* coming from the localities used in this study. F_v/F_m, SI, Sclerophylly Index. SUVAC and IUVAC, bulk levels of, respectively, soluble and insoluble UV-absorbing compounds (as the AUC, area under the absorbance curve, in the interval 280-400 nm, per Dry Mass, DM). TUVAC, the sum of SUVAC and IUVAC. I/S, the ratio between IUVAC and SUVAC. C1 to C7, individual UV-absorbing compounds already named in the text.

Locality	F_v/F_m	SI	SUVAC	IUVAC	TUVAC	I / S	C1	C2	C3	C4	C5	C6	C7	C1/C3	C6/C7
		mg cm^{-2}	$AUC_{280-400}$ mg^{-1} DM	$AUC_{280-400}$ mg^{-1} DM	$AUC_{280-400}$ mg^{-1} DM		µmol g^{-1} DM	µmol g^{-1} DM	µmol g^{-1} DM	µmol g^{-1} DM	µmol g^{-1} DM	µmol g^{-1} DM	µmol g^{-1} DM		
Lu	0.65 ± 0.00	2.75 ± 0.17	29.05 ± 0.75	10.55 ± 0.24	39.60 ± 0.74	0.36 ± 0.01	14.72 ± 0.59	48.78 ± 2.63	4.65 ± 0.15	7.00 ± 0.40	19.57 ± 1.04	0.62 ± 0.02	4.18 ± 0.14	3.07 ± 0.05	0.15 ± 0.01
Se	0.70 ± 0.01	1.91 ± 0.09	37.80 ± 1.28	11.91 ± 0.41	48.66 ± 1.18	0.32 ± 0.01	16.61 ± 0.77	116.58 ± 3.32	9.01 ± 0.10	11.06 ± 0.38	8.13 ± 0.33	0.82 ± 0.03	3.68 ± 0.25	1.84 ± 0.08	0.23 ± 0.01
Lv	0.60 ± 0.00	1.94 ± 0.11	35.73 ± 1.53	10.49 ± 0.48	46.57 ± 1.88	0.30 ± 0.00	15.13 ± 1.52	101.75 ± 6.77	8.45 ± 0.48	15.11 ± 1.36	5.43 ± 0.86	0.58 ± 0.05	3.75 ± 0.16	1.84 ± 0.07	0.15 ± 0.01
Ca	0.68 ± 0.01	2.32 ± 0.18	33.78 ± 1.91	10.54 ± 0.26	44.32 ± 2.00	0.32 ± 0.02	21.15 ± 1.15	78.96 ± 7.90	9.01 ± 0.62	24.44 ± 3.54	5.63 ± 0.82	0.82 ± 0.04	4.79 ± 0.50	2.59 ± 0.31	0.17 ± 0.02
Pi	0.67 ± 0.01	2.13 ± 0.06	34.63 ± 0.96	10.33 ± 0.34	44.96 ± 1.19	0.30 ± 0.01	9.76 ± 0.46	101.90 ± 4.54	8.48 ± 0.43	9.38 ± 0.42	5.12 ± 0.32	0.73 ± 0.04	4.70 ± 0.22	1.16 ± 0.07	0.15 ± 0.00
Cn	0.61 ± 0.02	2.59 ± 0.10	21.72 ± 0.60	9.38 ± 0.44	31.09 ± 1.03	0.43 ± 0.01	6.73 ± 0.20	51.53 ± 1.76	4.50 ± 0.10	3.48 ± 0.48	3.07 ± 0.24	0.48 ± 0.02	2.31 ± 0.25	1.50 ± 0.05	0.22 ± 0.02
Pa	0.66 ± 0.02	2.32 ± 0.11	43.14 ± 1.46	14.94 ± 0.69	58.08 ± 2.05	0.35 ± 0.01	27.25 ± 1.14	34.49 ± 0.87	6.47 ± 0.21	13.59 ± 1.74	35.78 ± 1.91	1.32 ± 0.08	6.79 ± 0.44	4.23 ± 0.25	0.19 ± 0.00
Vi	0.61 ± 0.03	2.09 ± 0.21	37.54 ± 1.46	13.21 ± 0.42	51.12 ± 1.61	0.36 ± 0.01	19.71 ± 1.27	39.86 ± 1.85	6.57 ± 0.47	8.65 ± 1.43	16.68 ± 1.39	1.22 ± 0.05	6.13 ± 0.37	2.83 ± 0.07	0.20 ± 0.01
Be	0.59 ± 0.03	2.58 ± 0.20	37.80 ± 1.74	11.34 ± 0.18	49.00 ± 1.73	0.30 ± 0.02	26.41 ± 1.47	55.73 ± 2.50	7.99 ± 0.56	17.46 ± 1.22	13.37 ± 0.83	1.09 ± 0.05	6.16 ± 0.28	3.51 ± 0.06	0.18 ± 0.00
J1	0.61 ± 0.03	3.24 ± 0.22	20.83 ± 2.07	6.75 ± 0.32	27.69 ± 2.37	0.33 ± 0.02	10.57 ± 1.23	84.41 ± 7.95	6.45 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	0.42 ± 0.03	2.36 ± 0.16	1.70 ± 0.04	0.18 ± 0.01
J2	0.61 ± 0.01	2.50 ± 0.18	31.08 ± 1.58	9.37 ± 0.24	40.45 ± 1.76	0.30 ± 0.01	15.73 ± 0.70	121.15 ± 5.57	11.21 ± 0.39	4.82 ± 0.32	1.88 ± 0.14	0.63 ± 0.03	3.70 ± 0.11	1.40 ± 0.02	0.17 ± 0.01
S1	0.65 ± 0.03	2.01 ± 0.09	40.11 ± 2.30	13.07 ± 0.63	55.10 ± 1.98	0.31 ± 0.01	13.26 ± 0.74	70.41 ± 4.50	9.23 ± 0.52	25.89 ± 2.22	8.06 ± 0.82	0.62 ± 0.04	3.98 ± 0.38	1.37 ± 0.03	0.16 ± 0.01
S2	0.65 ± 0.01	2.05 ± 0.09	37.75 ± 1.01	9.43 ± 0.34	47.18 ± 1.28	0.25 ± 0.01	15.41 ± 0.45	64.64 ± 3.10	9.46 ± 0.21	24.75 ± 0.88	8.69 ± 0.36	0.75 ± 0.05	2.98 ± 0.26	1.63 ± 0.03	0.26 ± 0.02
D1	0.56 ± 0.02	3.04 ± 0.04	25.41 ± 1.60	7.50 ± 0.34	32.82 ± 1.95	0.29 ± 0.01	14.52 ± 0.99	42.21 ± 3.08	5.98 ± 0.36	8.94 ± 0.63	20.48 ± 1.29	0.59 ± 0.01	3.61 ± 0.20	2.42 ± 0.04	0.17 ± 0.01
D2	0.60 ± 0.04	2.80 ± 0.03	38.69 ± 1.91	8.59 ± 0.46	47.64 ± 2.20	0.23 ± 0.01	21.63 ± 1.08	64.47 ± 4.20	9.62 ± 0.56	19.80 ± 1.61	22.88 ± 1.09	0.73 ± 0.05	4.19 ± 0.33	2.25 ± 0.03	0.17 ± 0.01
Cl	0.63 ± 0.04	2.08 ± 0.10	36.46 ± 1.19	11.40 ± 0.68	47.86 ± 1.83	0.31 ± 0.01	16.61 ± 0.88	61.24 ± 1.39	7.06 ± 0.21	3.02 ± 0.04	18.61 ± 0.94	0.81 ± 0.04	5.34 ± 0.20	2.35 ± 0.07	0.15 ± 0.01
SG	0.62 ± 0.01	1.86 ± 0.08	36.76 ± 1.77	9.90 ± 0.48	46.21 ± 1.95	0.26 ± 0.01	8.75 ± 0.43	66.20 ± 4.09	5.21 ± 0.39	10.60 ± 1.17	3.22 ± 0.27	0.56 ± 0.05	3.67 ± 0.17	1.82 ± 0.17	0.15 ± 0.01

The order of abundance of the individual compounds was similar to the orders found in other studies using fresh (Núñez-Olivera *et al.*, 2009) or herbarium (Otero *et al.*, 2009; Chapter 4) samples of *J. cordifolia*, and also in laboratory studies (Arróniz-Crespo *et al.*, 2008a, 2008b; Fabón *et al.*, 2010). In all the studies, C2 has clearly been the most abundant compound, but has hardly been affected by enhanced UV (Arróniz-Crespo *et al.*, 2008a, 2008b). Thus, C2 would be, at most, a constitutive non-UV-inducible UVAC, probably playing in the cell other additional roles not directly related to UV protection.

Correlations between the variables used

The correlations between the environmental and physiological variables used are shown in Table 5.3. All the radiation variables, including irradiances and doses, were positively correlated between them. In particular, all the variables indicative of UV radiation were strongly and positively correlated, as occurred in Chapter 4. This is logical because all of them were derived from the same model (Engelsen & Kylling, 2005). It is interesting to note that the UV variables calculated either with the spectral weighting functions of Caldwell (1971) or Flint & Caldwell (2003) were strongly correlated. Hence, for our aims, it was unimportant to estimate UV levels with either model.

Radiation variables, except UV-B maximum irradiance and UV-B_{BE} (Plant Damage) dose, were positively correlated with ozone. Given that stratospheric ozone absorbs UV-B radiation, it could be expected a negative relationship between ozone and UV-B levels. However, it must be taken into account that the ozone values in the different localities were relatively similar (as well as UV levels), and that other factors, such as latitude and altitude, interacted with ozone in the determination of UV levels. This complex interaction obscured the otherwise simple (and negative) relationship between UV-B and ozone, and may be responsible for the positive correlations between ozone and some UV variables. On the other hand, the lack of correlation between some UV-B variables and ozone may be surprising, but again the interaction between ozone, altitude and latitude may complicate the relationship between ozone and UV-B.

Table 5.3. Correlation coefficients among environmental and physiological variables. For identification of variables, see Tables 5.1 and 5.2. Significant correlations are differentiated in different colours depending on the significance level.

Alt	Lat	Long	UV-A	UV-B	UV_{BE}	PAR	UV-A Dose	UV-B Dose	$UV\text{-}B_{BE}$ Dose	Ozone	Water temp	F_v/F_m	SI	SUVAC	IUVAC	I/S	TUVAC	C1	C2	C3	C4	C5	C6	C7	C1/C3	C6/C7	
1	-0.59	-0.05	0.74	0.73	0.75	0.65	0.86	0.78	0.49	0.78	-0.92	-0.23	0.02	0.58	0.18	-0.39	0.48	0.28	-0.59	0.04	0.50	0.80	0.26	0.20	0.35	-0.05	**Altitude**
	1	0.45	-0.90	-0.74	-0.84	-0.98	-0.72	-0.69	-0.52	-0.74	0.47	0.01	-0.09	-0.14	0.15	0.30	0.00	0.18	0.16	-0.15	-0.48	-0.35	0.10	0.20	0.16	-0.02	**Latitude**
		1	-0.36	-0.23	-0.22	-0.39	-0.23	-0.26	-0.19	-0.41	-0.09	-0.12	0.07	0.23	0.36	0.31	0.35	0.56	-0.07	0.11	0.06	0.12	0.45	0.35	0.49	0.38	**Longitude**
			1	0.78	0.85	0.94	0.89	0.79	0.60	0.72	-0.58	0.07	-0.03	0.32	0.00	-0.29	0.17	-0.09	-0.25	0.10	0.43	0.55	-0.01	-0.12	-0.05	-0.07	**UV-A**
				1	0.94	0.80	0.89	0.98	0.88	0.48	-0.77	-0.06	-0.13	0.33	-0.01	-0.46	0.18	0.09	-0.12	0.23	0.62	0.53	0.02	-0.07	0.14	-0.22	**UV-B**
					1	0.90	0.90	0.91	0.77	0.58	-0.71	-0.04	-0.04	0.35	-0.03	-0.49	0.18	0.06	-0.10	0.25	0.66	0.51	0.03	-0.06	0.08	-0.16	**UV_{BE}**
						1	0.80	0.76	0.61	0.69	-0.52	0.05	0.09	0.21	-0.10	-0.30	0.03	-0.10	-0.16	0.17	0.52	0.44	-0.05	-0.16	-0.08	-0.03	**PAR**
							1	0.94	0.75	0.61	-0.76	-0.07	-0.13	0.46	0.07	-0.42	0.31	0.07	-0.27	0.11	0.50	0.65	0.03	-0.06	0.14	-0.19	**UV-A Dose**
								1	0.87	0.50	-0.78	-0.06	-0.16	0.38	0.02	-0.45	0.23	0.09	-0.17	0.18	0.56	0.58	0.01	-0.08	0.16	-0.26	**UV-B Dose**
									1	0.14	-0.60	-0.08	-0.02	0.05	-0.22	-0.38	-0.11	-0.07	0.07	0.20	0.33	0.31	-0.23	-0.29	0.02	-0.35	**$UV\text{-}B_{BE}$ Dose**
										1	-0.60	-0.13	0.03	0.43	0.02	-0.40	0.33	0.00	-0.47	0.08	0.46	0.49	0.12	0.05	-0.07	0.13	**Ozone**
											1	0.30	0.00	-0.54	-0.11	0.46	-0.44	-0.33	0.46	-0.15	-0.53	-0.68	-0.26	-0.15	-0.36	0.02	**Water temp**
												1	-0.42	0.21	0.47	0.32	0.18	-0.04	0.27	0.12	0.18	-0.09	0.26	0.12	-0.13	0.06	**F_v/F_m**
													1	-0.50	-0.52	0.14	-0.53	0.05	-0.33	-0.24	-0.37	0.15	-0.18	-0.07	0.23	0.06	**SI**
														1	0.62	-0.25	0.94	0.56	-0.12	0.44	0.68	0.49	0.63	0.51	0.21	0.16	**SUVAC**
															1	0.43	0.80	0.45	-0.26	0.00	0.26	0.39	0.67	0.69	0.42	0.01	**IUVAC**
																1	-0.06	-0.01	-0.27	-0.50	-0.46	-0.06	0.07	0.10	0.27	0.20	**I/S**
																	1	0.61	-0.21	0.34	0.53	0.50	0.72	0.65	0.31	0.14	**TUVAC**
																		1	-0.25	0.40	0.35	0.62	0.86	0.73	0.70	0.22	**C1**
																			1	0.57	0.09	-0.69	-0.26	-0.31	-0.66	-0.06	**C2**
																				1	0.56	-0.03	0.34	0.16	-0.32	0.15	**C3**
																					1	0.26	0.33	0.23	0.05	0.10	**C4**
																						1	0.58	0.54	0.72	-0.08	**C5**
																							1	0.77	0.58	0.32	**C6**
																								1	0.60	-0.21	**C7**
																									1	-0.01	**C1/C3**
																										1	**C6/C7**

$p<0.05$
$p<0.01$
$p<0.001$

In a 3-year temporal study (Núñez-Olivera *et al.*, 2009), a positive correlation between ozone and UV-B levels was found, due to the rather parallel seasonal variation of both variables in the mid-latitudes of northern hemisphere: ozone maxima are usually reached in March-May and minimum values in October-November, whereas UV peaks around the summer solstice and then decreases (Häder *et al.*, 2007). In contrast, in Antarctica, ozone and UV-B are negatively correlated, in a more intuitive way (Newsham *et al.*, 2002). In conclusion, in our study, UV-B levels were not mainly determined by ozone levels but by an interaction of different factors, explaining the positive correlation between both variables.

Radiation variables were positively correlated with altitude and negatively with latitude, as occurred in the herbarium samples of *J. cordifolia* (see Chapter 4). These are logical correlations. UV levels, especially UV-B, generally increase with increasing altitude due to a shorter pathlength and a higher atmospheric purity, which reduce UV attenuation in mountain areas (Björn *et al.*, 1998; Arróniz-Crespo *et al.*, 2006). This happens at least with UV maximum irradiances, because total annual UV doses may be influenced by the higher cloudiness associated with high altitudes (Núñez-Olivera *et al.*, 2011). UV levels decrease with increasing latitude because sun rays are received less perpendicularly at higher latitudes (Björn, 1999; Vanicek *et al.*, 1999). In our study, altitude and latitude were negatively correlated because the localities situated at higher altitudes were the southernmost ones (Sierra Nevada), whereas northern localities were located at either low (Jaizquíbel) or high (Picos de Europa, Pyrenees) altitudes. Radiation variables were negatively correlated with water temperature because temperature and altitude were logically negatively correlated, and radiation levels increased with increasing altitude.

The bulk levels of SUVACs and IUVACs were positively correlated, which contrasted with the results found in herbarium samples (Chapter 4). This positive correlation would suggest a similar variation pattern, but the reason underlying this fact remains unexplained. The bulk levels of SUVACs and IUVACs were strongly and positively correlated with the bulk level of TUVACs, which was logical because both SUVACs and IUVACs contributed to TUVACs. SUVACs were better correlated with TUVACs than IUVACs, due to the fact that the contribution of SUVACs to TUVACs was more important than that of IUVACs. The bulk levels of SUVACs, IUVACs and TUVACs were negatively correlated with SI, probably because those levels were obtained per DM, and, if DM increases, SI increases but those levels decrease.

The bulk levels of SUVACs and TUVACs were positively correlated with all the individual compounds except C2 and C3, whereas the bulk level of IUVACs was only positively correlated with the compounds located in the insoluble fraction (C6 and C7). These correlations were notably different to those found in herbarium samples (Otero *et al.*, 2009; Chapter 4), in which the bulk level of SUVACs was significantly and positively correlated with all the individual soluble UVACs (C1 to C5) and the bulk level of IUVACs was not positively correlated with any individual compound. Thus, the relationships between the bulk levels of SUVACs and IUVACs, and between those bulk levels and the concentrations of the individual compounds, were strikingly different to the relationships of those variables in herbarium samples of the same species collected in (approximately) the same locations on similar dates (Chapter 4). These differences may be due to the different sampling in the temporal scale, because collection of fresh samples was completed in all the localities in a few days of a specific year, whereas collection of herbarium samples was carried out in different months along a prolonged period of years (1913-2006). Thus, herbarium samples integrated a higher seasonal and interannual variability, and consequently the correlations between the different UVACs could be different. In addition, it cannot be discarded that each UVAC in herbarium samples may have been modified (for example, broken down) in a different way due to storage, thus altering the relationships among all the UVACs.

The individual compounds showed a few and not very solid correlations between them. In the soluble fraction, there were positive correlations between C1 and C5, C2 and C3, and C3 and C4. There was also a negative correlation between C2 and C5. In the insoluble fraction, the two compounds (C6 and C7) were strongly and positively correlated between them, and also with two soluble compounds (C1 and C5). This pattern of correlations was different to that found in herbarium samples, in which, for example, all the individual soluble UVACs were positively correlated (Otero *et al.*, 2009; Chapter 4). A possible explanation for these differences has been postulated above. Nevertheless, in both fresh and herbarium samples the two insoluble compounds (C6 and C7) were correlated. As occurred in the herbarium samples, the relationships between the different individual UVACs in fresh samples, as well as the relationships between the bulk levels of SUVACs and IUVACs and the individual compounds, were complex and remain to be completely elucidated.

Regarding the correlations between physiological and environmental variables, the bulk level of SUVACs, together with C4 and C5, were positively correlated with altitude and negatively with temperature. The bulk level of TUVACs was also positively correlated with altitude. These correlations could be a response of UVACs to increasing UV levels with increasing altitude, and are congruent with the increase in the bulk level of SUVACs, C4 and C5 that Arróniz-Crespo *et al.* (2006) found in a more modest altitudinal gradient (1140 to 1816 m), using also *J. cordifolia*. Thus, these three variables have consistently been demonstrated to be the most UV-responsive in spatial gradients in the field, and they could be used for the biomonitorization of ambient UV levels. In addition, C4 and C5 were positively correlated with most variables indicative of UV radiation levels except, mainly, the UV-B_{BE} (Plant Damage). These additional correlations corroborated the use of both compounds in *J. cordifolia* as UV biomonitors, as Arróniz-Crespo *et al.* (2006) anticipated using a more modest altitudinal gradient. Even in herbarium samples (Chapter 4), C5 was positively correlated with altitude. It was surprising that C1 and C6, the most UV-responsive UVACs of *J. cordifolia* under laboratory conditions (Fabón *et al.*, 2010, 2012a) and in temporal gradients (at least C1: Núñez-Olivera *et al.*, 2009), did not respond to UV in the present study. This could be due to the relatively narrow UV gradient used, or to the fact that the concentration of C1 had reached its maximum values (sample collection was done around midday and around the summer solstice, when C1 concentrations are the highest: Núñez-Olivera *et al.*, 2009; Fabón *et al.*, 2012a). Other phenolic compounds have responded to UV levels along altitudinal gradients in tracheophytes (Murai *et al.*, 2009; Jaakola & Hohtola, 2010). The negative correlation between water temperature and the bulk level of SUVACs, C4 and C5, could be a collateral effect derived from the fact that altitude and temperature were negatively correlated. Nevertheless, a direct effect of temperature cannot be discarded, since low temperatures can increase the synthesis of phenolic compounds (Jaakola & Hohtola, 2010).

F_v/F_m was not correlated with UV levels or any other environmental variable. At first sight, this seemed surprising because F_v/F_m is a UV-sensitive physiological variable in *J. cordifolia* and decreases under enhanced UV (Martínez-Abaigar *et al.*, 2003; Fabón *et al.*, 2010). However, it must be taken into account that, under field conditions, F_v/F_m usually shows fairly homogeneous values, for example 0.63–0.70 in Núñez-Olivera *et al.* (2009). This range is similar to that found in the present study (0.56-0-70). This means that F_v/F_m maintained relatively high values throughout the spatial gradient

considered. This was not surprising because F_v/F_m may be an indicator of vitality (Maxwell & Johnson, 2000), and all the samples analysed could have a similar vitality state because, in aquatic bryophytes, water has a strong buffering capacity for adverse environmental factors, such as extreme temperatures or desiccation. Thus, *J. cordifolia* was well adapted to the environmental conditions in all the streams sampled, having physiologically active apices with relatively high values of F_v/F_m, and this variable did not respond to the relatively narrow UV gradient considered in our study. Probably, one of the factors allowing a good adaptation to the environment was the possession of sufficient levels of UVACs.

As occurred with F_v/F_m, SI was not correlated with any environmental variable, although its range was relatively wider than that of F_v/F_m. SI may represent an indirect measurement of growth, because young or newly grown shoots are softer and less sclerophyllous than mature or old shoots (Martínez-Abaigar *et al.*, 2003). Given that all the environmental variables considered in our study were macroenvironmental variables, probably the growth (and thus the SI) of *J. cordifolia* was more determined by microenvironmental variables inherent to the strongly dynamic nature of mountain streams (Núñez-Olivera *et al.*, 2010): the water velocity and turbulence directly affecting the liverwort patch which was sampled, the topographic or canopy shading which could limit the sun exposure, etc. Harris (2009) also pointed out the importance of "fine-scale ecological features" (*i.e.* microenvironmental factors) in the determination of physiological characteristics of bryophytes, specifically flavonoid content in the moss genus *Plagiomnium*.

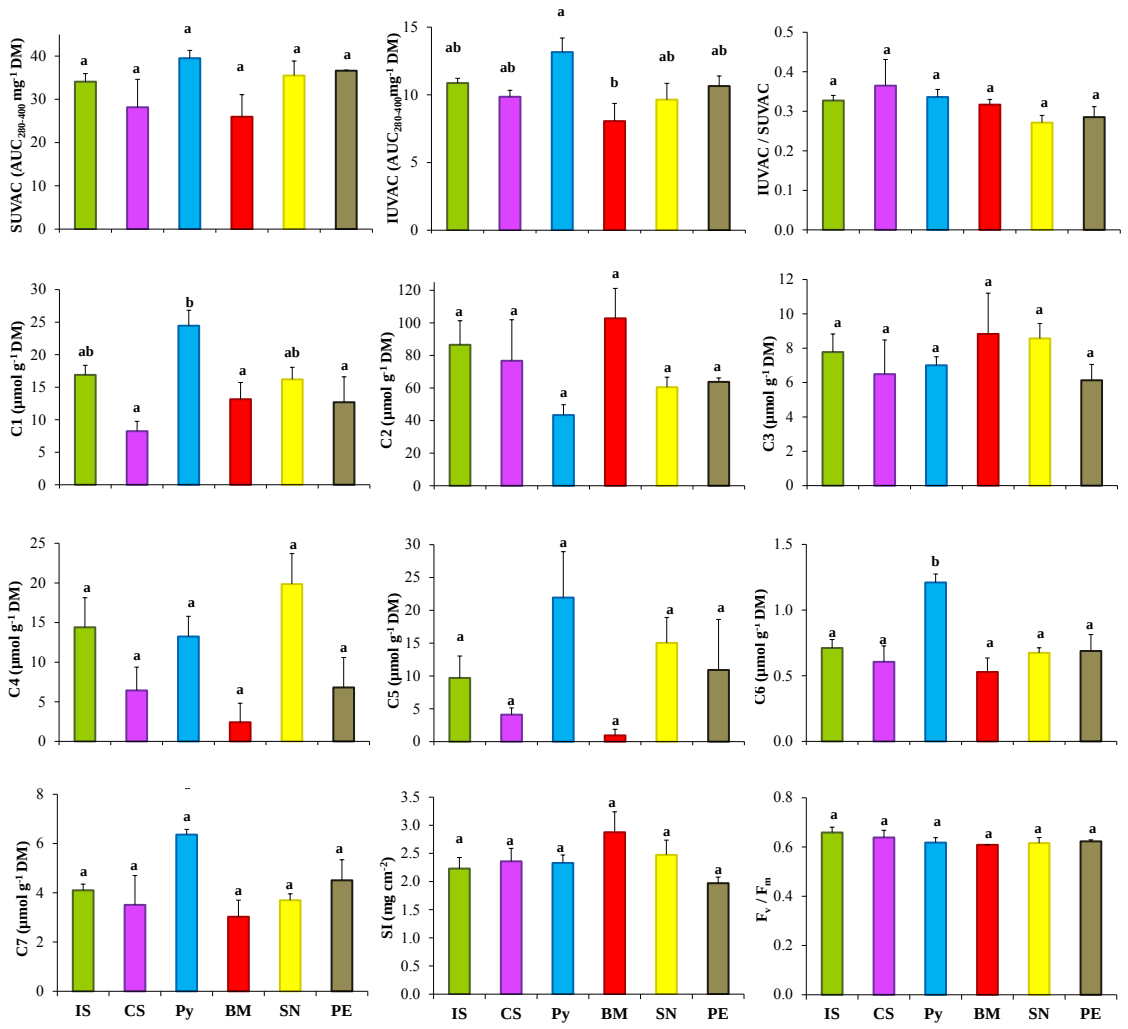


Figure 5.2. Differences in the physiological variables between the samples located in the six mountain chains used in this study. For identification of variables, see Table 5.2. Codes of mountain chains are defined in Figure 5.1 and Table 5.1 For each variable, different letters mean significant differences at least with $p<0.05$ (see the text for details of the statistical tests carried out).

Differences among mountain chains and within each mountain chain

Fig. 5.2 shows the differences in the physiological variables among the samples located in the six mountain chains used in this study. Significant differences only appeared in 3 out of the 12 variables: the bulk levels of IUVACs and the concentrations of C1 and C6. These differences consisted in that samples from the Pyrenees had higher bulk levels of IUVACs than those from the Basque Mountains, higher concentrations of C1 than samples from the Central System, the Basque Mountains and Picos de Europa, and higher concentrations of C6 than the remaining mountain chains. Surprisingly, the three variables showing significant differences among the mountain chains were not correlated with any environmental variable considered in our study (Table 5.3), and hence those differences were due to other factors, probably (as it was explained above) microenvironmental factors not assessed in our study. In particular, two of the variables showing significant differences among mountain chains were C1 and C6, the most UV-responsive UVACs in *J. cordifolia* (Núñez-Olivera *et al.*, 2009; Fabón *et al.*, 2010, 2012a), but neither C1 nor C6 were correlated with UV levels in our study.

The remaining variables did not show any significant difference among the mountain chains studied, although in some variables the differences were noticeable (for example, the bulk level of SUVACs and the concentrations of C2, C4, C5 and C7). This lack of significance could be explained by the high variability of the variables within each mountain chain (Table 5.4). For example, samples from the Central System, the Iberian System, the Basque Mountains and Sierra Nevada showed differences in 10-11 physiological variables (out of 13). In contrast, samples from the Pyrenees and Picos de Europa showed differences only in 5 and 7 variables, respectively. Other variables (IUVAC/SUVAC, C3, SI and F_v/F_m) showed values much more homogeneous among the mountain chains (Fig. 5.2), and thus it was logical these variables were not significantly different among chains. Nevertheless, in all the physiological variables except F_v/F_m, differences within each mountain chain were significant at least in half the chains, and C1 and C5 showed significant differences in every chain (Table 5.4).

In conclusion, 1) the high variability of physiological variables among the localities within each mountain chain (Table 5.4) could obscure the differences among mountain chains (Fig. 5.2); and 2) the differences among and within mountain chains could be due not only to the macroenvironmental factors assessed in our study but also to microenvironmental factors.

Table 5.4. Significance results of the statistical analyses comparing the localities within each mountain chain for each physiological variable. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

Mountain Chain	F_v/F_m	SI	SUVAC	IUVAC	I / S	TUVAC	C1	C2	C3	C4	C5	C6	C7
Iberian System	NS	**	**	NS	*	**	**	***	*	**	***	***	NS
Central System	NS	**	***	NS	***	***	**	***	***	***	**	**	***
Pyrenees	NS	NS	NS	**	NS	*	**	*	NS	NS	***	NS	NS
Basque Mountains	NS	NS	*	***	NS	**	**	**	***	***	***	**	***
Sierra Nevada	NS	**	***	***	***	***	***	***	***	***	**	NS	NS
Picos de Europa	NS	NS	NS	NS	**	NS	***	NS	**	***	***	**	***

Ordination of localities and mountain chains by PCA

The localities were ordinated by PCA using both the environmental and physiological variables. The accumulated variance by the first three axes was 77.5% (39.4% for axis I, 25.8% for axis II and 12.3% for axis III). The plot using the first two axes is shown in Fig. 5.3. The most significant loading factors for the positive part of axis I were altitude, all the radiation variables, ozone and C4, whereas water temperature and latitude were the most significant loading factors for the negative part of axis I. The most significant loading factors for the positive part of axis II were diverse variables related with UVACs (the bulk levels of SUVACs, IUVACs and TUVACs, the concentrations of C1, C5, C6 and C7, and the ratio C1/C3), together with the longitude, whereas no significant loading factor appeared for the negative part of axis II. Thus, axis I ordinated the localities on the basis of (almost exclusively) environmental variables, whereas axis II was much more influenced by physiological variables (exclusively UVACs).

Most localities were clearly grouped forming consistent units that corresponded to their respective mountain chains. The localities from the Pyrenees appeared in the central positive part of axis II, and therefore they were mainly characterized by their UVACs (and not by environmental variables): high values of bulk levels of SUVACs, IUVACs and TUVACs, together with high concentrations of several soluble (C1 and C5) and insoluble (C6 and C7) individual compounds. The clear differentiation of the Pyrenees on the basis of UVACs was in line with the particular characteristics shown by

this mountain chain in Fig. 5.2. Nevertheless, within the Pyrenees, the locality of Panticosa was separated from the other two, given that it showed the highest values of most UVACs (Table 5.2). The localities from the Basque Mountains appeared on the third quadrant, and were characterized by high water temperatures (in accordance with their low altitudes), high latitudes, low radiation values and relatively low bulk levels of IUVACs and concentrations of C1, C4, C5, C6 and C7. The localities from Sierra Nevada appeared in the second quadrant, opposite to the localities from the Basque Mountains with respect to axis I, thus showing contrary environmental characteristics. With respect to their UVACs, localities from Sierra Nevada showed relatively low bulk levels of IUVACs and concentrations of C1, C6 and C7, but very high concentrations of C4. The relationship between C4 and altitude towards the positive part of axis I is in line with the correlation between both variables (Table 5.3) that has been described above, and also coincides with the finding of Arróniz-Crespo *et al.* (2006) using a more modest altitudinal gradient. Finally, most of the remaining localities (except Candelario in the Central System) were grouped in the centre of the PCA plot, thus showing intermediate values of both environmental variables and UVACs.

The PCA showed that both environmental and physiological (UVACs) variables were important to ordinate the mountain chains and the different localities within each mountain chain. The importance of UVACs may be surprising in the sight of the great variability they showed among the mountain chains (Fig. 5.2) and within each mountain chain (Table 5.4), but, in spite of their variability, UVACs as a whole were still useful for ordination. Also, it is important to note that the environmental variables responsible for the PCA ordination were all macroenvironmental variables, but microenvironmental variables could partly justify the great variability of most physiological variables shown in Fig. 5.2 and Table 5.4, and for example could be responsible of within-chain differences in the case of localities sharing the same macroenvironmental characteristics (because of close neighbourhood) but showing different UVAC composition (Dílar-1 and Dílar-2, Jaizquíbel-1 and Jaizquíbel-2, etc.). The importance of microenvironmental factors in the determination of certain physiological characteristics of bryophytes has also been highlighted by Harris (2009).

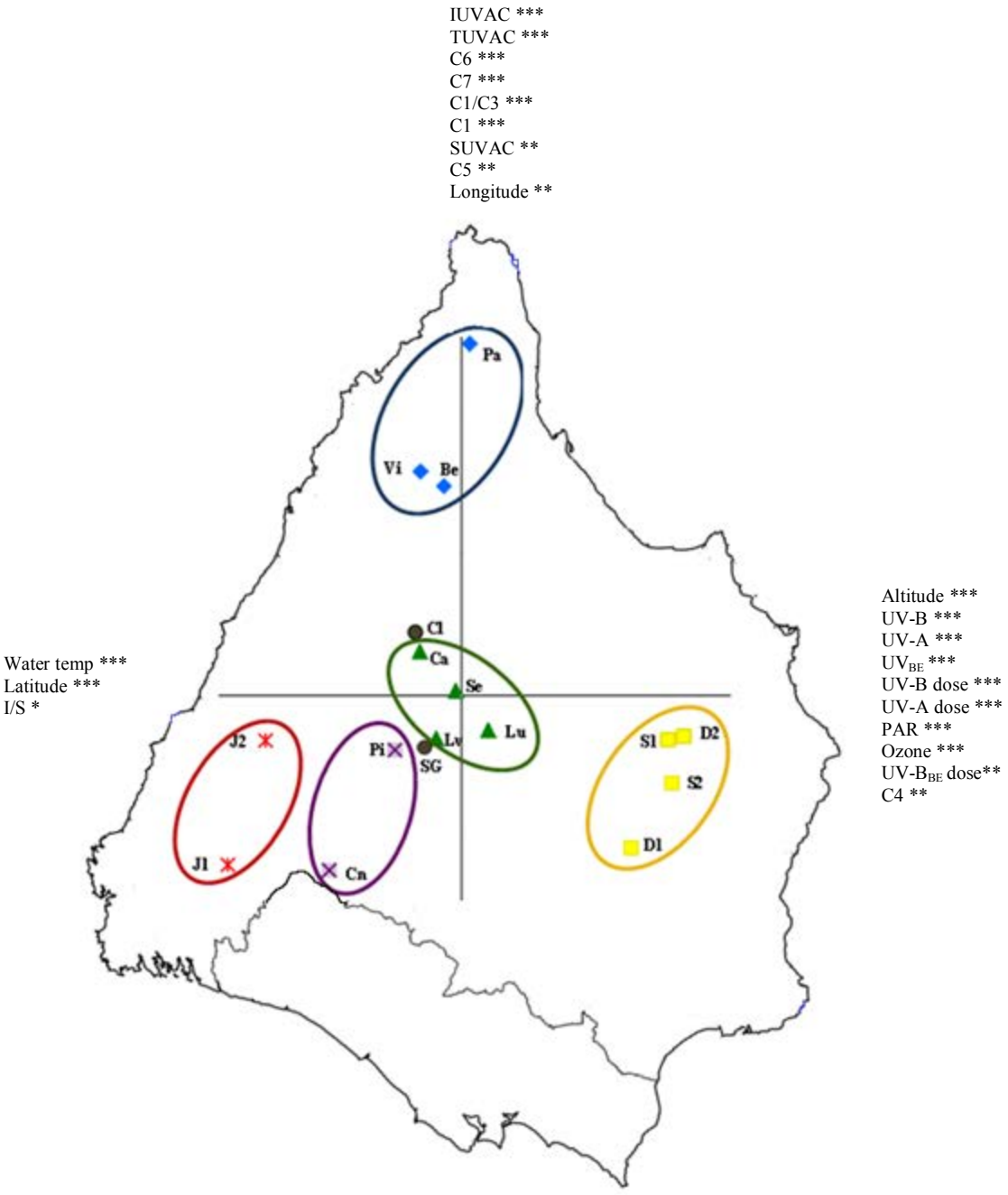


Figure 5.3. Ordination of the sampling localities used in this study through Principal Components Analysis (PCA), using both environmental and physiological variables. Significant loading factors for the positive and negative parts of each axis, together with their corresponding significance levels, are shown. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$. For identification of variables, see Tables 5.1 and 5.2. Codes of mountain chains are defined in Figure 5.1 and Table 5.1. Axis 1 is the horizontal one, and axis 2 is the vertical one. Each tic-mark on the axes represents one unit. Samples collected in the different mountain chains are distinguished using different symbols: triangle, Iberian System; cross, Central System; diamond, Pyrenees; asterisk, Basque Mountains; quadrat, Sierra Nevada; and circle, Picos de Europa. The outline of the Iberian Peninsula has been overlapped on the PCA plot.

In conclusion, macroenvironmental variables and UVACs were important sources of variation underlying the variability existing both among the mountain chains (Fig. 5.2) and within each mountain chain (Table 5.4), and thus were important in their ordination (except Picos de Europa). Nevertheless, microenvironmental factors not assessed in our study would also be responsible for some differences. Interestingly, the ordination shown by the PCA plot reproduced (to a certain extent) the location of the mountain chains considered within the Iberian Peninsula, as it can be noticed when its outline is overlapped on the PCA plot (Fig. 5.3): northern chains (except Picos de Europa) are clearly differentiated from inland chains (Iberian and Central Systems) and southern chains (Sierra Nevada).

DNA damage

No DNA damage was detected in any of the fresh samples of *J. cordifolia* from the different localities considered in the present study, although it was checked that the method applied detected DNA damage using positive controls of plasmid pBSK, pink salmon sperm and *J. cordifolia* irradiated with UV-C (Fig. 5.4). This is in line with previous results showing that no DNA damage was produced in three mosses and the liverwort *J. cordifolia* itself when they were exposed to ambient UV-B levels under field conditions (Lud *et al.*, 2003; Núñez-Olivera *et al.*, 2009, 2010). However, DNA damage was detected in *J. cordifolia* in response to artificially enhanced UV-B under laboratory conditions (Otero *et al.*, 2006; Fabón *et al.*, 2010, 2011, 2012b), and also in several mosses (*Bryum pseudotriquetrum*, *Ceratodon purpureus*, *Fontinalis antipyretica*, *Schistidium antarctici* and *Sanionia uncinata*) exposed to similar artificial UV-B supplements in both the laboratory (Lud *et al.*, 2002; Turnbull *et al.*, 2009; Fabón *et al.*, 2012a) 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).

The two types of results that have been described (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 short periods, in which 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 influenced by 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, 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 cyclobutane pyrimidine dimers (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 withstand, especially low temperature (-18.2ºC to 4.9ºC in the period studied).

In conclusion, the lack of DNA damage in the samples of *J. cordifolia* exposed to natural ambient UV-B levels along our spatial gradient was logical, because of the adaptation to those levels through the action of both protection mechanisms and repairing systems. This is congruent with previous results obtained in other bryophytes exposed to natural ambient UV-B levels.

Comparison of fresh and herbarium samples of *J. cordifolia*

The UVACs of the herbarium samples of *J. cordifolia* used in Chapter 4 were compared to those of the fresh samples used in this Chapter, using only the herbarium samples collected in June-July (as the fresh samples) in the same mountain chains. The herbarium samples used had been stored for 12-98 years at the moment of analysis. A total of 43 herbarium samples and 17 fresh samples were analysed. Soluble UVACs (both the bulk levels and the concentrations of C1-C5) were significantly higher in fresh than in herbarium samples, whereas insoluble UVACs (both the bulk level and the concentration of C6) showed the contrary (Fig. 5.5). C7 concentration was similar in both herbarium and fresh samples.

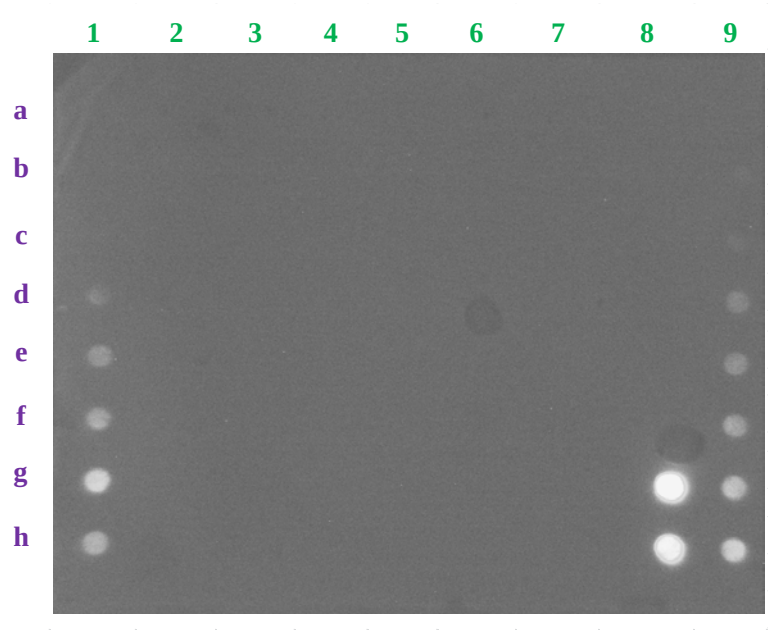


Figure 5.4. Results of the immunoassay performed to determine DNA damage (presence of thymine dimers) in the samples of *Jungermannia exsertifolia* subsp. *cordifolia* collected in the field. The dot blot shows: in columns 1 and 9 (lanes a-h) increasing amounts (0, 1, 2, 3, 4, 5, 8 and 10 ng) of DNA from plasmid pBSK irradiated with UV-C (calibration columns); in column 8, lanes a and b, unirradiated DNA samples of pink salmon sperm (negative controls); in column 8, lanes g and h, DNA samples of, respectively, pink salmon sperm and *Jungermannia exsertifolia* subsp. *cordifolia* irradiated with UV-C (positive controls); in columns 2-7, samples of 1 µg of liverwort DNA per dot corresponding to the different localities (columns 2-4 lane a, Jaizquíbel; columns 2-4 lane b, Caldevilla; columns 2-4 lane c, San Glorio; columns 2-4 lane f, Panticosa; columns 2-4 lane g, Benasque; columns 2-4 lane h, Viella; columns 5-7 lane a, Dílar; columns 5-7 lane b, San Juan; columns 5-7 lane c, Pico; columns 5-7 lane d, Candelario; columns 5-7 lane e, Lumbreras; columns 5-7 lane f, Senestillos; columns 5-7 lane g, Lavieja; columns 5-7 lane h, Calamantío).

There are two different hypotheses that may explain the higher values of soluble UVACs in fresh than in herbarium samples: 1) soluble UVACs are broken down with storage; and 2) soluble UVACs have really increased in response to the increased UV-B levels caused by stratospheric ozone degradation in recent decades (in Spain, ozone has decreased around 5% in the period 1980-2006, which would mean around 7% increase in UV-B: Antón *et al.*, 2007). This dichotomy has already been discussed in Chapter 4. Phenolic derivatives can be broken down during storage, but breakdown velocity depends on the type of compound. For example, *p*-coumaric acid and other HCA derivatives seem to be chemically stable because they have been found in fossil pollen, mostly in cell walls (Rozema *et al.*, 2001, 2009; Blokker *et al.*, 2005, 2006; Lomax *et al.*, 2008; Ryan *et al.*, 2009; Willis *et al.*, 2011). However, soluble UVACs in vacuoles

might be more prone to breakdown than UVACs in cell walls. In addition, degradation may be stronger if samples are not stored correctly (Ryan *et al.*, 2009), and it is impossible to control the storage conditions in the different herbaria. The second hypothesis matches well with the fact that some soluble UVACs from *J. cordifolia* (C1, C4, and C5) increased with increasing UV, under both laboratory and field conditions (Martínez-Abaigar *et al.*, 2003; Arróniz-Crespo *et al.*, 2006, 2008a, 2008b; Núñez-Olivera *et al.*, 2009). However, C2 and C3 were not UV-responsive, and they were also found in higher concentrations in fresh than in herbarium samples of *J. cordifolia*. On the other hand, it is not easy to explain why the bulk level of IUVACs and the concentration of one insoluble individual compound (C6) were higher in herbarium than in fresh samples, particularly considering that both variables increase under enhanced UV-B, at least in the laboratory (Fabón *et al.*, 2012a). Therefore, there are uncertainties concerning both hypotheses, and it is difficult to unequivocally confirm any of them.

The herbarium and fresh samples of *J. cordifolia* from the different chains were ordinated by PCA on the basis of their UVACs. Fig. 5.6 shows the distribution of samples in the plot obtained using the first two axes, that explained 81.4% of the variance (47.3% for axis I and 34.1% for axis II). The most significant loading factors for the positive part of axis I were the bulk level of SUVACs, together with the concentrations of three soluble compounds (C1, C4 and C5) and one insoluble compound (C7). There was no significant loading factor for the negative part of axis I. The most significant loading factors for the positive part of axis II were the concentrations of two soluble compounds (C2 and C3), and the bulk level of IUVACs and the concentration of one insoluble compound (C6) for the negative part. There was a clear separation between herbarium and fresh samples, the former with higher bulk levels of IUVACs and higher concentrations of the insoluble C6, and the latter with higher bulk levels of SUVACs and higher concentrations of the soluble C1-C5. Thus, the plot in Fig. 5.6 summarized the differences between both types of samples shown in Fig. 5.5. These differences appeared in every chain, in spite of the different macro- and microenvironmental conditions at the moment of collection, and also in spite of the possible degradation of UVACs caused by storage in herbarium. Interestingly, the most distant chains, both in herbarium and fresh samples, were the Pyrenees and the Basque Mountains. Thus, the temporal evolution of UVACs was relatively homogeneous in the different localities. In addition, IUVACs were much more stable than SUVACs, and thus the former would be more adequate for retrospective UV biomonitorization.

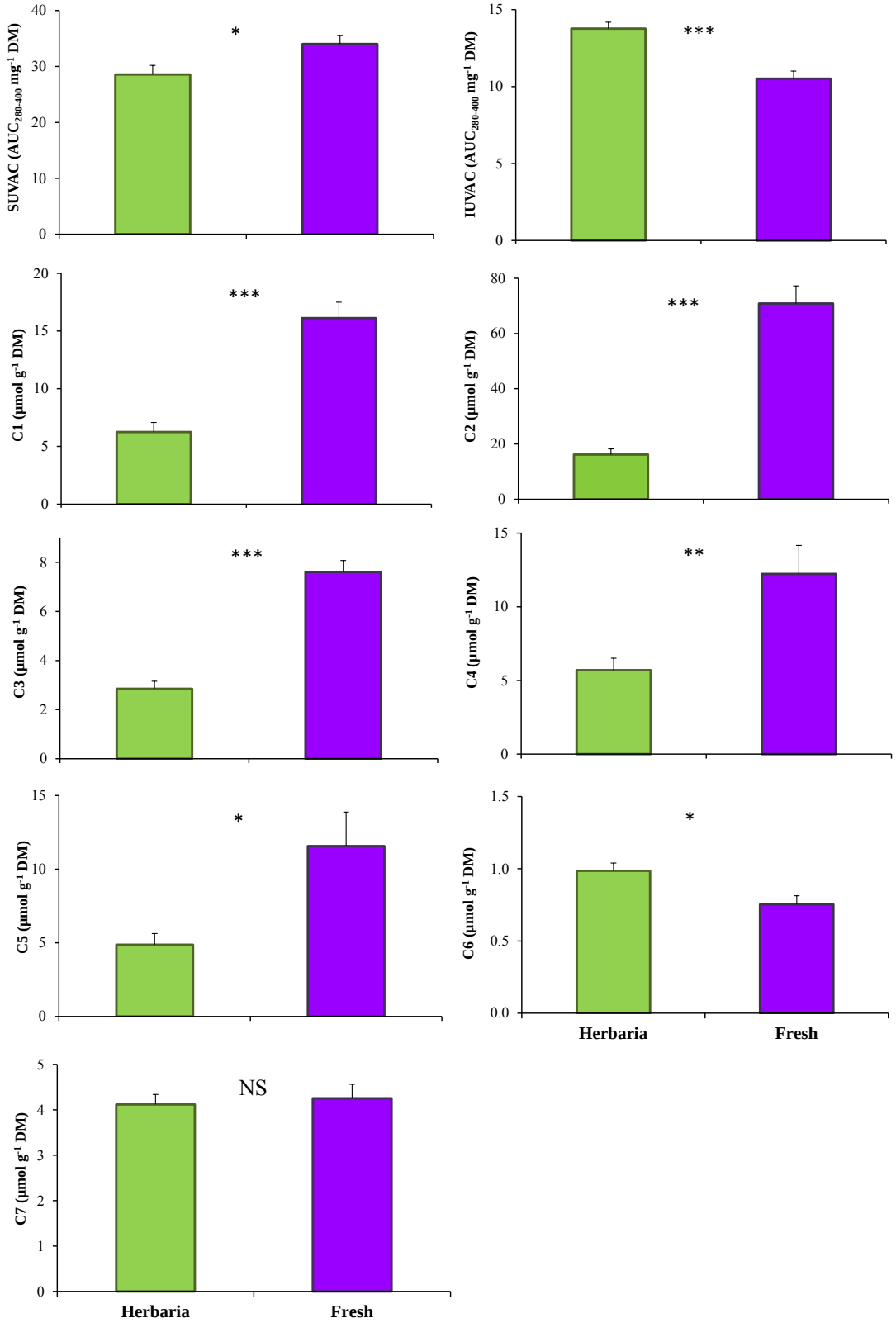


Figure 5.5. Comparison (mean ± SE values) of the UVACs analysed in samples of *Jungermannia exsertifolia* subsp. *cordifolia* either deposited in herbaria (Herbaria; see Chapter 4) or collected in the field (Fresh; this Chapter). See the text for the details considered for the comparison. For identification of variables, see Table 5.2. Significance levels for Student's *t* tests are shown: ***, $p<0.001$; **, $p< 0.01$; *, $p<0.05$; NS, non-significant.

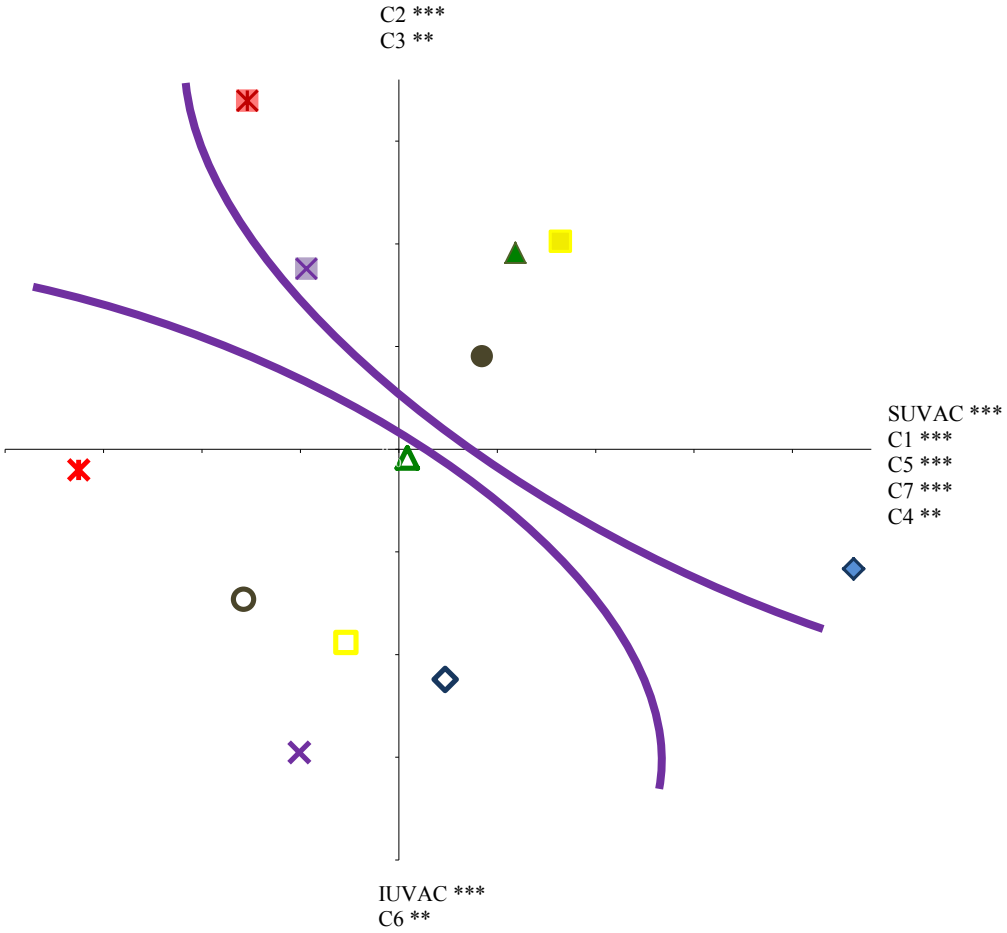


Figure 5.6. Ordination, through Principal Components Analysis (PCA), of the herbarium (Chapter 4) and fresh (this Chapter) samples of *Jungermannia exsertifolia* subsp. *cordifolia* coming from the different mountain chains, on the basis of their UVACs. Significant loading factors for the positive and negative parts of each axis, together with their corresponding significance levels, are shown. ***, $p<0.001$; **, $p<0.01$. For identification of variables, see Table 5.2. Symbols for mountain chains: triangle, Iberian System; cross, Central System; diamond, Pyrenees; asterisk, Basque Mountains; quadrat, Sierra Nevada; and circle, Picos de Europa. Solid symbols correspond to fresh samples and open symbols to herbarium samples. Axis 1 is the horizontal one, and axis 2 is the vertical one. Each tic-mark on the axes represents one unit.

5.5. CONCLUSIONS

1) The ranges of UV radiation and ozone in the spatial gradient used in the present study were relatively narrow because the sampling was completed in only 18 days. This allowed to strengthen the influence of other remarkable gradients, such as those regarding latitude (more than 6º), and, especially, altitude (almost 2500 m, the widest altitudinal gradient to date using one only bryophyte) and water temperature (11ºC).

2) The ranges of the physiological variables analysed (F_v/F_m, SI, the bulk levels of SUVACs and IUVACs, and the concentrations of 7 individual UVACs) were concordant with the values found in previous studies using *J. cordifolia*. In particular, the homogeneous and relatively high values of F_v/F_m found suggested that *J. cordifolia* was well adapted to the environmental conditions in all the streams sampled. This was also corroborated by the lack of DNA damage in any of the samples analysed.

3) The bulk level of SUVACs, together with C4 and C5 concentrations, were the most UV-responsive variables in spatial gradients in the field and they could be used for the biomonitorization of ambient UV levels.

4) The high variability of physiological variables among the localities within each mountain chain could obscure the differences among mountain chains. In addition, the differences among and within mountain chains could be due not only to the macroenvironmental factors assessed in our study but also to microenvironmental factors inherent to the strongly dynamic nature of the mountain streams.

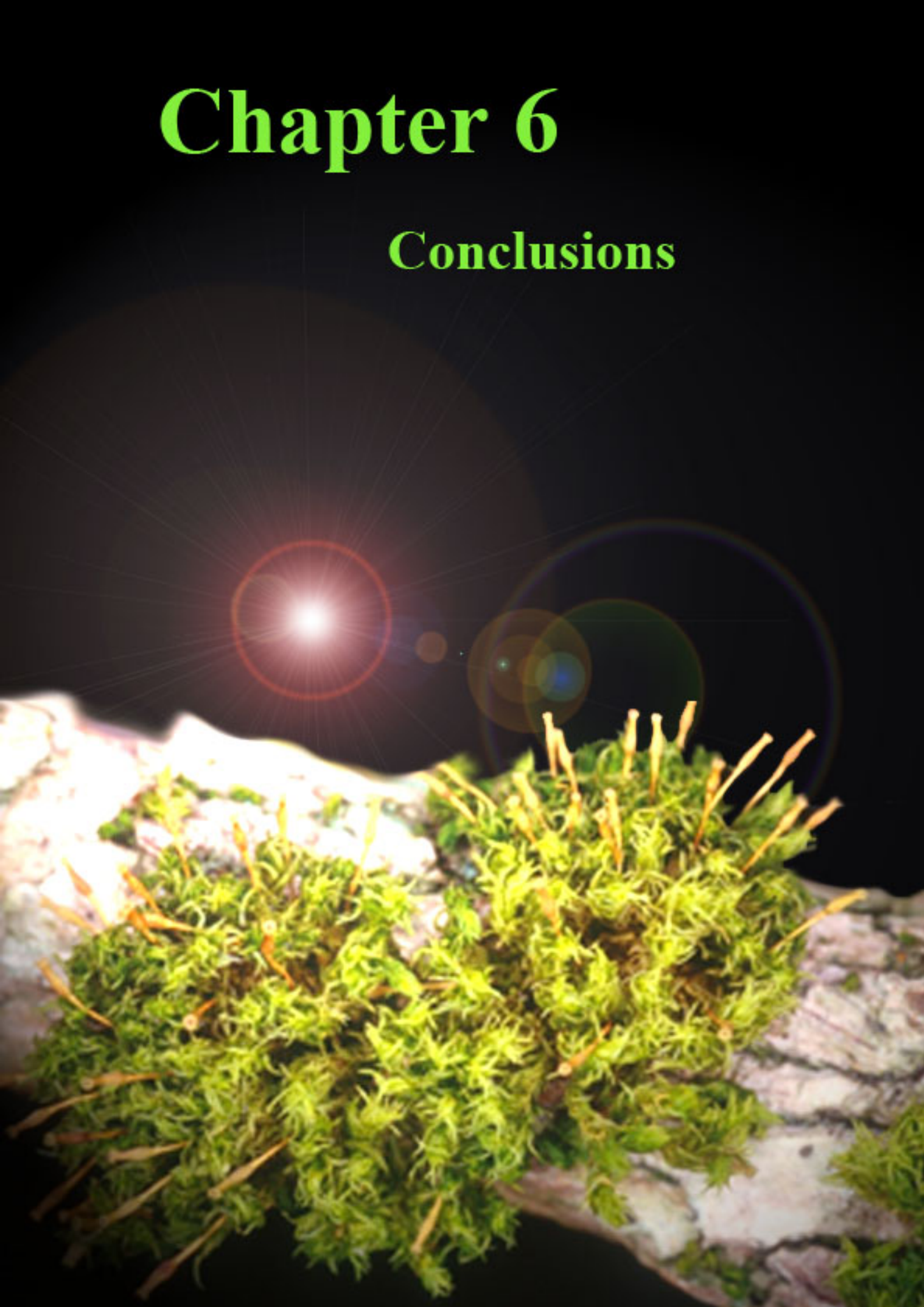
5) The PCA conducted using both the environmental and physiological variables showed that macroenvironmental variables and UVACs were able to ordinate, in a reasonably congruent manner, both the different mountain chains and the different localities within each mountain chain, although microenvironmental factors would be responsible for some differences. The ordination shown by the PCA plot reproduced the location of the mountain chains considered within the Iberian Peninsula.

6) Correlations between UVACs in recently collected fresh samples were notably different to those found in herbarium samples, probably because the different sampling pattern (herbarium samples were collected in different months along a prolonged period of years) and the possible degradation of UVACs during storage. Thus, the relationships between the bulk levels of SUVACs and IUVACs and the individual compounds were complex and remain to be completely elucidated.

7) Soluble UVACs were significantly higher in fresh than in herbarium (stored for 12-98 years) samples of *J. cordifolia*, whereas insoluble UVACs showed the contrary. These results, which were summarized by PCA, could be due to the degradation of soluble UVACs during storage or to their increase in response to the increased UV-B levels caused by stratospheric ozone degradation in the last decades. It is difficult to unequivocally confirm any of these hypotheses, but it is clear that IUVACs were much more stable than SUVACs, and thus the former would be more adequate than the latter for retrospective UV biomonitorization.

Chapter 6

Conclusions



6. CONCLUSIONS

In relation with the third Chapter of this Thesis (objectives 1 and 2):

1) Liverworts and mosses differ in the levels and compartmentation of UVACs. In general, liverworts had higher levels of SUVACs and lower levels of IUVACs than mosses, and vice-versa. This difference represents an additional evidence of the phylogenetic distance between both groups, that nowadays is considered to be deeper than previously thought. Thus, UVACs compartmentation represents a new ecophysiological trait that could be evolutionarily important in the colonization of new UV-rich environments after the conquest of land by plants. More data are needed regarding hornworts to place them appropriately in this context, but the first data suggest they are more related to liverworts than to mosses.

2) Exceptions to the general rule described above may appear in cases of species with a peculiar UVAC composition or compartmentation, such as *Atrichum undulatum*, *Bartramia pomiformis*, and species of *Bryum*, *Porella* and *Frullania*.

3) UVAC compartmentation may help differentiate the main evolutionary lineages in mosses (acrocarpous and pleurocarpous), and also peculiar mosses as *Sphagna*, but still not the main evolutionary lineages in liverworts or the different Orders of mosses and liverworts. Plerocarpous mosses constituted the most homogeneous phylogenetic group, regarding UVACs levels and compartmentation, among the groups differentiated in our study.

4) Assuming that cell wall-bound compounds are more efficient UV screens than vacuolar compounds, the higher amount of cell wall-bound UVACs in mosses than in liverworts would have allowed mosses to be more competitive in sunny UV-rich environments. Thus, mosses and liverworts may have evolved differently in this regard and thus they have tended to occupy different environments.

5) The lack of clear and solid relationships between UVACs accumulation and compartmentation, and the environmental variables (in particular those determining the UV amount received by the samples), suggests that UVACs are, at least to a certain extent, constitutive and privative of each species, and may not be usually induced by the natural ambient UV levels.

6) Liverworts and mosses also differed in their sclerophylly index, since mosses (in particular, Polytrichaceae) were significantly more sclerophyllous than liverworts (especially species constituted by simple leaves or simple thalli).

In relation with the fourth Chapter of this Thesis (objective 3):

1) The bulk level of SUVACs was not correlated with that of IUVACs in the 90 herbarium samples of *J. cordifolia* that were analysed for this study. This suggests that both types of compounds may play different roles in the cells: efficient UV screening for IUVACs, due to their location in the cell walls, and an additional antioxidant role for SUVACs.

2) *p*-Coumaroylmalic acid (C1) was the only UVAC of *J. cordifolia* showing significantly higher concentrations in the years after the onset of stratospheric ozone degradation with respect to the previous years. This indicated its usefulness in the biomonitorization of the UV-B increase associated to ozone degradation.

3) The PCA conducted using the environmental and physiological variables considered, clearly separated the samples collected in summer from those collected in autumn, mainly due to the different UV levels experienced in both seasons and the higher concentrations of protecting UVACs in summer than in autumn. In addition, the PCA ordinated the samples on the basis of their geographical origin, and demonstrated that the different storage conditions in the different herbaria did not affect much that ordination.

4) The bulk level of SUVACs in *J. cordifolia*, together with the concentrations of three individual soluble UVACs (C1, C3 and C5), were higher in Spain than in northern Europe, suggesting a better UV protection which is in line with the higher UV levels received in Spain.

5) The UV-B_{BE} (Plant Damage) was reconstructed in Spain using a model composed by the collection month and the bulk level of IUVACs of *J. cordifolia*, and did not show a clear trend in the period 1913-2006. This lack of trend might be influenced by several factors (short sampling period within the year, uncertainties about UV exposure of the samples at the moment of sampling, possible breakdown of UVACs during sample storage in herbaria, and limited availability of UV climatic data), but was coincident with other UV reconstructions that have been carried out using other biological variables, species, sampling periods and geographical regions.

6) Overall, UVACs of herbarium samples of *J. cordifolia* have demonstrated a wide variety of relationships with UV radiation in the temporal and spatial scales. This is in line with the fact that UVACs of fresh samples of *J. cordifolia* also respond consistently to UV radiation both temporally and spatially. Thus, they are good biomonitors of changes in past and present UV levels.

In relation with the fifth Chapter of this Thesis (objectives 4 and 5):

1) The ranges of UV radiation and ozone in the spatial gradient used in the present study were relatively narrow because the sampling was completed in only 18 days. This allowed to strengthen the influence of other remarkable gradients, such as those regarding latitude (more than 6º), and, especially, altitude (almost 2500 m, the widest altitudinal gradient to date using one only bryophyte) and water temperature (11ºC).

2) The ranges of the physiological variables analysed (F_v/F_m, SI, the bulk levels of SUVACs and IUVACs, and the concentrations of 7 individual UVACs) were concordant with the values found in previous studies using *J. cordifolia*. In particular, the homogeneous and relatively high values of F_v/F_m found suggested that *J. cordifolia* was well adapted to the environmental conditions in all the streams sampled. This was also corroborated by the lack of DNA damage in any of the samples analysed.

3) The bulk level of SUVACs, together with C4 and C5 concentrations, were the most UV-responsive variables in spatial gradients in the field and they could be used for the biomonitorization of ambient UV levels.

4) The high variability of physiological variables among the localities within each mountain chain could obscure the differences among mountain chains. In addition, the differences among and within mountain chains could be due not only to the macroenvironmental factors assessed in our study but also to microenvironmental factors inherent to the strongly dynamic nature of the mountain streams.

5) The PCA conducted using both the environmental and physiological variables showed that macroenvironmental variables and UVACs were able to ordinate, in a reasonably congruent manner, both the different mountain chains and the different localities within each mountain chain, although microenvironmental factors would be responsible for some differences. The ordination shown by the PCA plot reproduced the location of the mountain chains considered within the Iberian Peninsula.

6) Correlations between UVACs in recently collected fresh samples were notably different to those found in herbarium samples, probably because the different sampling pattern (herbarium samples were collected in different months along a prolonged period of years) and the possible degradation of UVACs during storage. Thus, the relationships between the bulk levels of SUVACs and IUVACs and the individual compounds were complex and remain to be completely elucidated.

7) Soluble UVACs were significantly higher in fresh than in herbarium (stored for 12-98 years) samples of *J. cordifolia*, whereas insoluble UVACs showed the contrary. These results, which were summarized by PCA, could be due to the degradation of soluble UVACs during storage or to their increase in response to the increased UV-B levels caused by stratospheric ozone degradation in the last decades. It is difficult to unequivocally confirm any of these hypotheses, but it is clear that IUVACs were much more stable than SUVACs, and thus the former would be more adequate than the latter for retrospective UV biomonitorization.

Chapter 7

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Chapter 8

Acknowledgements

8. ACKNOWLEDGEMENTS

Formal Acknowledgements

I benefited from a PhD grant of the Gobierno de La Rioja (PRED2010/16). In addition, the Gobierno de La Rioja funded my 3-month stay in the Laboratory of Genome and Epigenome Evolution (Max Planck Institute for Plant Breeding Research, Cologne, Germany) which has allowed to get the International Mention for my PhD.

I am also grateful to the Ministerio de Ciencia e Innovación and Ministerio de Economía y Competitividad of Spain, and to the Fondo Europeo de Desarrollo Regional (FEDER), for the financial support of the research (Projects CGL2008-04450 and CGL2011-26977).

I gratefully acknowledge Ales Pecinka (Max Planck Institute) and Pedro Aphalo (University of Helsinki), who kindly acted as my supervisors in my short stays in Cologne and Helsinki, respectively.

The present work was integrated in the COST (European Cooperation in Science and Technology) Action FA0906 of the European Union "UV-B radiation: a specific regulator of plant growth and food quality in a changing climate", whose collaboration in funding short stays in University of Helsinki and Max Planck Institute (Cologne), and also in meetings expenses, is greatly appreciated. In particular, I warmly thank the chair of the Action (Marcel Jansen, University College Cork, Ireland) for his personal and institutional collaboration with my research.

The Departamento de Agricultura y Alimentación (Universidad de La Rioja) provided the infrastructure to carry out my research.

Herbaria curators are gratefully acknowledged for sample loaning: ARAN-VIT, BC (BCB), BCB, FCO-Briófitos, GDAC, LISU, MA-Hepat, MACB, MUB, PAMP, SALA-Bryo, and VIT.

The Gobierno de La Rioja (Dirección General de Medio Natural) kindly authorized our work in the Natural Park of Sierra Cebollera and other parts of La Rioja, and other institutions authorized sample collection in protected areas of Spain: Parque Nacional Picos de Europa, Gobierno de Cantabria (Dirección General de Biodiversidad), Diputación Foral de Álava (Departamento de Medio Ambiente, Sección de Parques Naturales), Diputación Foral de Guipúzcoa (Departamento de Desarrollo del Medio Rural), Instituto Aragonés de Gestión Ambiental, and Parque Nacional Sierra Nevada. All of them are gratefully acknowledged.

Ozone data were obtained thanks to the Atmospheric Chemistry and Dynamics Branch, (NASA Goddard Space Flight Center).

Prof. D.-P. Häder (Erlangen, Germany) and Prof. Osamu Nikaido (Kanazawa University, Japan) kindly provided materials for the analysis of DNA damage.

Personal Acknowledgements / Agradecimientos personales

En primer lugar, agradecer a mis directores de tesis Encarnación Núñez-Olivera y Javier Martínez-Abaigar este trabajo, sin vosotros hubiera sido imposible. No sé cómo agradeceros la oportunidad que me brindasteis cuando un día de finales de verano de 2009, toqué a la puerta del despacho de Encarna diciéndole que me gustaría hacer la tesis con ella, pero que no sabía qué debía hacer. No era consciente de que estaba comenzando una etapa espectacular en mi vida. Entonces comenzamos con la solicitud de becas, la matrícula en el Programa de Doctorado… Ahí empezó lo que ahora está terminando para continuar. Habéis sido unos magníficos directores, como mil y una vez os he dicho, unos segundo padres, por la cantidad de horas que hemos pasado juntos, trabajando en la Universidad, en el monte de muestreos, en congresos, reuniones, cenas y eventos varios…No sé cómo agradeceros todo lo que me habéis enseñado (y me enseñaréis), la oportunidad para probar si este mundo me gustaba, y no es que me guste, me encanta. Gracias por estar siempre ahí, por tener siempre buena cara, sobre todo cuando hay algún problema, porque en vez de reñir buscáis soluciones y alternativas para enfrentar el problema; por ser tan buenos transmisores de vuestro enorme conocimiento, por saber explicar lo que puede ser sencillo para vosotros y entender que a veces cueste. En fin, que os agradezco todo lo hecho durante este tiempo, os aprecio mucho, sois un referente y los "leader" de grupo que a todos nos gustaría tener siempre. No cambiéis nunca.

Continúo con la gente que durante este tiempo ha pasado por el laboratorio de botánica y ecofisiología de la Universidad de La Rioja. Mutuamente hemos aprendido los unos de los otros y nos hemos ido enriqueciendo. Paula estaba cuando yo llegué, después se nos fue al área microbiológica que le gustaba, aunque no le guste que la felicitemos. Más tarde estuvo Belén, que ahora se va a hacer el doctorado a América, y Marian y Gonzalo (más tarde hablaré de ellos). También Sandra durante un poco de tiempo, hasta que se fue con sus primates. María e Inma, siempre pelando bayas a oscuras. También a oscuras Helga, para sus análisis de pigmentos. Leti y Olga en el "labo" de al lado, que solo nos vemos en el pasillo. Por supuesto las estancias que han hecho Meritxell, con su achuchón incorporado, Helena, Belén, y Fidel, siempre tranquilo, paciente, sereno, no prisas… Cuántas horas hemos pasado juntos en el "labo".

Agradecer a Gabi, mi compañero durante 3 años y medio, que me enseñara lo que no se debe hacer, cómo no se debe trabajar, y además cómo comenzar a moverme en el laboratorio, cómo usar todo el instrumental (sobre todo los aparatos y en especial el HPLC), cómo descubrir este mundo que tanto me apasiona, la INVESTIGACIÓN. También me enseñó a darme cuenta y auto-demostrarme que amo lo que hago por encima de muchas cosas, pero no por encima del respeto hacia la intimidad y privacidad de los demás, de los compañeros, ni de las relaciones personales y sociales, que tan importantes son.

Agradecer a todo el Departamento de Agricultura y Alimentación, tanto profesores como doctorandos, compañeros de cursos de doctorado, de alguna cañita en la cafetería, y de saludos por los pasillos… Por supuesto a Nines y Ernesto por los ratos que hemos pasado intentando saber qué compuestos pueden tener los briófitos, y a los técnicos de laboratorio que me han ayudado en ciertos momentos.

A Rafa, gracias por enseñarme muchas cosas en el laboratorio, por esas horas y horas que hemos pasado extrayendo ADN, hibridando membranas, cruzando los dedos cuando íbamos a revelar un gel. Por todas las horas de coche en los muestreos por toda la Península Ibérica. Me costó, pero entendí tu humor, más vale tarde que nunca, actualmente hago yo los chiste, jejeje…

Cómo no, a mi actual equipo, infinitamente agradecida estoy de mis chicos Marian y Gonzalo, sois los mejores compañeros que he tenido (en mi corta vida en este mundo), probablemente que tendré. Sois fantásticos, geniales, risueños, alegres, divertidos, siempre ayudando, apoyando, hombro con hombro, estando ahí cuando se os necesita, escuchando cuando he necesitado hablar y aconsejando cuando os lo he pedido. Cuántas horas hemos pasado en el "labo" con las extracciones de CARUV, pigmentos, algo de uva (guisantillos a veces, ¿verdad Marian?). En fin, con vosotros también aprendí que no todo es laboratorio, que existe algo llamado cafetería dos pisos más abajo del nuestro, y es un lugar a investigar y visitar una vez por semana (at least), por esas cañas de los viernes al mediodía que espero continúen por mucho tiempo, qué más decir que no os haya dicho ya, no cambiéis, os aprecio muchísimo.

Agradecer a toda las personas que he conocido durante este tiempo en las estancias que he realizado, tanto en Helsinki (Pedro Aphalo, Luis Morales, Matthew…) como en Colonia (Ales Pecinka, Petra, Andreas, Thomas, Phoebe, Björn, Navratan, Mariana, Barbara, Tom, Ahmed…), a todos los que allí conocí. A la gente que he conocido y me he encontrado en los congresos, reuniones, workshops, coloquios…

Agradecer a mis amigos, que siempre están ahí cuando se necesita, y que entienden que llegues tarde por no salir a tiempo del laboratorio.

Y cómo no a mi familia, a mis 4 magníficos hermanos, mis cuñadas y cuñado, mis 8 sobrinos, que habéis entendido mi ausencia en algún evento y me apoyáis en lo que hago, os quiero. Por supuesto (los últimos como la guinda del pastel), agradezco mucho el apoyo incondicional de mis padres, su capacidad para saber entender alguna salida de tono y su apoyo en mis decisiones para encauzar mi carrera profesional. Os quiero, mucho no, muchísimo. En especial agradecer a mi madre, sabes que eres muy especial para mí, eres el pilar central en la familia, pero para mí eres los cimientos; siempre estás ahí cuando te necesitamos, escuchando como poca gente sabe hacer, da igual que entiendas lo que te están contando o no, lo importante es que escuchas, comprendes, aconsejas, apoyas, abrazas, quieres, amas, arropas como una SUPER MAMÁ que eres, y lo haces a la perfección. Esto va por vosotros, por ti, mamá, en especial, os quiero mucho.

“ Los científicos se esfuerzan por hacer posible lo imposible. Los políticos por hacer lo posible, imposible.”
(Bertrand Russell)

“Usa el método científico: probando varias veces, llegarás a la verdad.”
(Marco Tulio Cicerón)