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Age-specific physiological responses to UV radiation in the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*

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Abstract – In the UV-tolerant aquatic liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váña, increased accumulation of certain hydroxycinnamoylmalic acid (HCM acid) derivatives has been observed under natural and artificial increases of UV radiation. We hypothesized that, under an artificial UV enhancement, the newly grown shoot apices would develop more physiological protection against UV radiation than the basal old parts.

Both the UV increase and the tissue age affected the distribution of HCM acid derivatives after 82 days of culture. Two coumarins (5˝-(7˝,8˝-dihydroxycoumaroyl)-2-caffeoylmalic acid, and its glucosyl derivative) significantly increased under the UV treatment, but their distribution between the apical and basal parts were opposite, where the former was more concentrated in the apical segments and the latter in the basal segments. *p*-Coumaroylmalic acid also showed a significant increase due to UV, and increased by the same proportion in both types of segments. In contrast, the main compound phaselic acid decreased in the apical parts under UV, whereas feruloylmalic acid only accumulated in the basal parts under UV. Physiological variables indicative of vitality, such as the maximum quantum yield of PSII (F_v/F_m) and chlorophyll concentration, did not show any damage caused by the UV enhancement in the liverwort studied. The different distribution of the abovementioned compounds between apical and basal parts – together with the increase of carotenoids in the UV treatment – may indicate changes in metabolic pathways to increase photoprotection in young apical shoots.

UV radiation / Physiological profile / UV-absorbing compounds / Hydroxycinnamic acid derivatives / Liverworts

INTRODUCTION

Tissue age affects the physiology and biochemistry of plant leaves and therefore their sensitivity to the environment. Variations between old and young shoots are also commonly found in bryophytes (Bates, 1979). Compared to old

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shoots, young shoots usually present higher physiological activity that is normally linked to high respiration rates, high concentration of chlorophylls and carotenoids and high maximum quantum yield of photosystem II (Sestak & Siffel, 1997; García-Álvaro, 1999). Differences in nutrient distributions have been also studied in detail where N, P, and K are typically more concentrated in the young apical segment, and Ca, Mg, and Fe in the old basal segments (Brown, 1982; Martínez-Abaigar *et al.*, 2002). However, the distribution of phenolic compounds along the profile of bryophyte shoots has received less attention, despite its importance as protection mechanism against various stresses (Waterman & Mole, 1994).

Solar ultraviolet radiation (UV-B: 280-315 nm and UV-A: 315-400 nm) is an important ecological factor to which plants have to cope within their environment (Day & Neale, 2002). The amount of UV irradiance that reaches the Earth's surface is not uniform and can increase naturally (by changes in altitude, zenith angle and season), or due to anthropogenic ozone depletion in the stratosphere (Björn, 1999). The former is starting to reach importance in ecological research whereas the latter has been a major subject since the "ozone hole" was discovered in Antarctica in the middle 1980's. In particular, the effects of UV-B radiation on plants have been mostly studied by means of manipulative experimental approaches that increase UV-B radiation using lamps or that reduce or exclude it using filters (Day & Neale, 2002; Rousseaux *et al.*, 2004). The first approach is more appropriate for addressing the ozone depletion issue, whereas the second is more suited to assess effects of current UV-B levels (Rousseaux *et al.*, 2004). Most of these studies have been focused on higher plants – especially crops –, phytoplankton and macroalgae, whereas bryophytes have received less attention. Nevertheless, our understanding of the effect of UV radiation on bryophytes from both aquatic and terrestrial ecosystems, has been improved over the last decade (Boelen *et al.*, 2006; Martínez-Abaigar *et al.*, 2006; Björn, 2007), where some species have been demonstrated to be more tolerant than others. However, the protecting mechanisms that provide this tolerance are still poorly characterized, and thus further studies are required under both controlled and field conditions (Martínez-Abaigar *et al.*, 2006).

Jungermannia exsertifolia Steph. subsp. *cordifolia* (Dumort.) Váña is one of the few liverworts that have been studied in detail regarding its physiological responses to UV radiation (Martínez-Abaigar *et al.*, 2006). This bryophyte has shown consistent UV tolerance to artificial increases of UV-B radiation. Moreover the physiology of the plant seemed to be improved under UV-B exposition in the light of increases in photosynthesis rate, chlorophyll concentration, different vitality variables based on the chlorophyll fluorescence technique and the concentration of UV-absorbing secondary metabolites (Martínez-Abaigar *et al.*, 2003; Núñez-Olivera *et al.*, 2004; Núñez-Olivera *et al.*, 2005). This response is not exclusive to this species and beneficial effects of UV-B exposition have been also reported in other bryophytes (Schipperges & Gehrke, 1996; Phoenix *et al.*, 2001).

Accumulation of UV-absorbing compounds is considered to be one of the most common acclimation responses of vascular plants to enhanced UV radiation (Searles *et al.*, 2001) and it has been extensively characterized (Caldwell *et al.*, 2007). In particular, the accumulation of certain flavonoids and hydroxycinnamic acids has been reported to be one of the main protection mechanisms against UV in higher plants and algae (Cockell & Knowland, 1999; Kolb *et al.*, 2001; Reifenrath & Muller, 2007). In bryophytes, the accumulation of methanol-extractable ultraviolet absorbing compounds is related to the higher UV tolerance of certain liverwort species (Newsham *et al.*, 2002; Newsham, 2003; Martínez-

Abaigar *et al.*, 2003). On this respect, specific increases of some hydroxycinnamoylmalic acid (HCM acid) derivatives have been observed under natural and artificial increases of UV radiation in *Jungermannia exsertifolia* subsp. *cordifolia*, suggesting that the accumulation of these compounds may serve as a protection mechanism against the harmful effect of UV exposure. In particular, the concentration of two coumarins (5˝-(7˝,8˝-dihydroxycoumaroyl)-2-caffeoylmalic acid, and 5˝-(7˝,8˝-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid), together with *p*-coumaroylmalic acid, increased along a natural gradient of UV-B radiation (Arróniz-Crespo *et al.*, 2006) and also under artificially enhanced UV radiation in controlled conditions (Arróniz-Crespo *et al.*, 2008).

The aim of this work was to study, under controlled conditions, the physiological responses to increased UV radiation in tissues of different ages along the profile of the UV-tolerant liverwort *Jungermannia exsertifolia* subsp. *cordifolia*. We wanted to test if young apices would be more stressed than the basal parts of the shoots or if, *per contra*, apices would develop effective and rapid protective mechanisms against UV radiation in this tolerant species. From our knowledge, this is the first study that analyses UV tolerance in shoots of different ages in bryophytes.

MATERIALS AND METHODS

Plant material

Specimens of the aquatic foliose liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váña (hereafter *J. cordifolia*) were collected at the oligotrophic stream Senestillos (La Rioja, northern Spain; 1350 m a.s.l.; 42º02' N, 02º37' W). The liverwort grew submerged and was exposed to full sun. Samples were collected on 18 Jan 2002, rinsed with stream water, and transported to the laboratory in a portable icebox. The material was then rinsed again with stream water and healthy apices were selected and cultivated.

Experimental design

Six mats of *J. cordifolia* (8 g of fresh mass each) were placed into separate plastic tubes with a basal net which prevented material losses. The six tubes were placed in a circulating bath system within a growth chamber. The bath was filled with stream water at a constant temperature of 10ºC. The radiation was provided by a combination of three types of lamps: photosynthetically active radiation (PAR) lamps (True-Lite, True Sun, Steubenville, Ohio, USA), UV-A lamps (QP-340, Q-Panel, USA) and UV-B lamps (Philips TL 40W/12, Philips Lighting, Eindhoven, The Netherlands). The liverwort was submerged at 1-2 cm depth, which attenuated less than 0.01% the photosynthetic and UV wavelengths. Each group of three tubes was covered with a different UV cut-off foil to establish two radiation regimes: (1) "P" treatment control (PAR alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which cut off UV radiation below 395 nm; (2) "PAB" treatment, (PAR + UV-A + UV-B) using Ultraphan 295 (Digefra GmbH, Munich, Germany), which cut off UV radiation below 295 nm (in particular, all UV-C radiation). This set-up was replicated 3 times. The plastic

tubes in the bath systems were moved on a daily basis to prevent possible place-dependent differences in the irradiance received by the plants. PAR lamps gave a photon flux of around 100 μmol m^{-2} s^{-1} (LI-190SA quantum sensor, LI-COR, Lincoln, NE, USA) with a 10:14 photoperiod (light:dark). UV-A and UV-B lamps were switched on around noon for 4 h each day (square-wave). UV-A lamps gave an irradiance of 10.9 W m^{-2}. The biologically effective UV-B irradiance applied was 0.67 W m^{-2} (equivalent to a daily integrated irradiance of 9.6 kJ m^{-2}), as estimated using the generalized plant damage action spectrum of Caldwell (1971) normalized to 300 nm. This extra UV-B irradiance was required to mimic a 20% ozone depletion at the latitude of the sampling site as calculated with a computer model for clear sky conditions and aerosol level zero (Björn & Teramura, 1993; Björn, pers. comm.). The spectral irradiances were measured, and the transmission characteristics of the filters were regularly checked with a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland). The liverwort was cultivated for 82 days.

Physiological variables

Physiological variables were measured along the profile of the liverwort at the end of the culture period when new growth tissue (young shoots) was distinguished from the old basal tissue (old shoots). Colour difference – light-green for the former and dark-green for the latter – was used to differentiate the two segments. For each physiological variable one sample was taken from each tube. Before the analysis, it was microscopically confirmed that the specimens had few algal epiphytes.

The sclerophylly index (SI) was calculated as the quotient between the dry mass (DM: 80 ºC for 24 h) and the surface area of the prostrate apex (LI-COR LI-3000 area meter, Lincoln, NE, USA). Chlorophylls and carotenoids were extracted on fresh samples with cold 80% acetone and mortar and pestle, and quantified spectrophotometrically (Perkin-Elmer λ3B UV/Vis, Perkin-Elmer, Wilton, CT, USA) as in Martínez-Abaigar *et al.* (2003). Total chlorophyll and carotenoid concentrations were expressed per unit of surface area (measured with a LI-COR LI-3000 area meter). *In vivo* chlorophyll fluorescence of PSII was measured with a portable pulse amplitude modulation fluorometer (MINI-PAM, Walz, Effeltrich, Germany) following Schreiber *et al.* (1995). Minimal and maximal fluorescence (F_0 and F_m) were measured in samples dark-adapted for 20 min, using a 600 Hz modulated beam at 0.03 μmol m^{-2} s^{-1} photon flux, and white saturating flashes of 12000 μmol m^{-2} s^{-1} photon flux. The maximum quantum yield of PSII was given by the ratio F_v/F_m, where $F_v = F_m - F_0$. After extraction in methanol: water: 7M HCl (70:29:1 v/v/v), the bulk amount of methanol-extractable UV-absorbing compounds (MEUVAC) was quantified spectrophotometrically and expressed in arbitrary units as the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) calculated per unit of surface area. Five individual UV-absorbing compounds of *J. cordifolia* were extracted and analyzed by HPLC (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA, USA), following Arróniz-Crespo *et al.* (2006). The five compounds are: compound 1 (*p*-coumaroylmalic acid), compound 2 (caffeoylmalic acid – or phaselic acid –), compound 3 (feruloylmalic acid), compound 4 (5″-(7″,8″-dihydroxycoumaroyl)-2-caffeoylmalic acid), and compound 5 (5″-(7″,8″-dihydroxy-7-O-β-glucosyl-coumaroyl)-2- caffeoylmalic acid). The chemical structures of the five compounds are shown in Fig. 1 and were described

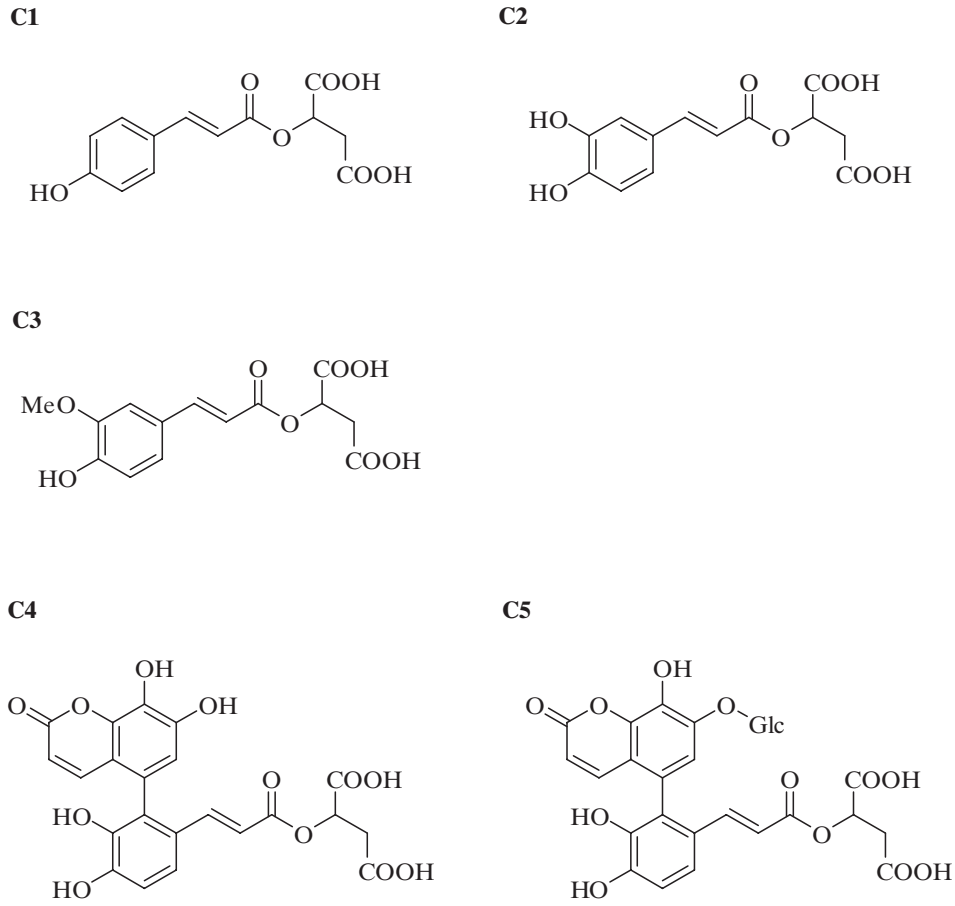

Fig. 1. Chemical structures of the five compounds analyzed in *Jungermannia cordifolia*. The compounds are as follows: C1, *p*-coumaroylmalic acid; C2, phaselic acid; C3, feruloylmalic acid; C4, (5″-(7″,8″-dihydroxycoumaroyl)-2-caffeoylmalic acid), and C5, (5″-(7″,8″-dihydroxy-7-O-β-glucosyl-coumaroyl)-2-caffeoylmalic acid). These chemical structures are described in Arróniz-Crespo *et al.* (2006).

in Arróniz-Crespo *et al.* (2006). They will be onwards referred as C1 to C5. Concentrations were expressed per unit of surface area.

Statistical analysis

The effects of the tissue age and the radiation regime on the responses of the liverwort were tested using a 2-way analysis of variance (ANOVA) after applying tests for normality and homoscedasticity of the data. For paired samples analysis (effects of the tissue age on P and PAB treatments and of the radiation regime on old and young shoots), means were compared by Student's *t* tests. All the statistical procedures were performed with SPSS 10.0 for Windows (SPSS Inc., Chicago, Illinois).

RESULTS

The physiology of *J. cordifolia* was significantly affected by both the age of the tissue and the radiation regime (Table 1, global statistics test). Some physiological variables, such as the sclerophylly index (SI), F_v/F_m, and C2 and C3 concentrations, were more influenced by the age of the tissue, whereas the radiation regime was associated to changes in all the variables except F_v/F_m.

Similar physiological differences between young and old shoots were shown in samples exposed to both radiation regimes for most of the physiological variables (Figs 2-3, Table 1 paired analyses). Regarding this, in both radiation regimes newly grown shoots showed a significant increase in F_v/F_m, chlorophylls concentration and MEUVAC ($AUC_{280-400}$), together with decreases in SI and the concentration of C3 and C5. On the other hand, independently of the age of the tissue, exposure to UV radiation caused increases in chlorophyll and carotenoids concentration and remarkable increases of MEUVAC ($AUC_{280-400}$) and compounds C1, C4 and C5 in both segment types.

Table 1. Overall effects (global) of the tissue age (old and young shoots) and the radiation regime (P and PAB) on the physiological variables of *Jungermannia cordifolia*, tested by two-way ANOVA. The specific effects of the tissue age on P and PAB treatments and of the radiation regime on old and young shoots (paired analyses), tested by Student's t tests, are also indicated. Figs 2-3 show the mean values (+ SE) of paired analyses.

Variables	*Global*		*Paired analyses*			
	Tissue age	*Radiation regime*	*Tissue age (P)*	*Tissue age (PAB)*	*Radiation regime (old)*	*Radiation regime (young)*
Sclerophylly index (SI)	***	*	**	**	ns	ns
F_v / F_m	***	ns	**	**	ns	ns
Chlorophyll per area	***	***	*	**	**	**
Carotenoids per area	**	***	ns	**	**	**
MEUVAC per area	ns	***	*	*	***	**
C1	ns	***	***	ns	**	**
C2	***	*	***	ns	ns	***
C3	***	**	*	**	*	ns
C4	ns	***	ns	**	**	**
C5	ns	***	***	**	**	***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns: non-significant; F_v/F_m: maximum quantum yield of PSII; MEUVAC: methanol-extractable UV-absorbing compounds; C1 to C5: five individual UV-absorbing compounds (see their chemical names in Fig. 1 and Materials and Methods); P: control treatment (PAR alone); PAB: UV treatment (PAR + UV-A + UV-B).

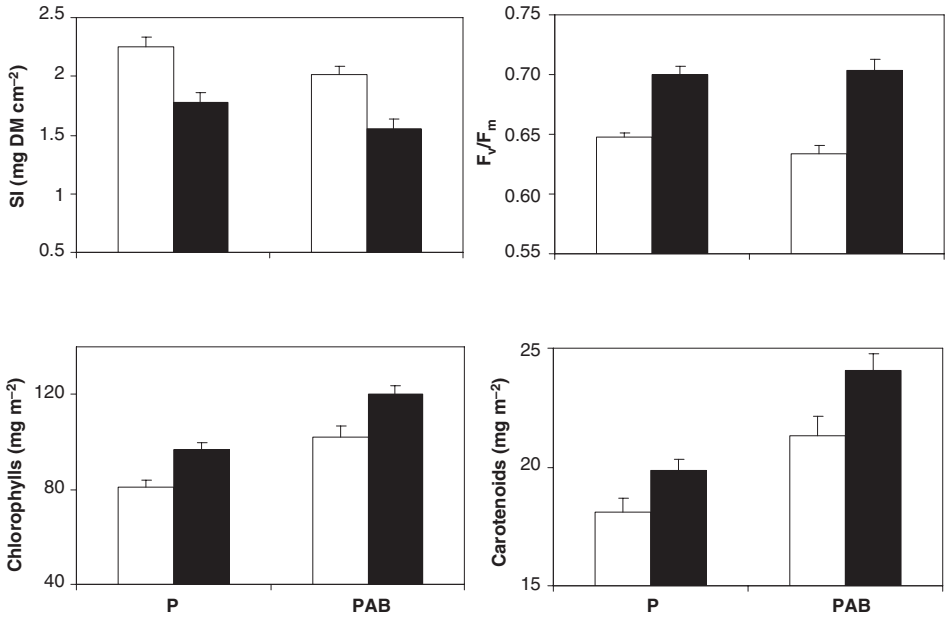

Fig. 2. Differences between old shoots (open bars) and young shoots (filled bars) in Sclerophylly Index (SI), maximum quantum yield of PSII (F_v/F_m), and chlorophylls and carotenoids concentrations per surface unit of *Jungermannia cordifolia* after 82 days growing under control (P: PAR only) and UV treatment (PAB: PAR+ UV-A + UV-B). Means (+SE) are shown. Global and paired statistical analyses as indicated in Table 1.

Changes in physiological patterns due to the interaction between the age of the tissue and the radiation regime were observed in some variables. In particular, the concentration of carotenoids increased in young shoots only under the PAB treatment (Fig. 2). The two coumarins C4 and C5 significantly increased under the PAB treatment but their distribution between the apical and basal parts were opposite, where the former was more concentrated in the apical segments and the latter in the basal segments. C1 also showed a significant increase due to UV, and increased by the same proportion in both types of segments. C3 only accumulated in the old tissue when exposed to UV. In contrast, the accumulation of the main compound C2 was not induced by UV radiation and was even reduced in young shoots under PAB compared to the P regime (Fig. 3). At the end of the culture period, newly grown shoots exposed to UV radiation showed the highest concentration of chlorophylls and carotenoids, MEUVAC ($AUC_{280\text{-}400}$) values and concentration of C4 (Figs 2-3).

DISCUSSION

The UV-tolerant aquatic liverwort *J. cordifolia* exhibited differences in the short-term physiological responses to UV radiation between young and old shoots after 82 days of UV exposition under laboratory conditions.

The measured physiological variables indicative of vitality, such as F_v/F_m and chlorophyll concentration, did not decrease due to UV exposition, and thus

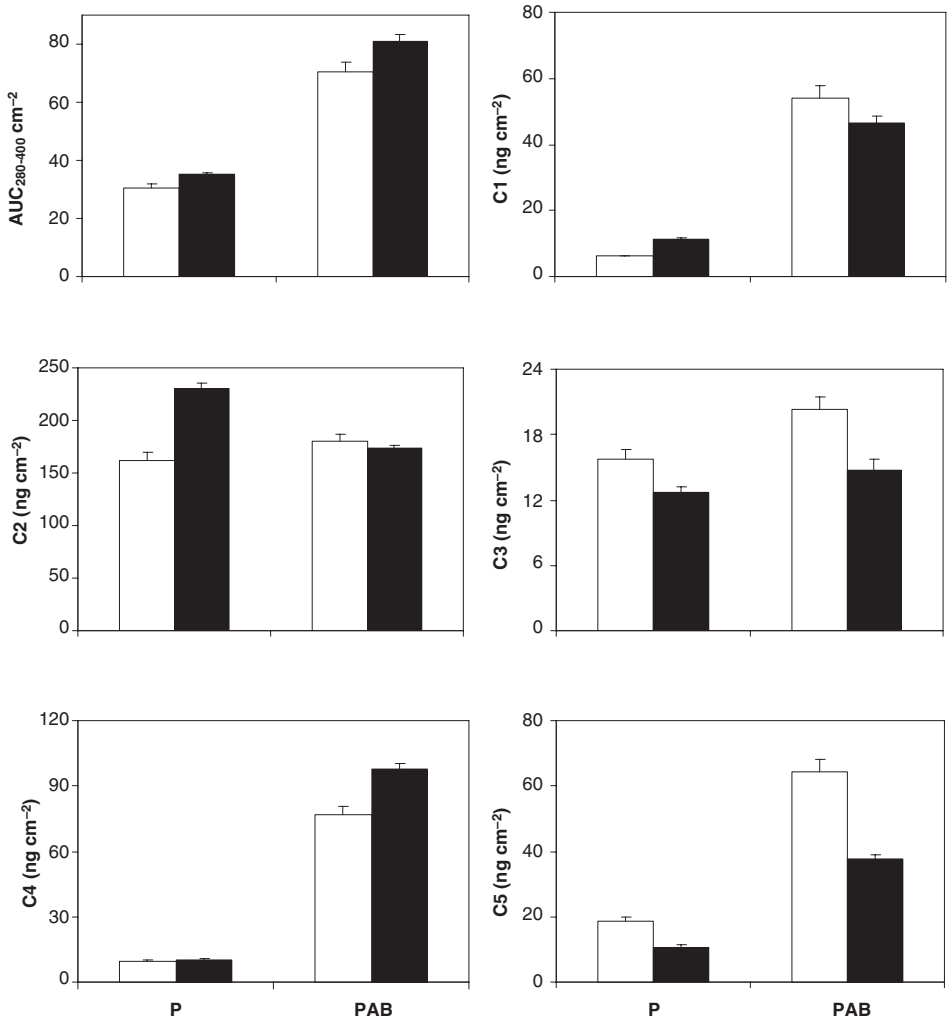

Fig. 3. Differences between old shoots (open bars) and young shoots (filled bars) in the bulk absorbance of methanolic extracts of *Jungermannia cordifolia* ($AUC_{280-400}$) and in the concentration of the five individual UV-absorbing compounds analyzed (C1-C5: see Fig. 1 and Materials and Methods) after 82 days growing under control (P: PAR only) and UV treatment (PAB: PAR + UV-A + UV-B). Means (+ SE) are shown. Global and paired statistical analyses as indicated in Table 1.

this liverwort did not show any UV caused damage. At the end of the culture period, F_v/F_m values were around 0.65 and 0.70 for old and young shoots respectively – independently of the radiation regime – (Fig. 2). This chlorophyll fluorescence variable has been used extensively as a stress index, since it decreases under different adverse conditions (Maxwell & Johnson, 2000), so these values indicated a good physiological state of both segments in both control and UV-treated plants. Moreover, increases in chlorophyll and carotenoid concentrations

and the bulk amount of MEUVAC ($AUC_{280-400}$) were also observed under the UV treatment (Figs 2-3). These results were expected since the liverwort responded in a similar way in previous studies under controlled conditions (Martínez-Abaigar *et al.*, 2003; Núñez-Olivera *et al.*, 2004). Additionally, beneficial effects of UV radiation have been previously reported in several bryophytes. UV-B exposure caused an increase in quantum yield (based on chlorophyll fluorescence measurements) and maximum net photosynthesis in *Hylocomium splendens* (Schipperges & Gehrke, 1996), and stimulated growth in different bryophyte species (Johanson *et al.*, 1995; Searles *et al.*, 1999; Phoenix *et al.*, 2001).

Sensitivity to a given stress factor can vary with the age of the tissue (Housti *et al.*, 2002). In the UV-tolerant liverwort studied, this different sensitivity was detected by an increase in the physiological activity in the young shoots and changes in UV protection mechanism, so that UV radiation may not be properly a stress factor for this liverwort, that rather displays an acclimation response to the new conditions of enhanced UV. In this respect, newly grown shoots exposed to UV radiation showed the highest concentration of chlorophylls, carotenoids and the bulk amount of MEUVAC ($AUC_{280-400}$). However, the individual HCM acids that form the bulk UV-absorbing compounds did not respond uniformly and thus different accumulation and distribution were observed along the liverwort profile.

In plants, the accumulation of HCM acid derivatives seems to play a role as a cellular protection mechanism in response to stress factors in general (Housti *et al.*, 2002). However, the specific accumulation of certain HCM in response to a particular environmental factor has been also reported, for example for wounding and salicylic acid stress effects (Housti *et al.*, 2002) and UV-B radiation exposure (Arróniz-Crespo *et al.*, 2006; Arróniz-Crespo *et al.*, 2008). In our study, three compounds (C1, C4 and C5) showed a remarkable and specific increase after UV exposure in both young and old parts of the shoots. C3 increased slightly in UV-exposed old parts but not in young parts. The main compound C2 was not affected by UV radiation in old shoots but its concentration was reduced in young shoots under UV. All these changes would appear to be a metabolic shift in compound composition. In this respect, the five compounds identified in *J. cordifolia* are all phenylpropanoids – secondary metabolites biosynthesized from the amino acid phenylalanine – and so, they all are present in the phenylpropanoid metabolic pathway. In particular, phenylalanine is first converted to cinnamic acid and subsequently to coumaric acid. Then, coumaric acid can act as a precursor for the biosynthesis of caffeic acid and ferulic acid derivatives (similar to our C2 and C3 compounds) in one direction, and to coumarins -such as compounds C4 and C5- in another direction (SRI International, 2007).

These two different metabolic pathways could be distinguished in the present study, given that UV exposure did not cause a clear stimulation of C2 and C3 but significantly induced the accumulation of C1 and the two coumarins C4 and C5. This metabolic shift may indicate different compounds function. C1, C4 and C5 may act as a protection mechanism against UV radiation, whereas C2 and C3 might have other physiological functions. In this sense, caffeic acid and ferulic acid derivatives are intermediates in the biosynthesis of the lignin precursors in higher plants (Van Doorsselaere *et al.*, 1995) and may have similar function in bryophytes where they have been found bound to the cell walls (Asakawa, 1995), so these compounds may be important structurally as precursors of lignin-like molecules. The decrease in C2 concentration in young shoots under UV could be due to the formation of another compound from C2. In this case, the identity of

that compound is presently unknown, given that C3, a further step in the metabolic pathway after C2, did not increase in young shoots under UV. C3 increased in old shoots under UV, and it could be suggested that C2 might be transported downwards along the liverwort profile. However, the limited transport capacity of foliose liverworts (Raven, 2003) makes this possibility highly improbable. Further investigation is needed to establish both the unequivocal links between the different compounds analyzed and their respective functions, and these issues cannot be concluded from the present study.

The main chemical functions of glycosylation processes are stabilization, storage, detoxification and solubilization of the substrates (McNally *et al.*, 2002; Ko *et al.*, 2006). It is possible that glycosylation also decreases the antioxidant capacity of phenolic compounds, however this may depend on the glycosylation position (Braham *et al.*, 2005). In our study C5 was the only glycosylated compound. It was significantly induced by UV radiation and was also affected by the age of the tissue since concentrations were always higher in old segments than in young ones. These results suggest that glycosylation may serve to stabilize and to store compound C5 in the old tissue.

It was interesting to observe that there was a close relationship between the different physiological variables and the effects of the tissue age or radiation treatment. In this respect, and under a global perspective (Table 1, global statistics test), some variables were better associated to the age of the tissue, such as SI, F_v/F_m and C2 and C3 concentrations, whereas MEUVAC ($AUC_{280\text{-}400}$), C1, C4 and C5 concentrations showed remarkable increases due to UV exposure. This distinction may be important when particular indicators of the tissue age, or the bioindication of UV radiation, are considered. SI and F_v/F_m may have a special interest as indicators of tissue age, given that low SI and high F_v/F_m values indicate, respectively, higher softness and higher physiological activity (Martínez-Abaigar *et al.*, 2003), and both characteristics were clearly typical of young shoots. The variables related to tissue age could have been influenced by the canopy structure of the liverwort mat, since young shoots received higher irradiances than old shoots. However, this influence seems to be improbable because, although the liverwort grew well under the experimental conditions applied, the production of young shoots was not enough to provide a significant shade on the old parts.

To conclude, this study has shown that young shoots of the liverwort *J. cordifolia* are well adapted to increased UV radiation and even show higher physiological activity than old shoots under these conditions (as indicated by higher values of F_v/F_m and higher chlorophyll concentrations). UV radiation caused a different accumulation and distribution of the individual HCM derivatives studied, indicating a possible metabolic shift to compounds with UV protection functions. Thus, *p*-coumaroylmalic acid and the two coumarins (C4 and C5) seem to act as a protection mechanism against UV in this liverwort. Our results are directly related to the experimental conditions applied, and thus the ecological consequences of a hypothetic UV enhancement in nature on the populations of *J. cordifolia* are difficult to predict. However, our study suggests that both old and young shoots of this liverwort have a good series of protecting compounds to cope with increased levels of UV radiation.

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