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First data on the effects of ultraviolet radiation on phenolic compounds in the model hornwort *Anthoceros agrestis*

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Abstract – Hornworts are the least species-rich bryophyte lineage, but represent a key group to understand the evolution of plants because, together with the remaining bryophyte lineages (mosses and liverworts), they constitute the earliest diverging land plants. The responses of hornworts to ultraviolet (UV) radiation are unknown, but they may be important to infer how primitive hornworts (and bryophytes in general) coped with UV upon land colonization. In this context, our aim was to show the first data on the effects of UV radiation on the accumulation patterns of phenolic UV-absorbing compounds (UVACs) in the emerging model hornwort *Anthoceros agrestis*. Thalli of 52 days age were exposed to photosynthetically active radiation (PAR) alone (P regime) and to a combination of PAR + UV-A + UV-B radiation (PAB regime) for 21 days, using realistic UV doses (equivalent to the natural ambient doses received in summer at mid-latitudes). At the end of the culture period, we measured the bulk levels and individual contents of phenolic UV-absorbing compounds (UVACs), differentiating in both cases the UVACs located in the methanol-soluble (mainly vacuolar) and -insoluble (cell wall-bound) fractions (SUVACs and IUVACs, respectively). Three soluble and one insoluble compounds were identified, among which the soluble rosmarinic and anthocerotonic acids are not present in any other bryophyte lineage. The bulk levels of SUVACs were higher than those of IUVACs, a physiological trait more typical of liverworts than of mosses. None of the variables measured responded significantly to UV exposure, but all of them showed an increasing trend under the PAB regime. Given that UV responsiveness of phenolic compounds depends on the UV levels used and the thallus age (with decreasing responsiveness as age increases), further research using higher UV levels and younger thalli should be conducted to more reliably establish the UV reactiveness of *Anthoceros agrestis*.

Bryophytes / hornworts / ultraviolet radiation / phenolic compounds / model species

INTRODUCTION

Ultraviolet (UV) radiation is a noteworthy environmental factor influencing photosynthetic organisms. It has traditionally been considered 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

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is rather considered as a general regulator inducing a number of acclimation responses in the plant (Jansen & Bornman, 2012). 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 radiation have been much less studied in bryophytes than in cormophytes, in accordance with the ecological relative importance of both plant groups. Among the three evolutionary lineages of bryophytes (mosses, liverworts and hornworts), mosses have been much more studied than liverworts (Martínez-Abaigar & Núñez-Olivera, 2011), and, curiously, no study has been carried out on hornworts yet. Hornworts (Division Anthocerotophyta: Goffinet & Shaw, 2009) are a minor group within bryophytes, comprising only 200-250 species worldwide (Villarreal *et al.*, 2010) in comparison with around 25.000 mosses and liverworts. Hornworts are characterized by a rosette-like thalloid gametophyte that harbors endosymbiotic cyanobacteria of the genus *Nostoc*, and sporophytes with potentially indeterminate growth because of a basal meristem. Interestingly, hornwort cells have one only algal-like chloroplast with a pyrenoid that contains the enzyme RuBisCO, and therefore exhibits a carbon concentration mechanism not seen in other land plants (Li *et al.*, 2017). These peculiar characteristics, together with the fact that bryophyte lineages are considered to be the earliest diverging land plants (Qiu *et al.*, 2007; Wickett *et al.*, 2014; Bowman *et al.*, 2016; Plackett & Coates, 2016), make hornworts an evolutionarily interesting group. Consequently, a model species for hornworts is emerging in recent years: *Anthoceros agrestis* (Szövényi, 2016). This species is a summer annual growing naturally on disturbed moist or wet, circumneutral or mildly base-rich soils: arable fields, marshy pastures, waste ground and ruts in woodland tracks (Paton, 1999). *Anthoceros agrestis* has been found in Europe (from Fennoscandia, Russia and the British Isles south to Bulgaria, France and Portugal), North America, northern Africa and Asia (Paton, 1999). Its distribution is incompletely known due to confusion with *A. punctatus* L.

The most frequent acclimation response of bryophytes to increased UV radiation is the accumulation of UV-absorbing compounds (UVACs), mainly of phenolic nature (Newsham & Robinson, 2009; Martínez-Abaigar & Núñez-Olivera, 2011). Bryophyte UVACs are usually measured globally by spectrophotometry, but much less attention has been paid to the analysis of individual compounds. This is important because each compound may respond in a different manner to UV, and these specific responses cannot be identified by merely measuring the bulk levels of UVACs. In addition, a simple methanol extraction exclusively renders the UVACs present in the soluble fraction of the extract (mainly located in the vacuoles), whereas the methanol-insoluble cell wall-bound compounds are overlooked. Yet, the location of UVACs in different cell compartments is crucial to properly interpret their diverse functions as, for example, UV screens and antioxidants (Agati *et al.*, 2012). In bryophytes, the cell wall-bound and vacuolar UVACs fractions may represent different modalities of UV tolerance, because the former constitutes a continuous more efficient UV screen than the latter (Clarke & Robinson, 2008). With respect to soluble compounds, they may have a preferential antioxidant function, especially when they are located in the nucleus or the chloroplast (Agati *et al.*, 2012).

Within the context described, the aim of the present study was to provide the first data on the effects of UV radiation on hornworts (specifically, in the model species *Anthoceros agrestis*). As response variables, we used the accumulation of UVACs, paying attention to both global and individual compounds, and also

differentiating the methanol-soluble and -insoluble fractions. Understanding the responses of hornworts to UV radiation is potentially useful to infer how they, as the rest of bryophytes, could cope with a new UV regime upon land colonization, because UV exposure in the terrestrial environments was higher than that present in the primordial aquatic habitats. In addition, our study can contribute to increase the physiological knowledge of an emerging model organism and its responses to environmental factors.

MATERIALS AND METHODS

Plant material and culture conditions

Thalli of the hornwort *Anthoceros agrestis* Paton (Oxford strain: Szövényi *et al.*, 2015) were cultivated in Petri dishes using ½ Gamborg's B5 medium in a growth chamber (Fitoclima 1200, Aralab, Portugal) under 22°C, 50% relative humidity and only photosynthetically active radiation (PAR, 60 μmol m^{-2} s^{-1}). After 52 days of growth, thalli were placed in a growth room at 22°C with 75% relative humidity and a 10:14 photoperiod (light:darkness). PAR, UV-A and UV-B radiations were provided, respectively, by LED-PAR tubes (LED T8 Tube, AOSZX Brilliant Crystal Co., Shenzhen, China), UV-A lamps (Actinic BL 40W RS, Philips, Amsterdam, Netherlands) and narrowband UV-B lamps (TL40W/01 RS UV-B Narrowband, Philips, Amsterdam, Netherlands). Two different radiation regimes (in three replicates) were imposed using different cut-off filters:

– P (only PAR), using XT Vitroflex 395 Solarium Incoloro (Polimer Tecnic, Girona, Spain), which cut off all UV radiation.

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

Table 1 shows the radiation conditions in the two regimes, including the biologically effective UV-B and UV irradiances ($UV\text{-}B_{BE}$ and UV_{BE}, respectively), which were calculated according to Caldwell (1971) and Flint & Caldwell (2003), respectively. The spectral irradiances were measured using a spectroradiometer (Macam SR9910, Macam Photometrics Ltd, Livingstone, Scotland). The plants

Table 1. Radiation conditions applied in the two radiation regimes under which the samples were cultivated: P (only photosynthetically active radiation, PAR) and PAB (PAR + UV-A + UV-B). Biologically effective UV-B radiation ($UV\text{-}B_{BE}$) and biologically effective UV radiation (UV_{BE}) were calculated on the basis of the action spectra by Caldwell (1971) and Flint & Caldwell (2003), respectively

	P	*PAB*
PAR (μmol m^{-2} s^{-1})	67.1	66.5
PAR (W m^{-2})	13.9	13.9
UV-A (W m^{-2})	0.06	3.27
UV-B (W m^{-2})	0.00	1.38
$UV\text{-}B_{BE}$ (W m^{-2})	0.00	0.17
UV_{BE} (W m^{-2})	0.00	0.19

under PAB regime received UV-B_{BE} and UV_{BE} daily doses of 6.21 and 6.92 kJ m^{-2}, respectively. These are realistic doses which should not cause any harm to the plants, given that, for example, the UV-B doses applied in our experiment were equivalent to the natural ambient doses received in summer at mid-latitudes (Giordano *et al.*, 2003; Häder *et al.*, 2007; Núñez-Olivera *et al.*, 2009). Plants were cultivated under these conditions during 21 days. In the last day of treatment, samples were collected at midday for measuring all the variables described below.

Physiological analyses

UV-absorbing compounds (UVACs) were analyzed following Fabón *et al.* (2010) and Monforte *et al.* (2015). In brief, thalli were frozen in liquid nitrogen and were ground in a TissueLyser (Qiagen, Hilden, Germany), and then 2 ml of methanol; water – 7-M 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 & 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 ($AUC_{280\text{-}400}$), corresponding to the absorbance in the UV-B plus UV-A ranges, using an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). $AUC_{280\text{-}400}$ was expressed per dry mass unit (DM, obtained after 24 h at 60°C). Individual phenolic compounds were analyzed 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. 7,8-Dihydroxy-4-phenylcoumarin was used as internal standard. Individual UVACs were expressed in peak area per DM unit.

Statistical analysis

For each physiological variable (the bulk levels of SUVACs and IUVACs, and the contents of the individual phenolic compounds identified), differences between the two radiation regimes were tested using Student's *t* test. The statistical procedures were performed with SPSS 24.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Four UVACs were identified in *Anthoceros agrestis*, and all of them were cinnamic acid derivatives. Three compounds were present in the soluble fraction: rosmarinic acid (the most abundant compound), anthocerotonic acid and caffeic acid. One additional compound (methyl caffeate) was found in the insoluble fraction. Interestingly, two of the three soluble compounds, rosmarinic and anthocerotonic acids, have not been found in any other evolutionary lineage of bryophytes (Asakawa *et al.*, 2013). Rosmarinic acid is a phenolic ester derived from caffeic acid, and outside bryophytes, is widespread within the Boraginaceae and Lamiaceae, and is also present in other 26 families of plants, such as Zosteraceae, Potamogetonaceae, Cannaceae, Hydrophyllaceae, Acanthaceae and Apiaceae (Harborne *et al.*, 1999; Bulgakov *et al.*, 2012). This compound has a range of biological activities, exerting antioxidant, anti-inflammatory, and antimutagenic actions in human beings, and it is considered to be a defense compound in plants (Vostalova *et al.*, 2010; Bulgakov *et al.*, 2012; Luis *et al.*, 2013). Anthocerotonic acid is much more unknown than rosmarinic acid, and apparently has not been found in any other organism (Asakawa *et al.*, 2013). Regarding caffeic acid, it is widespread in bryophytes and cormophytes, and has also a number of biological activities (Harborne *et al.*, 1999; Staniforth *et al.*, 2006; Asakawa *et al.*, 2013). Its presence in the soluble fraction is somewhat surprising, because caffeic acid, as other hydroxycinnamic acid derivatives (such as *p*-coumaric and ferulic acids), are good candidates to be UV-B screens because of their efficient absorption in the UV-B range (Agati & Tattini, 2010), and this function would be better performed if they were deposited in the cell wall (Clarke & Robinson, 2008). Nevertheless, in cormophytes, caffeic acid can be located both in the soluble and the cell wall-bound fractions (Santiago *et al.*, 2009). Methyl caffeate, the last compound found in *Anthoceros agrestis* in our study, has a much more restricted distribution among plants than caffeic acid, being mostly confined to Asteraceae (Harborne *et al.*, 1999). It has also been found in bryophytes, but only in hornworts (Asakawa, 1995). Methyl caffeate shows a very strong antioxidant activity (Masuda *et al.*, 2008), antifungal activity, and weak antimicrobial activity (Harborne *et al.*, 1999). This is the first time it has been found in the insoluble fraction of bryophyte extracts.

Fig. 1 shows the absorbance spectra in the UV range for the soluble and insoluble fractions of *Anthoceros agrestis*. The spectrum of the soluble fraction presented two absorption maxima at 287 and 333 nm, which was consistent with the individual compounds identified, since this fraction was dominated by hydroxycinnamic acid derivatives (particularly rosmarinic acid), which have two maximum absorption peaks around 285 and 330 nm (Waterman & Mole, 1994). In the insoluble fraction, another hydroxycinnamic acid derivative was found, but no peak was defined and absorbance increased as wavelength decreased. This lack of defined peaks was coincident with other insoluble fraction spectra in bryophytes (Hespanhol *et al.*, 2014) and could be due to a major interference of the cell wall matrix. This would blur the peaks of specific compounds, especially if their concentrations were relatively low. This point merits further study.

By comparing the absorbance spectra of the soluble and insoluble fractions of *Anthoceros agrestis*, it was clearly seen that the bulk level of SUVACs was higher than that of IUVACs (see also Fig. 2). This fact places *Anthoceros agrestis* closer to liverworts than to mosses, since mosses show an inverse pattern (Fabón *et al.*, 2010, 2012; Hespanhol *et al.*, 2014). Although more studies using a higher number of

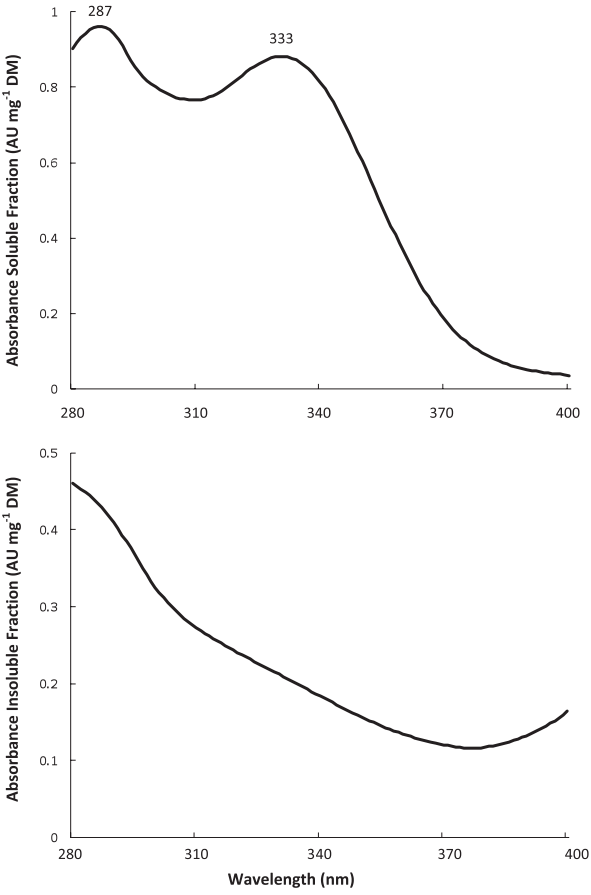

Fig. 1. Representative absorbance spectra of methanol extracts of *Anthoceros agrestis* in the UV-A + UV-B range (280-400 nm), differentiating the methanol-soluble (top) and methanol-insoluble (bottom) fractions. Absorption maxima are shown when possible.

hornworts are needed to confirm this point, the distribution of soluble and insoluble compounds in the different bryophyte lineages would imply that mosses are better protected than hornworts and liverworts against UV radiation, because insoluble cell wall-bound compounds constitute a more efficient UV screen than soluble vacuolar compounds (Clarke & Robinson, 2008). Nevertheless, it should also be taken into account that phenolic UVACs, particularly those located in the soluble fraction, may act as antioxidants (Agati *et al.*, 2012), being also protective against the potential harmful effects of UV radiation.

This is the first study presenting data on the effects of UV radiation on hornworts. No significant differences in the bulk levels of SUVACs and IUVACs were found between P and PAB treatments, and none of the four individual compounds showed significant between-treatment differences (Fig. 2). However, every variable, both global and individual, tended to have higher values in PAB than in P samples. Diverse factors can explain these results. The lack of significant differences between both treatments could be due, on one hand, to the excessive age of the thallus. This would be congruent with results obtained in the thalloid liverwort *Marchantia polymorpha* L., in which the UV-induced accumulation of phenolic

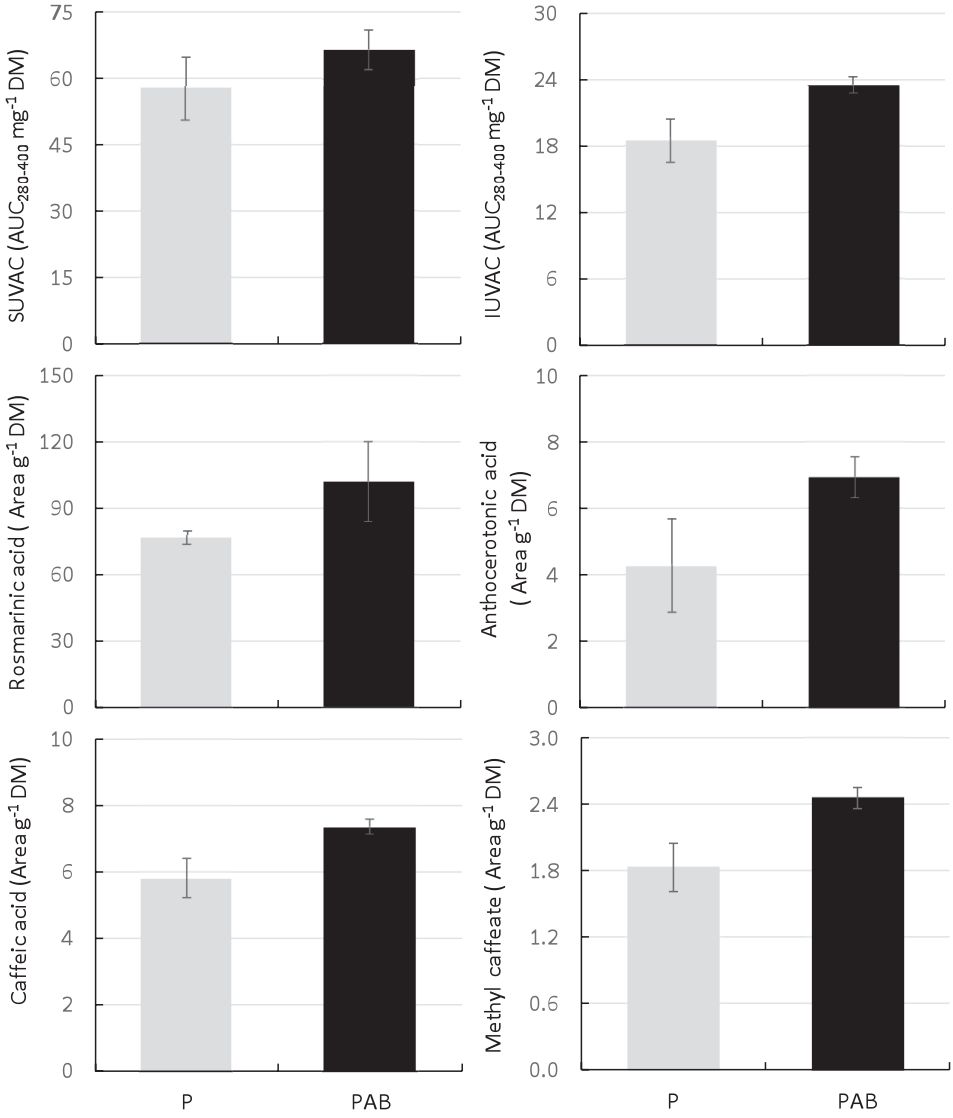

Fig. 2. Bulk levels of both soluble and insoluble UV-absorbing compounds (SUVAC and IUVAC, respectively, in terms of the area under the absorbance curve in the interval 280-400 nm ($AUC_{280-400}$) per DM unit), and contents of individual soluble and insoluble compounds, measured in samples of *Anthoceros agrestis* exposed for 21 days to two different radiation regimes: P (only photosynthetically active radiation (PAR), grey bars) and PAB (PAR + UV-A + UV-B, black bars).

compounds strongly decreased with increasing thallus age (data not shown), and in the leafy liverwort *Jungermannia exsertifolia* Steph., in which different UV-responsive compounds showed different accumulation patterns in younger or older shoot segments (Arróniz-Crespo *et al.*, 2008). Apparently, the functionality of this distribution was to increase the UV protection of younger shoots. On the other hand,

it could be possible that the compounds found in *Anthoceros agrestis* have UV-response thresholds higher than the levels used in the present study, and thus higher UV levels should be applied to find a clearer response. In addition, it cannot be discarded that these compounds were mainly constitutive and not especially inducible by environment, because the constitutive UVACs would be enough to protect the plant. This is supported by the contrasting responses of UVACs to UV radiation that have been found in other bryophyte lineages (Martínez-Abaigar & Núñez-Olivera, 2011; Robinson & Waterman, 2014). Nevertheless, it must be taken into account that the constitutiveness or inducibility of UVACs in bryophytes can be influenced by 1) the type of bryophyte considered, because, for example, UVACs seem to be more UV-responsive in liverworts than in mosses (Fabón *et al.*, 2010, 2012); and 2) the fraction in which UVACs are located, because SUVACs seem to be more UV-responsive than IUVACs, at least in mosses (Fabón *et al.*, 2012). New experiments using other hornwort species, younger thalli of *Anthoceros agrestis*, and higher UV doses, should be conducted to establish more reliably the reactiveness of UVACs to UV radiation in this particular species and in hornworts in general.

Obviously, no comparative data on the effects of UV on hornworts are available in the literature. Regarding the bulk levels of SUVACs and IUVACs, their responses to UV in other bryophytes have been diverse (Fabón *et al.*, 2010, 2012; Martínez-Abaigar & Núñez-Olivera, 2011). This diversity of results was probably due to the different experimental conditions used in the different studies (mainly, the UV levels applied). In addition, these variables may respond to UV in a relatively obscure manner, since they group together the responses of every specific compound contributing to the bulk absorbance, and each compound can respond to UV in a different manner (positive, negative or neutral). Thus, overall, it is not strange that the responses to UV of the bulk levels of SUVACs and IUVACs in *Anthoceros agrestis* were not more clearly defined.

As for the responses of the specific individual compounds found in *Anthoceros agrestis*, no comparative data for bryophytes exist in the literature. In cormophytes exposed to UV under controlled conditions, the content of rosmarinic acid increased in rosemary, oregano, red perilla and lavandin plants (Luis *et al.*, 2007; Kwon *et al.*, 2009; Iwai *et al.*, 2010; Usano-Alemany & Panjai, 2015), but not in *Plectranthus coleoides* L´Hér. (Vidovic *et al.*, 2015). Similar contradictory results were found for caffeic acid, with both positive (Luthria *et al.*, 2006; García-Macías *et al.*, 2007; Iwai *et al.*, 2010) and neutral or negative results (Berli *et al.*, 2010; Kovacik *et al.*, 2010; Vidovic *et al.*, 2015) in response to UV radiation. Controversy was particularly evident when considering the location of caffeic acid in the soluble or insoluble fractions (Ruhland *et al.*, 2005). These discrepancies were probably due to the different species and experimental conditions used, as well as to the effect of PAR wavelengths (Kolb *et al.*, 2001; Jaakola *et al.*, 2004). Moreover, Agati *et al.* (2011) pointed out that hydroxycinnamates in general were unresponsive to radiation treatments, which further complicates the global interpretation of the results obtained. Hence, as occurred with the bulk levels of SUVACs and IUVACs, the relatively unclear responses of rosmarinic and caffeic acids to UV in *Anthoceros agrestis* are within the range of results obtained in other species. There exist no previous data in the literature about the effects of UV radiation on anthocerotonic acid and methyl caffeate.

Overall, the concomitant increase of every variable measured (the bulk levels of SUVACs and IUVACs, and the contents of the four individual compounds) in response to UV radiation, although subtle, suggests that phenolic UVACs as a whole could act as a defense mechanism in *Anthoceros agrestis*, on the basis of their

recognized role as both UV screens and antioxidants (Dixon & Paiva, 1995; Masuda *et al.*, 2008; Agati & Tattini, 2010; Agati *et al.*, 2012; Brunetti *et al.*, 2013). These roles could also have contributed to UV tolerance in the colonization of land by primitive hornworts. Finally, it should be taken into account that the present research was a mechanistic study performed under controlled conditions, whose results cannot directly be extrapolated to the field.

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