



Research article

Phenolic compounds from different bryophyte species and cell compartments respond specifically to ultraviolet radiation, but not particularly quickly[☆]

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ABSTRACT

To study the potential quick responses to ultraviolet (UV) radiation of bryophyte phenolic compounds, we cultivated two thalloid liverworts, two leafy liverworts, and two mosses under three moderate realistic UV levels in the laboratory for 22 days. At the end of the daylight period on the first and last culture days, we measured the bulk levels and individual contents of phenolic UV-absorbing compounds (UVACs) of each species, differentiating in both cases the UVACs located in the methanol-soluble (mainly vacuolar) and -insoluble (cell wall-bound) fractions (SUVACs and IUVACs, respectively). The bulk levels of SUVACs and IUVACs mostly showed linear or hyperbolic relationships with the UV dose applied. Thirteen flavones (apigenin and luteolin derivatives) and two hydroxycinnamic acids (*p*-coumaric and ferulic acids) were identified in the soluble and insoluble fractions, respectively. Only two compounds (*p*-coumaric and ferulic acids) from the insoluble fraction of the leafy liverwort *Plagiochila asplenioides* showed a significant quick accumulation in response to UV radiation in the first day of culture, whereas six UVACs (mainly soluble apigenin and luteolin derivatives) from different species (mainly liverworts) were significantly accumulated at the end of the culture. In conclusion, the responses of bryophyte UVACs to UV radiation were influenced by the specific compound considered, the fraction in which each UVAC was located, the global or individual way of UVACs quantification, the bryophyte species and evolutionary lineage, and the experimental conditions used. Particularly, SUVACs were more UV-responsive than IUVACs and liverworts than mosses, and responses were not especially quick.

1. Introduction

Ultraviolet (UV) radiation is a noteworthy environmental factor influencing photosynthetic organisms. It has traditionally been considered as a harmful factor because of the diverse physiological damage that a UV excess can produce on photosynthetic organisms (Jansen et al., 1998). However, more recently, UV radiation is rather contemplated as a general regulator inducing a number of acclimation responses in the plant (Jansen and Bornman, 2012). The discovery of the UV specific photoreceptor UVR8 (Jenkins, 2009) has much contributed to consolidate this perspective. Both UV-B (280–315 nm) and UV-A (315–400 nm) wavelengths reach the Earth's surface, but UV-B effects have been more studied due to the relationship between UV-B and the stratospheric ozone depletion (Bais et al., 2015). Nevertheless,

UV-A effects on plants are important and have been recently reviewed (Verdaguer et al., 2017).

The effects of UV on photosynthetic organisms have mainly been studied in marine algae, crop plants and the model plant *Arabidopsis thaliana* (Martínez-Abaigar and Núñez-Olivera, 2011). UV responses have been much less studied in bryophytes, structurally simple and evolutionarily important plants that are considered the earliest diverging embryophytes and the first “true” plants colonizing land (Bowman et al., 2016). This relative scarcity of studies can be due to their limited contribution to the total biomass of the planet, with the important exception of peatland mosses, mainly *Sphagnum* (Vanderpoorten and Goffinet, 2009). Yet, it is interesting to know how bryophytes respond to UV radiation because these plants were the first embryophytes facing high UV levels during land colonization, in comparison with the lower

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levels present in the ancestral aquatic environment. Thus, considerable research has been performed on the responses of bryophytes to UV radiation (Newsham and Robinson, 2009; Martínez-Abaigar and Núñez-Olivera, 2011; Robinson and Waterman, 2014). Research has focused mainly on bryophytes (particularly mosses) from Antarctic habitats and circumpolar heathlands and peatlands, and the results obtained have been diverse, since UV has been found to stimulate, depress, or have no effect on the bryophyte performance. To a certain extent, this may have been caused by the diversity of species and experimental conditions used in the different studies. Globally, the responses of bryophytes to UV radiation and their protective systems are still poorly characterized, and thus further study is required under both controlled and field conditions.

The most frequent acclimation response of plants, including bryophytes, to increased UV radiation is the accumulation of UV-absorbing compounds (UVACs), mainly of phenolic nature (Searles et al., 2001; Newsham and Robinson, 2009). Regarding this point, many studies conducted on bryophytes and UV radiation have considered the responses of the bulk levels of UVACs, either measuring the absorbance of methanolic extracts at a specific wavelength in the UV range, or quantifying the area under the absorbance curve in this same range (Martínez-Abaigar and Núñez-Olivera, 2011). However, little attention has relatively been paid to: a) the responses of individual UVACs to UV radiation; and b) the responses of UVACs located in different cell compartments. UVACs compartmentation can determine their functions as UV screens and/or antioxidants (Agati et al., 2012), but cannot be assessed by the mostly used simple methanol extraction, which exclusively renders soluble (mainly vacuolar) but not insoluble cell wall-bound UVACs (Clarke and Robinson, 2008).

Another understudied topic in relation to bryophytes and UV is whether bryophytes may show quick responses to UV as tracheophytes do (Barnes et al., 2016a, 2016b). In this regard, several experiments have been conducted under both controlled and field conditions (Conde-Álvarez et al., 2002; Newsham et al., 2002, 2005; Newsham, 2003; Fabón et al., 2012a). However, inconclusive results, both positive and negative, have been obtained, and quick responses of individual UVACs located in the soluble and insoluble fractions only rarely have been measured (Fabón et al., 2012a).

Our aim was to study the responses of bryophyte UVACs to moderate realistic levels of UV radiation, considering important aspects such as: a) the responses of not only the bulk levels of UVACs, but also those of individual UVACs; b) the responses of UVACs located in both the soluble and insoluble fractions of the methanolic extracts; and c) the quick and delayed responses of UVACs. We used six species of diverse structure and taxonomic position (thalloid and leafy liverworts, and mosses) to have a wider perspective on the effects of UV on bryophytes.

2. Materials and methods

2.1. Plant material and experimental design

Six bryophytes were used for this study: two thalloid liverworts (one with a simple thallus, *Pellia endiviifolia* (Dicks.) Dumort., and one with a complex thallus, *Marchantia polymorpha* L. subsp. *ruderalis* Bischl. & Boisselier), two leafy liverworts (one with simple leaves, *Plagiochila asplenioides* (L.) Dumort., and one with plicate leaves composed of two parallel lobes, *Porella arboris-vitae* (With.) Grolle), and two mosses (*Cinclidotus fontinaloides* (Hedw.) P. Beauv. and *Fissidens grandifrons* Brid.). Samples were collected on 21 March 2013 at different localities in La Rioja (northern Spain), between 378 and 1117 m altitude (approximate latitude and longitude of the sampling points were 42° 19' N and -2° 30' E, respectively). The material was transported to the laboratory in a portable icebox. Green healthy shoot apices were selected, rinsed and precultured in a circulating bath system filled with air-bubbled stream water (pH 6.8, conductivity 21 $\mu\text{S cm}^{-1}$) in a growth chamber. Samples grew floating at the water surface, and thus were

continuously wet to prevent the interference of desiccation on the results. Plants were maintained at 10 °C with a 11:13 photoperiod (light:dark) for 45 days (acclimation period). The photosynthetically active radiation (PAR) was around 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) at the water surface (LI-190SA quantum sensor, LI-COR, Lincoln, NE, USA), and was provided by Sylvania Coolwhite (Osram-Sylvania, Madrid, Spain) lamps.

After the acclimation period, apices were distributed into 18 separate transparent plastic tubes of 12 cm of diameter (three tubes for each species) provided with a basal net to prevent material losses. PAR radiation was supplied by True-Lite Industrial F40T12/TL full spectrum fluorescent tubes (True Sun, Steubenville, OH, USA) and a Hönle SOL 1200RF2 lamp (Dr. Hönle AG UV-Technologie, Gräfelfing, Germany), and this last lamp also provided UV radiation (for spectral characteristics, see Gröniger et al., 1999). The day was divided in four different periods: the darkness period, the first two-hour-long low-PAR period (in which only fluorescent tubes were switched on, providing around 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$), the seven-hour-long high-PAR plus UV period (in which both fluorescent tubes and Hönle lamp were switched on), and a second two-hour-long low-PAR period (after switching off the Hönle lamp). After this last period, the fluorescent tubes were switched off and the darkness period started again. Plants were exposed to three different conditions of biologically effective UV radiation (UV_{BE}) by covering the tubes with specific UV cut-off filters (for transmittance spectra, see Gröniger et al., 1999):

- Low UV_{BE} (L), using Ultraphan 395 (DigeFra GmbH, Munich, Germany), which eliminated 5% PAR and 77.6% UV_{BE}
- Medium UV_{BE} (M), using Folex 320 (Folex GmbH, Dreieich, Germany), which eliminated 8% PAR and 48.5% UV_{BE}
- High UV_{BE} (H), using Ultraphan 295 (DigeFra GmbH, Munich, Germany), which eliminated 4.5% PAR and 11.6% UV_{BE}

One tube was set-up for each species and UV conditions. Each tube was internally divided into three different sectors using vertical partitions, each sector representing a replicate. The plants under the L, M and H regimes received a daily UV_{BE} dose of 4.28, 9.83 and 16.88 kJ m^{-2} , respectively, calculated using the action spectrum of Flint and Caldwell (2003). The daily UV_{BE} dose in the sampling localities around the collection day, estimated on the basis of the UV measurements taken at Valdezaray (approximately 35 km away from the sampling sites: Núñez-Olivera et al., 2009), was 14.77 kJ m^{-2} . The daily PAR doses received by the plants under the three radiation regimes were similar (2451–2540 kJ m^{-2}). The spectral irradiances for each condition were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingston, Scotland), and PAR was measured with a quantum sensor. The filters were pre-irradiated and replaced every week. Cultures were maintained for 22 days and water in the culture was replaced every week.

2.2. Physiological measurements

Maximum (F_m) and minimum (F_0) chlorophyll fluorescence values were daily measured before the first low-PAR period with a portable pulse amplitude modulation fluorometer (MINI-PAM, Walz, Effeltrich, Germany), following Núñez-Olivera et al. (2004). Then, the maximum quantum yield of PSII (F_v/F_m) was determined, where $F_v = F_m - F_0$.

UV-absorbing compounds (UVACs) were analysed following Fabón et al. (2010) at the end of the high-PAR plus UV period on the first and last days of culture. In brief, fresh shoot apices were frozen in liquid N_2 and ground in a TissueLyser (Qiagen, Hilden, Germany). Then 2 ml of methanol: water: 7M HCl (70:29:1 v/v/v) was added for extraction (24 h at 4 °C in the dark). The extract was centrifuged to differentiate two UVACs fractions: the methanol-soluble UVACs (SUVACs) in the supernatant and the methanol-insoluble UVACs (IUVACs) in the pellet. Subsequently, the pellet was subjected to alkaline digestion to extract

the insoluble compounds. Presumably, SUVACs are mainly located in the vacuoles whereas IUVACs are bound to the cell walls (Clarke and Robinson, 2008). Then, we measured the bulk levels of SUVACs and IUVACs as the area under the absorbance curve of each fraction in the interval 280–400 nm ($AUC_{280-400}$), using an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

Individual phenolic compounds were analysed by ultra-performance liquid chromatography (UPLC) using a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA). Solvents were: A, water/formic acid (0.1%), and B, acetonitrile with 0.1% formic acid. The gradient program employed was: 0–7 min, 99.5–80% A; 7–9 min, 80–50% A; 9–11.7 min, 50–0% A; 11.7–15 min, 0–99.5% A. The UPLC system was coupled to a microTOF II high-resolution mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an Apollo II ESI/APCI multimode source and controlled by the Bruker Daltonics DataAnalysis software. The electrospray source was operated in negative mode. The capillary potential was set to 4 kV; the drying gas temperature was 200 °C and its flow 9 l min⁻¹; the nebulizer gas was set to 3.5 bar and 25 °C. Spectra were acquired between m/z 120 and 1505 in negative mode. To quantify the different compounds, the following standards were used: apigenin, luteolin, coumaric acid and ferulic acid (Sigma-Aldrich, St. Louis, MO, USA).

The bulk levels of UVACs and the contents of individual UVACs were expressed per dry mass (DM) and surface area (LI-3000 area meter, LI-COR, Lincoln, NE, USA) of the bryophyte shoots. Given that both expressions rendered similar results, only the results per surface area will be shown.

2.3. Statistical analysis

Once tested the normality (Shapiro–Wilk's test) and homoscedasticity (Levene's test) of the data, the global effects of species, exposure time and UV_{BE} conditions on the variables that were measured in the six species were tested using a 3-way analysis of variance (ANOVA). Given that the global effect of species was very significant, the global effects of exposure time and UV_{BE} conditions, and their interactions, were separately tested for each variable of each species using 2-way ANOVAs. In addition, the specific global effect of UV_{BE} conditions on every measured variable for each sampling day was tested (1-way ANOVAs). In the case of significant effects, means were then compared by Tukey's test. Finally, regressions between the bulk levels of UVACs, both SUVACs and IUVACs, and UV_{BE} dose were conducted. All the statistical procedures were performed with SPSS 24.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

F_v/F_m values depended on the species. The highest values were found in *M. polymorpha* (0.743–0.776), whereas the lowest values were found in *P. arboris-vitae* (0.650–0.686). The global effects of exposure time and UV_{BE} conditions on F_v/F_m were not significant in any species.

The absorbance spectra of the methanolic extracts in the UV range in the six species studied showed different shapes depending on the species and the fraction considered (Fig. 1). In the insoluble fraction, absorption maxima were not clearly defined except for *F. grandifrons*, which showed maxima between 289 and 316 nm. The remaining species showed regularly increasing absorbances towards shorter wavelengths, without showing any defined maxima. In the soluble fraction, the location of maxima differed between liverworts and mosses. Liverworts generally showed well-defined maxima around 324–340 nm, whereas mosses showed almost flat spectra, although with slightly increasing absorbances towards shorter wavelengths (and even a little defined maximum at 290 nm for *F. grandifrons*). In mosses, the bulk levels of IUVACs were higher than those of SUVACs, whereas in liverworts it depended on the species (Table S1). *Porella arboris-vitae* showed

a similar pattern to mosses, *P. endiviifolia* and *P. asplenioides* showed the opposite pattern, and *M. polymorpha* showed almost equal levels of SUVACs and IUVACs.

Regarding the best regressions found between the bulk levels of UVACs, both SUVACs and IUVACs, and UV_{BE} dose, no regression was significant in *P. arboris-vitae* at the $p < 0.05$ level (Fig. 2). For the remaining species, either hyperbolic (saturation) or linear models showed the highest determination coefficients (R^2) and, concordantly, the lowest p values. The best regressions were generally obtained in liverworts (except *P. arboris-vitae*), where R^2 was in the range 0.877–0.976. In the two mosses, regressions were notably significant, but lower R^2 values were generally found (0.682–0.934). The bulk levels of both SUVACs and IUVACs showed a linear fitting in one liverwort (*P. asplenioides*) and the two mosses, and fitted to a hyperbolic curve in two liverworts (*P. endiviifolia* and *M. polymorpha*).

A total of 15 individual UVACs were identified (Table S1). Thirteen of them were flavones from the soluble fraction: apigenin, apigenin 7-O-glucuronide, apigenin 7,4'-di-O-glucuronide, apigenin 6,8-di-C-hexoside, luteolin, luteolin 7-O-glucuronide, luteolin 3'-O-glucuronide, luteolin 7,3'-di-O-glucuronide, luteolin 7,4'-di-O-glucuronide, luteolin 6,8-di-C-hexoside I, luteolin 6,8-di-C-hexoside II, luteolin 6-C-xyloside-8-C-glucoside, and luteolin 6-C-glucoside-8-C-xyloside. The remaining two UVACs were hydroxycinnamic acids from the insoluble fraction: *p*-coumaric and ferulic acids. The most diverse species regarding UVACs were *M. polymorpha* and *P. asplenioides* (Table S1). The major compounds in *M. polymorpha* at the beginning of the experiment were the soluble apigenin, apigenin 7-O-glucuronide and luteolin 7,3'-di-O-glucuronide, whereas the major compounds in *P. asplenioides* were the insoluble ferulic acid and the soluble apigenin 6,8-di-C-hexoside. The least diverse species regarding UVACs were the liverwort *P. endiviifolia* and the two mosses studied, where one only compound (*p*-coumaric or ferulic acids) was found.

The overall effect of exposure time on UVACs was mostly significant, whereas the influence of the UV_{BE} conditions was significant only on half of the compounds (Table S1). The most responsive species to UV_{BE} conditions were *P. asplenioides*, *P. arboris-vitae* and *F. grandifrons*, where most compounds were significantly affected by this factor. The least responsive species to UV_{BE} conditions were *C. fontinaloides* and *P. endiviifolia*. In *M. polymorpha*, most compounds showed an increasing trend (significant or not) with increasing UV_{BE}. Interactions between exposure time and UV_{BE} dose were significant on around half of the compounds, particularly in *P. asplenioides*, *P. arboris-vitae*, *C. fontinaloides* and *F. grandifrons*.

At the end of the first day of the culture period, only the contents of *p*-coumaric and ferulic acids in *P. asplenioides* increased significantly with increasing UV_{BE} levels (Table S1). The bulk levels of IUVACs in *P. endiviifolia*, and the bulk levels of SUVACs and IUVACs in *M. polymorpha*, showed a similar but non-significant trend.

At the end of the last day of the culture period, six individual UVACs showed significantly higher contents under higher UV_{BE} doses (Fig. 3). Three of them were found in *P. asplenioides* and the remaining three (one compound for each species) in *M. polymorpha*, *P. arboris-vitae* and *F. grandifrons*. Except the insoluble *p*-coumaric acid in *F. grandifrons*, the responsive compounds were soluble: luteolin 7-O-glucuronide in *M. polymorpha*, and 6,8-di-C-hexoside derivatives of apigenin or (mainly) luteolin in *P. asplenioides* and *P. arboris-vitae*. In most cases, the contents only increased under the highest UV_{BE} level (H regime). This was the only regime under which the samples received the shortest UV wavelengths (both UV-B and UV-A). Similar significant changes were found in the bulk levels of SUVACs in *P. endiviifolia*, *P. asplenioides* and *F. grandifrons*, and the bulk levels of IUVACs in *M. polymorpha*, *P. asplenioides* and *F. grandifrons* (Table S1). In addition, similar but non-significant trends were found in the bulk levels of SUVACs in *M. polymorpha*, the contents of *p*-coumaric acid in *P. asplenioides* and ferulic acid in *C. fontinaloides*, and the contents of several individual compounds in *M. polymorpha*, both soluble (apigenin 7-O-glucuronide,

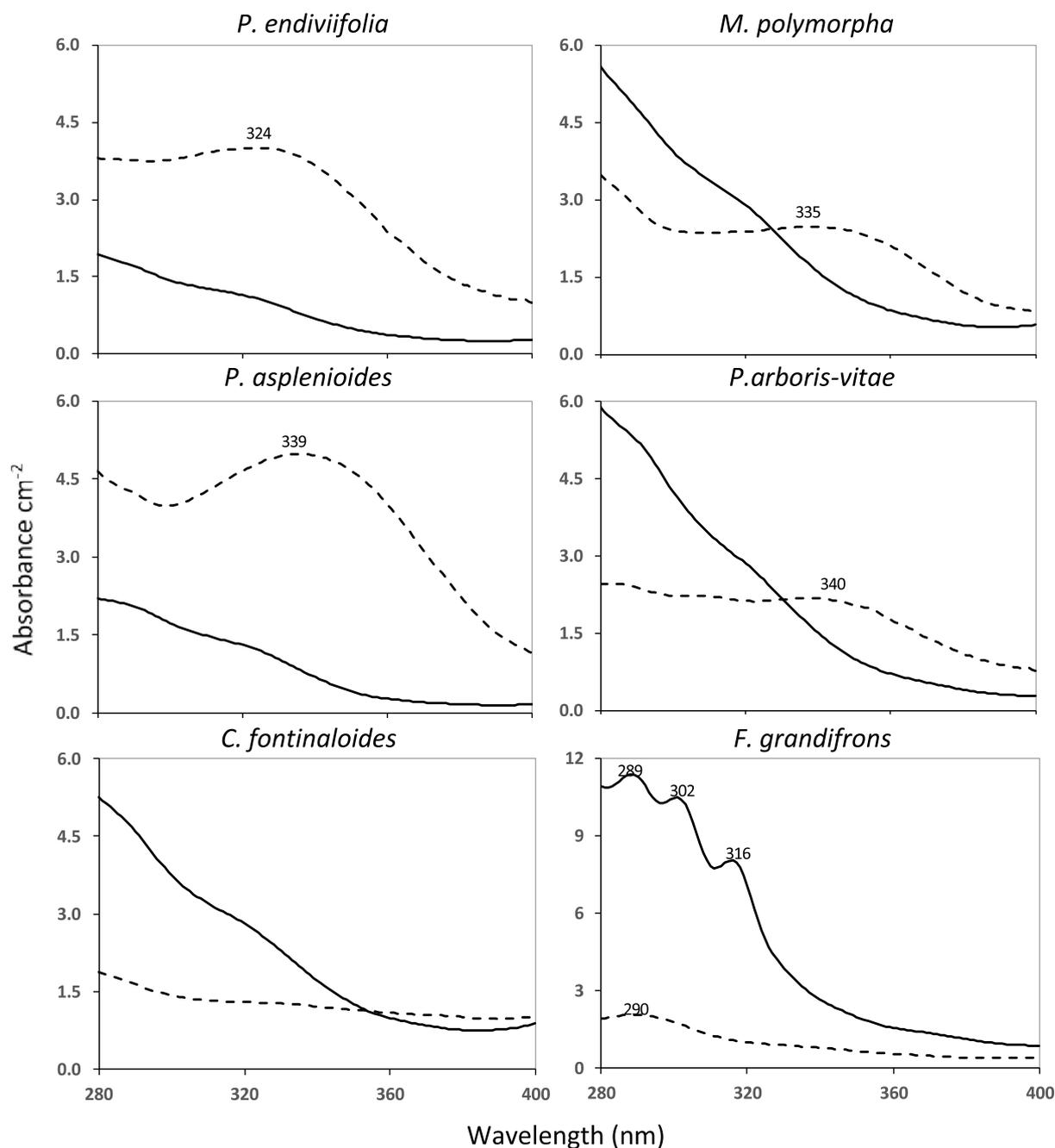


Fig. 1. Representative absorbance spectra of methanol extracts between 280 and 400 nm for the six studied species (*Pellia endiviifolia*, *Marchantia polymorpha*, *Plagiochila asplenioides*, *Porella arboris-vitae*, *Cinclidotus fontinaloides* and *Fissidens grandifrons*), differentiating the soluble (dashed line) and insoluble (solid line) fractions. Absorption maxima are shown when possible.

luteolin 3'-*O*-glucuronide, luteolin 7,3'-*di-O*-glucuronide and luteolin 7,4'-*di-O*-glucuronide) and insoluble (*p*-coumaric and ferulic acids) (Table S1).

4. Discussion

Although F_v/F_m varied among the species studied, it was not affected by exposure time or UV_{BE} conditions in any species. Given that F_v/F_m can be considered as an indicator of general physiological vitality (Maxwell and Johnson, 2000), these findings would mean that the culture conditions and UV_{BE} levels used in the present study were well tolerated by the plants. This could be expected because the highest level used was close to those received by the plants in their natural locations.

The two mosses had higher absorbances in the insoluble than the

soluble fraction of the methanolic extracts in the UV range, whereas two liverworts (*P. endiviifolia* and *P. asplenioides*) showed an inverse pattern and *M. polymorpha* showed almost equal absorbances in both fractions. This is congruent with previous data obtained in those species (Monforte et al., 2018), suggesting that mosses would be better protected than liverworts against UV radiation, because insoluble cell wall-bound compounds constitute a more efficient UV screen than soluble vacuolar compounds (Clarke and Robinson, 2008). Nevertheless, soluble phenolic UVACs may act as antioxidants (Agati et al., 2012), being also protective against excessive UV. *Porella arboris-vitae* represented a particular case within liverworts because its higher absorbance in the insoluble than the soluble fraction (probably due to cell wall-bound compounds conferring its bronze colour) puts this species closer to mosses (Monforte et al., 2018).

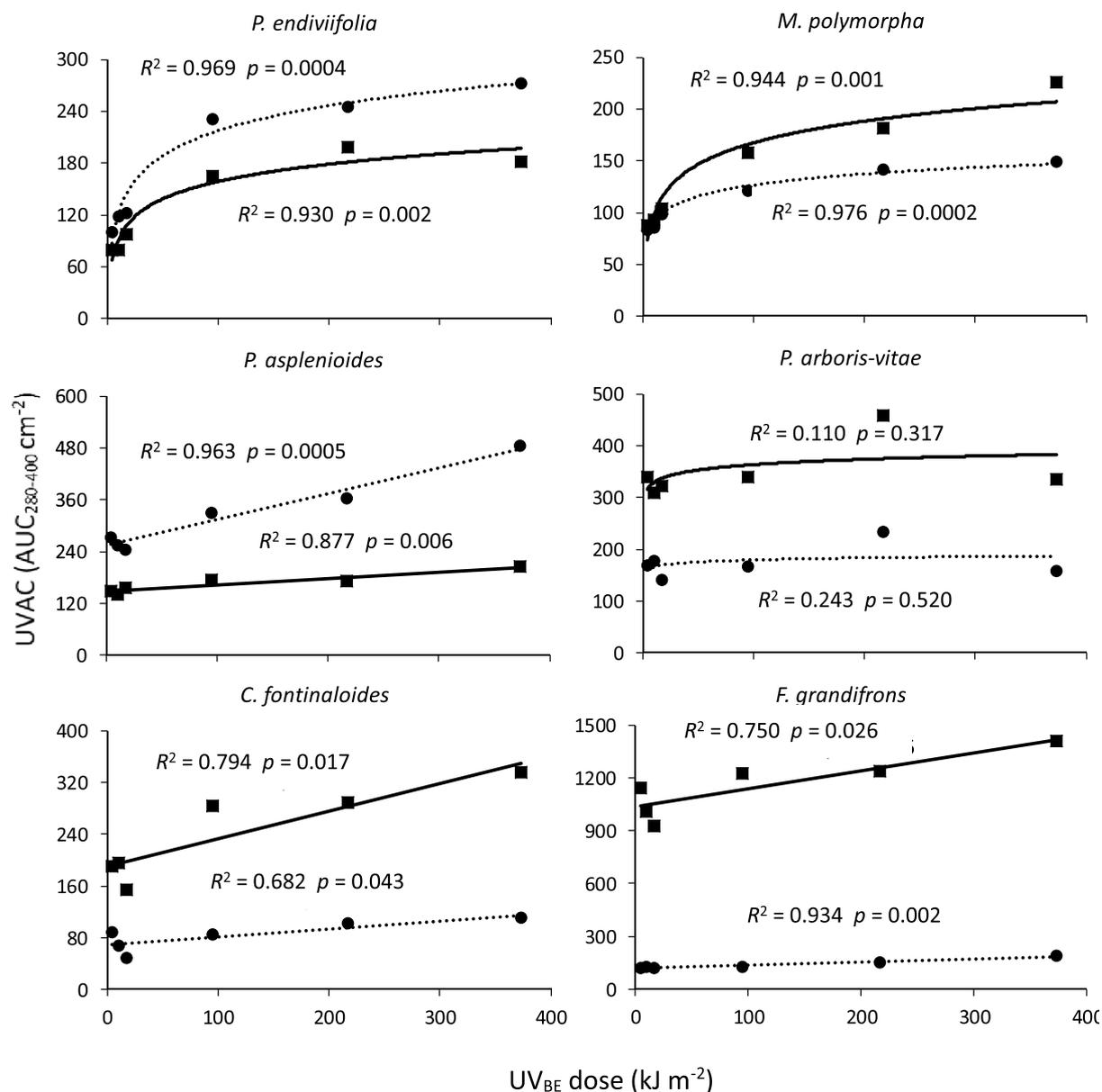


Fig. 2. Regressions between the bulk levels of UV-absorbing compounds (UVAC), both soluble (dotted line) and insoluble (solid line), and the total dose of biologically effective UV radiation (UV_{BE}) received by the samples of the six studied species (*Pellia endiviifolia*, *Marchantia polymorpha*, *Plagiochila asplenioides*, *Porella arboris-vitae*, *Cinclidotus fontinaloides* and *Fissidens grandifrons*). The best fits are shown, together with determination coefficients (R^2) and p values. UVAC levels of each fraction were measured as the area under the absorbance curve in the interval 280–400 nm (AUC_{280–400}) per surface area unit. Each point represents the mean of three replicates. Error bars are not shown for clarity reasons, but errors are shown in Table S1.

Absorbance spectra were generally consistent with the individual compounds identified in each fraction of each species (Table S1). In the insoluble fraction, cinnamic acids (with absorption maxima between 285 and 330 nm; Waterman and Mole, 1994) would predominate in every species, giving higher absorbances towards shorter wavelengths, although defined maxima were only found in *F. grandifrons*. This fact agrees with the presence of ferulic and/or *p*-coumaric acids, and the absence of flavonoids, in the insoluble fraction of every species. In the soluble fraction of liverworts, the absorption maxima found matched well with the presence of different flavones (apigenin and luteolin derivatives) in most species. Flavones have three bands of maximum absorption in the range 245–350 nm, with a clearly defined maximum in the 330–350 region (Waterman and Mole, 1994). Mosses hardly showed defined absorption maxima in the soluble fraction, which is congruent with the fact that no individual compound was identified in

this fraction.

Highly significant linear or hyperbolic regressions between the bulk levels of UVACs and UV_{BE} doses were found in most species. This was somewhat surprising, because this kind of global variables may not always respond consistently to UV radiation in bryophytes, given that each compound contributing to the bulk absorbance can respond in a different manner (Martínez-Abaigar and Núñez-Olivera, 2011). Linear regressions, shown by one liverwort (*P. asplenioides*) and the two mosses, meant that the bulk levels of UVACs increased with a constant slope and did not reach a maximum in response to the UV_{BE} doses used in this study. In the hyperbolic (saturation) regressions, the initial increase was steeper than that found in linear regressions, but afterwards UVACs levels reached a plateau representing the limit of UV protection through UVACs accumulation. The different patterns found, which were not apparently related with the diversity of individual compounds

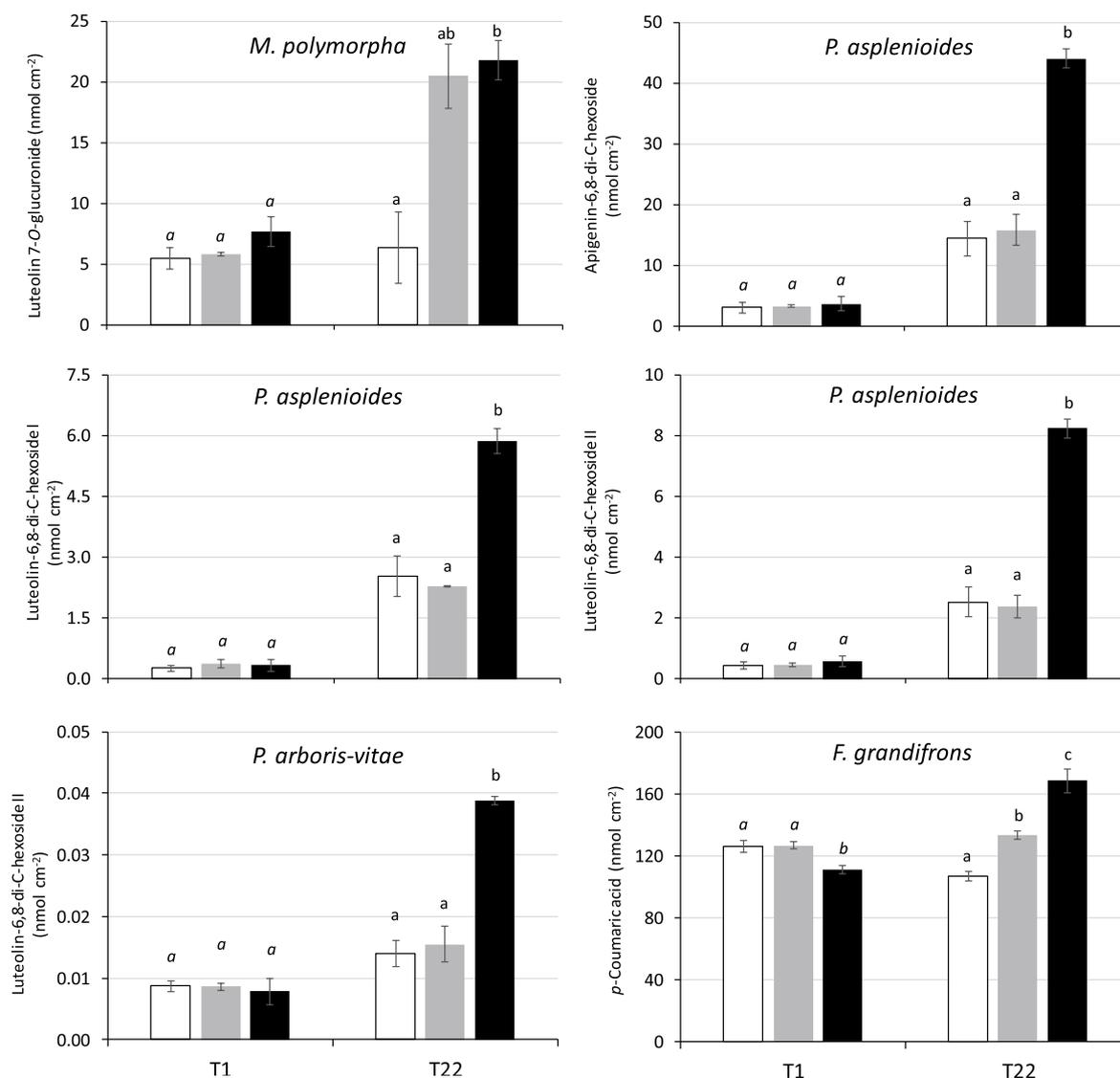


Fig. 3. Effects of exposure time (T1 and T22, first and last days of the culture period, respectively) and conditions of biologically effective UV radiation (UV_{BE} : low, white bars; medium, grey bars; high, black bars) on the contents of those individual UV-absorbing compounds that showed significantly higher contents under higher UV_{BE} doses in the last sampling day. Means \pm SE are shown. Different letters mean significant differences (at least $p < 0.05$) between UV_{BE} conditions for each sampling day separately (T1, italic letters; T22, roman letters).

identified for each species, strongly suggest that the responses of the bulk levels of UVACs to increasing UV_{BE} doses represented specific strategies of each bryophyte species. Thus, bryophytes should not be grouped as a single functional type with respect to UV effects (Martínez-Abaigar et al., 2003) and, particularly, to UVACs accumulation. In addition, the determination coefficients of the regressions were generally higher in liverworts (except *P. arboris-vitae*) than in mosses, suggesting that UVACs of liverworts are more UV-responsive than those of mosses (Fabón et al., 2010, 2012a, 2012b; Martínez-Abaigar and Núñez-Olivera, 2011). Liverwort species were clearly heterogeneous regarding UVACs accumulation in response to UV_{BE} doses. *Plagiochila asplenioides* showed a linear pattern of accumulation, whereas *P. endiviifolia* and *M. polymorpha* showed saturation patterns and *P. arboris-vitae* did not show any significant pattern. This exception can be attributable to the important constitutive accumulation of coloured IUVACs in this species (Monforte et al., 2018), which may mask any clear pattern of UVACs accumulation.

UVACs were more strongly affected by exposure time than by the UV_{BE} doses applied in the different treatments. This may be logical

considering that 22 days of exposure is a period long enough to induce UVACs (Arróniz-Crespo et al., 2008) in comparison to only one day of treatment. Nevertheless, the general lack of a quick response was surprising, because genes encoding key flavonoid biosynthesis enzymes can be induced in minutes (Jenkins, 2009; Soriano et al., 2018), and a quick accumulation of UVACs after a few hours of UV exposure had previously been found in bryophytes (Newsham et al., 2002, 2005; Newsham, 2003; Fabón et al., 2012a) and tracheophytes (Barnes et al., 2016a, 2016b). Yet, this quick response has not been found in every case in bryophytes (Conde-Álvarez et al., 2002; Fabón et al., 2012a). Hence, quick responses of UVAC to UV radiation in bryophytes may depend on the species and compound considered, as well as the UV dose applied and the associated wavelengths.

The only UVACs showing a significant quick accumulation in response to UV radiation were two insoluble hydroxycinnamic acids (*p*-coumaric and ferulic acids) of *P. asplenioides*, which also increased quickly in another leafy liverwort (*Jungermannia exsertifolia* subsp. *cordifolia*) under enhanced UV-B (Fabón et al., 2012a). Overall, IUVACs can be considered weakly UV-responsive in bryophytes because they

would be relatively immobilized in the cell wall, which might limit their reaction capacity. In addition, IUVACs seem to be more UV-responsive in liverworts than in mosses, because IUVACs of the moss species tested did not show any response (as in Fabón et al., 2012b).

More UVAC variables (up to six individual UVACs from different species, together with the bulk levels of SUVACs or IUVACs in four of the six species studied) significantly increased at the end of the 22nd day of culture. This response was especially found in liverworts and SUVACs, confirming their higher responsiveness as compared with mosses and IUVACs. UVACs accumulation particularly took place when samples were exposed to the highest UV_{BE} dose (Fig. 3), which also corresponded to the only radiation regime under which the samples received UV-B wavelengths. This suggests that the UV_{BE} threshold (and the wavelengths) needed to induce accumulation had been provided only under these specific conditions. This occurred with di-C-hexoside derivatives of luteolin and apigenin of *P. asplenoides* and *P. arboris-vitae*. There are no comparative data in the literature on the UV-induced accumulation of these compounds in bryophytes. Other compounds were accumulated with lower UV_{BE} thresholds, such as luteolin 7-O-glucuronide in *M. polymorpha*, whose content already increased under the Medium UV_{BE} dose. In this species, Markham et al. (1998) did not find any statistically significant increase in diverse apigenin and luteolin glucuronides with increasing UV-B levels, but they did find a strong positive correlation between UV-B levels and the ratio of luteolin to apigenin glycosides, presumably improving the level of antioxidant defence or the dissipation of absorbed UV energy. Thus, the specific accumulation of luteolin derivatives in *M. polymorpha* seems to be a protecting UV-response, but depends on the experimental conditions applied.

The only individual IUVAC increasing in response to increasing UV_{BE} at the end of the culture was *p*-coumaric acid of *F. grandifrons*. This compound, which is frequently found in the insoluble fraction of bryophytes (Rasmussen et al., 1995; Fabón et al., 2010, 2012a, 2012b; Liu et al., 2017; Table S1), increased under both the High and the Medium UV_{BE} doses, and also increased in the first day of culture in *P. asplenoides*. Although insoluble and thus potentially less UV-responsive than the soluble compounds, it was accumulated in another leafy liverwort (*Jungermannia exsertifolia* subsp. *cordifolia*) under enhanced UV in the laboratory (Fabón et al., 2010, 2012a), but not in the moss *Fontinalis antipyretica* (Fabón et al., 2012b). In tracheophytes, *p*-coumaric acid from cell walls has been found to be highly UV-responsive, and it has been used as a UV proxy (Rozema et al., 2001; Ruhland et al., 2005; Blokker et al., 2006; Lomax et al., 2008). Thus, species and experimental conditions may influence the accumulation of *p*-coumaric acid in response to UV, and further research is needed to fully understand these responses. The compounds unresponsive to the UV_{BE} doses applied in our study would probably need higher UV_{BE} doses to reach the thresholds to be accumulated, or would not be induced by UV at all.

The bulk levels of SUVACs and IUVACs responded quite well to the increasing UV_{BE} dose in the last day of culture, in line with the generally good regressions obtained between these variables and the UV_{BE} dose.

In conclusion, bryophyte UVACs showed different responses to UV radiation. Factors contributing to this variability were mainly 1) the specific compound considered and its associated threshold and velocity of response; 2) the soluble or insoluble fraction in which the UVACs were located; 3) the way in which UVACs were quantified, either globally or individually; 4) the species; 5) the evolutionary lineage of bryophytes (liverworts or mosses); and 6) the experimental conditions used, in particular the UV_{BE} dose applied and the associated wavelengths. The relatively moderate UV_{BE} levels used in our study were probably responsible for the lack of a clearer response in more compounds. Given that this mechanistic study was performed under controlled conditions, the results obtained cannot directly be extrapolated to the field.

Author contributions

ENO and JMA conceived the study and designed the experimental setup. GS, LM and MADCA collected, cultivated and analyzed the samples. GS and ENO analyzed the data. All authors discussed the results. JMA and ENO drafted the paper. All authors edited the paper and approved the final version.

Conflicts of interest

The authors declare no conflict of interest in the present research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.plaphy.2018.07.020>.

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