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Hill, Elizabeth M, Robinson, Lynne A., Abdul-Sada, Ali et al. (3 more authors) (2018) Arbuscular Mycorrhizal Fungi and Plant Chemical Defence:Effects of Colonisation on Aboveground and Belowground Metabolomes. Journal of chemical ecology. pp. 198-208. ISSN 0098-0331

https://doi.org/10.1007/s10886-017-0921-1

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Journal of Chemical Ecology

ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT CHEMICAL DEFENCE: EFFECTS OF COLONISATION ON ABOVEGROUND AND BELOWGROUND METABOLOMES

--Manuscript Draft--

Manuscript Number:	JOCE-D-17-00277R
Full Title:	ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT CHEMICAL DEFENCE: EFFECTS OF COLONISATION ON ABOVEGROUND AND BELOWGROUND METABOLOMES
Article Type:	Original Research
Keywords:	Arbuscular mycorrhizal symbiosis, Above-belowground interactions, Metabolomics, Pyrrolizidine alkaloids, Blumenols, Senecio jacobaea, Rhizophagus irregularis.
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Funding Information:	
Abstract:	Arbuscular mycorrhizal fungal (AMF) colonisation of plant roots is one of the most ancient and widespread interactions in ecology, yet the systemic consequences for plant secondary chemistry remain unclear. We performed the first metabolomic investigation into the impact of AMF colonisation, by Rhizophagus irregularis on the chemical defences, spanning above- and below-ground tissues, in its host-plant ragwort (Senecio jacobaea). We used a non-targeted metabolomics approach to profile, and where possible identify, compounds induced by AMF colonisation in both roots and shoots. Metabolomics analyses revealed that 33 compounds were significantly increased in the root tissue of AMF-colonised plants, including seven blumenols, plant-derived compounds known to be associated with AMF colonisation. One of these was a novel structure conjugated with a malonyl-sugar and uronic acid moiety, hitherto an unreported combination. Such structural modifications of blumenols could be significant for their previously reported functional roles associated with the establishment and maintenance of AM colonisation. Pyrrolizidine alkaloids (PAs), key anti-herbivore defence compounds in ragwort, dominated the metabolomic profiles of root and shoot extracts. Analyses of the metabolomic profiles revealed an increase in four PAs in roots (but not shoots) of AMF colonised plants, with the potential to protect colonised plants from below-ground organisms.

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ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT CHEMICAL DEFENCE: EFFECTS OF COLONISATION ON ABOVEGROUND AND BELOWGROUND METABOLOMES

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Abstract - Arbuscular mycorrhizal fungal (AMF) colonisation of plant roots is one of the most ancient and widespread interactions in ecology, yet the systemic consequences for plant secondary chemistry remain unclear. We performed the first metabolomic investigation into the impact of AMF colonisation, by *Rhizophagus irregularis* on the chemical defences, spanning above- and below-ground tissues, in its host-plant ragwort (*Senecio jacobaea*). We used a non-targeted metabolomics approach to profile, and where possible identify, compounds induced by AMF colonisation in both roots and shoots. Metabolomics analyses revealed that 33 compounds were significantly increased in the root tissue of AMF-colonised plants, including seven blumenols, plant-derived compounds known to be associated with AMF colonisation. One of these was a novel structure conjugated with a malonyl-sugar and uronic acid moiety, hitherto an unreported combination. Such structural modifications of blumenols could be significant for their previously reported functional roles associated with the establishment and maintenance of AM colonisation. Pyrrolizidine alkaloids (PAs), key anti-herbivore defence compounds in ragwort, dominated the metabolomic profiles of root and shoot extracts. Analyses of the metabolomic profiles revealed an increase in four PAs in roots (but not shoots) of AMF colonised plants, with the potential to protect colonised plants from below-ground organisms.

Key Words – Arbuscular mycorrhizal symbiosis, Above-belowground interactions, Metabolomics, Pyrrolizidine alkaloids, Blumenols, *Senecio jacobaea*, *Rhizophagus irregularis.*

INTRODUCTION

Plant-mediated interactions between organisms above- and below-ground are increasingly recognised as a structuring force in ecology, though in many systems there is

still a need for a more mechanistic understanding of the chemical basis of these effects (Johnson et al. 2012, 2016; Wardle et al. 2004). Microbial symbionts have significant impacts on the chemical composition of their host plant, so are potentially key drivers of these indirect interactions (Hartley and Gange 2009). One of the most ancient and widely occurring plant-symbiont relationships involves arbuscular mycorrhizal fungi (AMF) (Smith and Read 2008). AMF are obligate symbionts that, via specialised structures (arbuscules) within the roots of their hosts, enhance plant uptake of macronutrients, particularly phosphorus and nitrogen (Hodge 2016; Hodge and Storer 2015; Javot et al. 2007; Leigh et al. 2009; Thirkell et al. 2016). In return, AMF are wholly dependent on the host plant for their carbon provision, usually acquiring around 10% of host photosynthate (Bago et al. 2000). These nutrient and carbon exchanges modify the nutrient status of the host plant, but also affect the allocation to secondary metabolites and other resistance mechanisms (Cameron et al. 2013; Minton et al. 2016). For example, colonisation by AMF has been shown to increase plant resistance to below-ground antagonists such as parasitic plants (Li et al. 2012) and plant parasitic nematodes (Rodriguez-Echeverria et al. 2009), as well as to generalist below-ground herbivores (Vannette and Rasmann 2012). Plant interactions with above-ground herbivores can also be affected by AMF colonisation (van der Putten et al. 2001), although usually to a lesser degree than below-ground (Van Wees et al. 2008), and outcomes vary with AMF species identity, herbivore diet breadth and/or feeding mode (Koricheva et al. 2009).

Early studies examining the impact of AMF colonisation on the chemical protection of plant tissues usually targeted specific chemical groups, limiting their power to detect the full range of induced chemical responses to AMF presence (de Deyn et al. 2009; Eftekhari et al. 2012). More recently, non-targeted metabolomic approaches have been used to investigate the effects of AMF on a wide range of metabolites (the metabolome) in foliar

69 tissues (Fester et al. 2011; Schweiger et al. 2014; Schweiger and Muller 2015). Again, several
70 of these did not measure effects on secondary metabolites, so much remains to be discovered
71 about the potential mechanisms by which AMF colonisation of plant roots can affect above-
72 ground plant-herbivore interactions (Gange et al. 2012; Schweiger and Muller 2015).
73 Similarly, the impact of AMF colonisation on the root metabolome has been little studied,
74 although Schliemann et al (2008a) used a metabolomic approach to show that both primary
75 and secondary metabolites differed between the roots of *Medicago truncatula* L. individuals
76 with and without AMF colonisation, and Saia et al (2015) studied impacts of colonisation on
77 the amino acid content of wheat. However, neither of these studies examined above-ground
78 foliar impacts, whilst none of the foliar studies (Fester et al. 2011; Schweiger et al. 2014;
79 Schweiger and Muller 2015) examined root tissue. Therefore, hitherto there has been no
80 metabolomic investigation into the concomitant above- and below-ground changes in plant
81 secondary chemistry following AMF colonisation, an important omission given the growing
82 interest in above-belowground links and systemic defence responses (Johnson et al. 2016).
83 This study addresses this knowledge gap by using non-targeted metabolomics
84 profiling techniques to examine the simultaneous effects of arbuscular mycorrhizal (AM)
85 colonisation on plant secondary chemistry in foliar and root tissues of ragwort (*Senecio*
86 *jacobaea* L.), a species which forms symbiotic associations with AMF (Gange et al. 2002;
87 Reidinger et al. 2012). We used experimental and untargeted metabolomic approaches to test
88 the effect of AMF (*Rhizophagus irregularis*) colonisation on the secondary chemistry of
89 ragwort roots and shoots. We aimed to identify the chemicals induced or repressed in host
90 root and shoot tissues as a result of AM colonisation. Effects of colonisation on host
91 metabolites might be expected to differ between the two tissue types given the established
92 differences in local vs systemic responses to AMF (Van Wees et al. 2008), and, whilst recent

research suggests high specificity in leaf metabolomic responses to AMF (Schweiger et al. 2014), this is as yet untested in roots. Hence, we address two questions:

1) How does colonisation by AMF affect the profiles of secondary metabolites in ragwort and are the impacts more pronounced in root vs. foliar tissue?

2) Are the metabolites induced by AM colonisation similar to those identified in other plant-fungal systems, or is there evidence of novel compounds, potentially indicating a degree of host specificity?

MATERIALS AND METHODS

Microcosm experiment

Ragwort plants were grown from seeds collected from a single plant on the University of Sussex campus (Lat: 50°52'18.6366"N, Long: 000°04'59.6860"W), sterilised in a 5% bleach solution and germinated on double autoclaved vermiculite. Thirty-one pots (1.7 L) received a double autoclaved (121°C; 60 min) growth medium comprising a 1:1 mixture of silver sand:Terragreen® (an attapulgite clay; Turf-Pro Ltd, UK) and bone meal ($0.25 g/L^{-1}$), a complex N and P source to encourage mycorrhizal development (Hodge and Fitter 2010). After mixing the growth media, all pots were twice flushed to saturation with water, aiming to remove the pulse of nutrients released following sterilisation (Troelstra et al. 2001). Seedlings were assigned to replicate pots and randomly assigned to AMF (n = 16) and control (n = 15) treatments. Each replicate in the AMF treatment was inoculated with 50 g (dry weight) of granular *Rhizophagus irregularis* (see Redecker et al. 2013 for recent changes in nomenclature; obtained from PlantWorks, Kent, UK). Those replicates assigned to the control treatment also had 50 g of this granular inoculate added, but double autoclaved (121°C; 60 min) to kill the *R. irregularis*, to control for the input of organic material with the inoculation (see Hodge 2001). In addition, non-AM pots received 100 ml of filtered

118 washings of the *R. irregularis* inoculum, but with AMF propagules removed to limit initial
119 differences among pots in starter microbial communities (as Atkin et al. 2009). To account
120 for any differences caused by the addition of water, 100 ml of deionised water was applied to
121 the AMF-treated microcosms. Each microcosm received a weekly dose of 50 ml of half-
122 strength Rorison's solution (Hewitt 1966), but with the plant-available phosphate removed to
123 encourage AMF colonisation. The experiment ran for 10 weeks in a greenhouse maintained
124 at 15-25°C with supplementary lighting (400W, high pressure sodium lamps) on a 16:8 L:D
125 photoperiod. Microcosms were watered with tap water *ad libitum*.

126 Plants were destructively harvested after 10 weeks in treatment. From each replicate
127 three new leaves (fully mature closest to the centre of the rosette) and two old leaves (closest
128 to the edge of the rosette with no visible signs of senescence) were excised, weighed,
129 immediately snap frozen in liquid nitrogen to halt enzymatic processes and stored in a -80°C
130 freezer for metabolomic analysis. Two representative root samples (each ~10% of the total
131 root mass) per plant were weighed and used for: i) plant metabolomic analysis (snap frozen
132 then stored at -80ºC) and ii) to measure AMF colonisation (stored at 4°C until processed).
133 Root sections were cleared in a 10% potassium hydroxide solution, ink-stained (Vierheilig et
134 al. 1998), slide mounted and the presence or absence of AMF structures was recorded for a
135 minimum of 100 root intersections using a compound microscope (x 200 magnification).
136 Numbers of arbuscules, vesicles and root length colonized (RLC; the percentage of total
137 intercepts where hyphae or other AM fungal structures were present) were recorded for each
138 intersection (as Hodge 2003). The remaining shoot and root material was weighed and then
139 oven-dried (60°C, 72 hours) to obtain dry biomass.

140

141 *Plant extraction and non-targeted metabolomic profiling*

142 A homogenised sub-sample (0.1 ± 0.01g), of the root and shoot tissue taken from each
143 replicate was mixed with 2mL of a 3:3:2 solvent mix of isopropanol, acetonitrile and water
144 using a ball mill (Pulverisette 23. Fritsch, Germany). This solvent mixture extracts both polar
145 metabolites (carbohydrates, amino acids) and non-polar metabolites (lipids) (Sana et al.
146 2010). Samples were spiked with two deuterated internal standards; 17β-estradiol 2,4,16,16-
147 d_4 sodium 3-sulfate (E2-d_4-S, >99% D atom) and progesterone-2,2,4,6,6,17α,21,21,21-d_9 (P-
148 d_9, 98% D atom) (Cambridge Isotope Laboratories Inc. MA and CDN isotopes, Quebec,
149 Canada, respectively). The sample was vortexed (1 min) and after overnight extraction at -
150 20°C, samples were centrifuged, the supernatant removed and the pellet extracted with 2 mL
151 of 80% methanol for 12 hours. The supernatants were combined and a 3 mL aliquot was
152 evaporated to dryness under vacuum and redissolved in 160 µl of methanol:water (3:1, v/v).
153 The extract was filtered (0.2 µm) prior to MS analysis. All extraction chemicals used were
154 purchased from Rathburn Chemicals Ltd, Walkerburn, UK.

155 Metabolites were profiled using ultraperformance liquid chromatography quadrupole
156 time-of-flight mass spectrometry (UPLC-QTOFMS) (Waters, Manchester, UK). Aliquots of
157 plant extracts were injected on to an Acquity UPLC BEH C18 column (1.7 µm particle size,
158 2.1 x 100 mm, Waters, UK). Metabolites were separated using a water-formic acid and
159 acetonitrile gradient as follows: 0-9.0 min, from 0 to 30.0% acetonitrile; 9.0-15.0 min, from
160 30.0 to 100% acetonitrile; 15.0 to 23.0 min, 100% acetonitrile. The flow rate was 0.2 mL
161 min^{-1} and the column temperature was 30°C. The injection volume of plant extracts was 20
162 µl.

163 Metabolites were detected in both positive and negative ESI modes using a
164 Micromass TOF-MS system (Waters, Manchester, UK). The mass spectrometer was tuned to
165 9000 mass resolution and data collected in full scan mode from 100 to 1200 *m/z*. The

166 collision gas used was argon, a constant collision energy of 10eV was used for all
167 experiments, and the TOF penning pressures ranged from 4.63 x 10^{-7} to 4.83 x 10^{-7} mbar.
168 Capillary voltage was 2.6 in positive mode and -2.9 in negative mode. In positive mode, the
169 cone voltage was set at 36V, and the multiplier voltage was set at 654V. In negative mode
170 the cone and multiplier voltage were set at 35V and 550V respectively. Desolvation N_2 gas
171 flow was set at 401L h^{-1} for both ionisation modes.

172 MarkerLynx software (V 4.1, Waters, Manchester, UK) was used to align, normalise
173 and remove isotopic peaks from the metabolomic profiles. Each metabolite signal was the
174 description of an analyte using its specific retention time (r.t.) and mass-to-charge ratio (*m/z*).
175 Using SIMCA-P multivariate analysis software (Umetrics UK Ltd, Windsor, UK) the data
176 were pareto scaled, log-transformed and modelled using partial least square-discriminate
177 analysis (PLS-DA) for > 2 classes, or orthogonal partial least-square discriminate analysis
178 (OPLS-DA) for comparison of just 2 classes followed by examination of the loading plots to
179 detect MS signals associated with AMF colonisation (Liland 2011; Trygg and Wold 2002).
180 For all multivariate analyses, the explained variation (R^2Y) and the predictive power of the
181 model (Q^2) were examined to assess the performance of the models.

182 Biochemical markers associated with AM colonisation were extracted from 'S'-plots
183 derived from the OPLS-DA models (Wiklund et al. 2008). The identity of biochemical
184 markers was determined from their accurate mass composition and isotopic fit using the
185 elemental composition tool from the MassLynx software (V 4.1, Waters, Manchester, UK).
186 Fragmentation data, obtained from collision induced dissociation (CID) using QTOFMS
187 (collision energy between 20 and 50eV) was used to confirm the putative identity of the
188 markers.

189

190 *Analyses of pyrrolizidine alkaloids in metabolomic profiles of tissue extracts.*

191 Metabolomic profiles of root extracts acquired in positive electrospray ionization (ESI) mode
192 were dominated by highly abundant pyrrolizidine alkaloids (PAs) saturating the detector
193 response. PAs are the principal secondary metabolites in *Senecio* species, synthesised in the
194 roots (Hartmann et al. 1989) before being transported to the shoots (Hartmann and Dierich
195 1998) where they are effective defences against generalist herbivores (Macel et al. 2005;
196 Narberhaus et al. 2005; Thoden et al. 2009). In order to determine whether PA profiles
197 changed as a result of AM colonisation, an additional analysis of a 0.5 µl injection of root
198 extracts was undertaken to reduce the highly abundant PA signals. The chromatograms (of
199 both root and shoot extracts) were manually searched, and the relative abundance of observed
200 signals corresponding to PA metabolites was determined relative to the internal standard
201 using MassLynx peak integration software. PAs were assigned putative identities as
202 described above. Where possible, authenticated standards were purchased to confirm PA
203 identity: a mixed standard of PAs seneciphylline and senecionine (Carl Roth, GmbH & Co,
204 Karlsruhe, Germany) and a retrorsine N-oxide standard (PhytoLab GmbH & Co. KG,
205 Nürnberg, Germany). Details of the MS fragmentation data used for the identification of
206 each metabolite are outlined in the supporting information.
207
208 *Data analysis*
209 Adjusted fresh weights ((total fresh weight/100)*water content) of plant tissues were
210 calculated to account for a significant between-treatment difference in root and shoot water
211 content (water content = (fresh weight-dry weight)/(fresh weight)*100). Statistical analyses
212 of differences in plant biomass, biochemical markers and AM colonisation between
213 treatments were performed in SPSS v11. Where the residuals of the data met the
214 assumptions, either before or following transformations, of parametric models (normality,
215 homogeneity of variance), then one-way ANOVA (for plant biomass) or t-tests (for

216 percentage RLC, metabolite and PA concentrations) were used. For non-parametric data,
217 Mann-Whitney U tests were employed. Bonferroni corrections were used to account for the
218 increased false discovery rate (FDR) associated with the multiple testing of metabolic
219 datasets containing many thousands of metabolite signals (Broadhurst and Kell 2006). A less
220 conservative Benjamini and Hochberg FDR correction was applied to the univariate tests of
221 PA concentrations (Benjamini et al. 2001), as this was more appropriate for these much
222 smaller datasets. The association between blumenol metabolites and percentage RLC were
223 investigated using Spearman rank-order correlations, as the data did not meet the assumptions
224 of parametric tests.
225

226 RESULTS

227 *AMF colonisation and plant biomass*

228 Mean percent RLC was 47 ± 4% in the *R. irregularis* colonised plants. Frequency of
229 arbuscules was 16 ± 1% and vesicles 13 ± 2% respectively. There was no AMF colonisation
230 of the non-AM controls. Shoot and root water content were higher in control plants than in *R.*
231 *irregularis* colonised plants, though plant biomass was unaffected by *R. irregularis*
232 colonisation (Table S1).
233

234 *Root metabolome*

235 The metabolite profiles of root extracts from AM colonised and non-AM control plants were
236 clearly separated, indicating a shift in chemical composition of plants in the different
237 treatment groups; (see Figure 1a for the positive ESI mode dataset, and a similar scores plot
238 was obtained for the negative ESI mode in Figure S1a). OPLS-DA of both datasets (positive
239 and negative ESI modes) produced models that explained a large amount of the variation in
240 metabolite profiles between treatments (negative: $R^2Y= 0.99$, positive: $R^2Y= 0.98$) with good

predictability (negative: $Q^2 = 0.53$, positive: $Q^2 = 0.48$). This separation between treatment classes was driven by increased concentrations of 33 metabolites associated with AM colonisation (threshold values after Bonferroni adjustment: positive mode: $P < 5.20 \times 10^{-7}$; negative mode: $P < 5.01 \times 10^{-7}$). The fold increase in concentration of the 33 metabolites associated with AM roots was between two to many thousands (up to 37,995-fold in one instance). Eight of these metabolites were assigned identities (Table 1); the remaining metabolites (n = 25) are listed in Table S2. Blumenol standards are not commercially available, so identifications are derived from comparisons with published mass spectra (cited in Strack and Fester 2006; Peipp et al. 1997; Schliemann et al. 2008b); see supporting information (Note 1, Figure S2 and Table S3) for further details.

Seven of the identified metabolite signals were conjugates of C_{13} cyclohexenones, i.e. blumenol apocarotenoids (metabolites 1-7, Table 1, Figure 2). Metabolites 1 and 4 were identified as blumenols with a 13-hydroxyblumenol C moiety ($C_{13}H_{23}O_3$). Metabolites 2, 3, 5, 6 and 7 were identified as blumenols with a blumenol C moiety ($C_{13}H_{23}O_2$). Whilst identified metabolites 1-7 share common blumenol structures, they differed in the nature of conjugation (Figure 2). Metabolites 1, 2 and 5 were conjugated with glycoside sugars, whereas metabolites 4 and 7 were conjugated with malonylglycoside sugars. Metabolites 3 and 6 were conjugated with an uronide group consistent with a glucuronide (Strack and Fester 2006), and so were identified as a glycosyl-glucuronide and a malonylglycoside-glucuronide conjugate respectively. Metabolite 6, blumenol C malonylglycosyl-glucuronide, is a hitherto unreported structure, whereas metabolites 1-5 and 7 have been found to be associated with AM colonisation in other species (Peipp et al. 1997; Schliemann et al. 2008b). All blumenols were present as their molecular ions, which were either $[M+H]^+$ or $[M-H]^-$ in positive or negative ESI, respectively. In positive ESI, some metabolites were also present as the sodium adduct of the molecular ion (Table S3). Of those blumenols identified,

266 two were observed in both positive and negative ESI modes (metabolites 1 & 6, Table 1).
267 The concentration changes of the identified blumenols corresponded to large differences,
268 with fold increases ranging from 250 to over 8000 in AM plants. The final identified
269 metabolite (number 8) was determined to be hexadecenoyl-glycero-phosphocholine, a
270 lysophospholipid.

271 All blumenol apocarotenoids were positively associated with increasing percentage
272 RLC in the AM colonised plants (Spearman correlation coefficient 0.523-0.711, $P \leq 0.03$-
273 0.002, Table 2). Levels of hexadecenoyl-glycero-phosphocholine were not significantly
274 correlated with RLC ($P \geq 0.11$). A number of unidentified metabolites were detected that
275 were associated with AM colonisation (Table S2) and these were likely to be conjugated
276 phenolic compounds, two additional conjugated blumenols and (in one case) a phospholipid.
277 Based on their empirical formula, none of the metabolites (except for the phospholipid) were
278 present in the plant metabolite databases and so potentially represent new structures.
279 However, their concentrations were too low to allow further structural characterisation.
280 Changes in the concentrations of primary metabolites were not observed as a result of AM
281 colonisation, but this was most likely due to the fact that some structures such as lipids and
282 carotenoids were not have been detected by ESI and small organic molecules, including
283 alkaloid precursors, would not have been retained on the HPLC column.
284

285 *Shoot metabolome*

286 PLS-DA models of the shoot dataset from positive ESI analysis revealed good predictability
287 ($Q^2 = 0.66$) and a high degree of explained variation (total $R^2Y = 0.99$ for six latent variables)
288 (Figure 1b). PLS-DA recorded in negative ESI mode revealed similar models that explained
289 a high level of variation in shoot chemistry ($R^2Y = 0.62$, 4 latent variables) with lower
290 predictability ($Q^2 = 0.36$) than in positive mode (Figure S1b). In both positive and negative

291 modes, the first latent variable separated the metabolomes of old and new leaves. The
292 second, whilst separating AM and non-AM control groups, only explained a relatively small
293 amount of the observed variation in metabolite profiles between these treatments. OPLS-DA
294 models with the highest predictability (R^2Y= 0.989, Q^2=0.431) were associated with the
295 positive ESI profiles of old leaves from the different treatment groups. However, in all
296 OPLS-DA models of shoot tissues, no metabolites were found to vary significantly in
297 association with AMF colonisation of ragwort.
298

299 *Concentrations of PA metabolites in the root and shoot metabolome.*

300 Analysis of the UPLC-QTOFMS chromatograms from the metabolomics study
301 revealed that signals of PA metabolites were saturated in samples of the root extracts.
302 Analysis of lower volumes of samples revealed 19 PA signals that could be resolved in the
303 metabolomic profiles of roots, however more PA structures (potentially up to 33 metabolites,
304 Kostkeno et al. 2013) were likely to be present, which were not detected due to masking of
305 their signals by the most abundant PA signals. The concentration of individual PAs within a
306 species or genotype varies greatly in both roots and shoots (Cheng et al. 2011a). Of the
307 detected PAs, four were significantly increased in AM plants (Table 3). The PAs that
308 responded to AMF colonisation were identified as senecionine, jacoline N-oxide, jaconine N-
309 oxide, and usaramine N-oxide. Of these four PAs, only levels of senecionine were
310 significantly correlated with RLC (Spearman correlation coefficient -0.560, $P \leq 0.02$).
311 Details of the identification of the different PA structures are given in supporting information.

312 The relative abundance of PAs was also determined in the metabolomics datasets of
313 the shoot extracts. A total of 17 abundant PA structures were resolved in the metabolomics
314 profiles of the shoot extracts out of a potential of 29 PA metabolites (Kostkeno et al. 2013).
315 However, there were no significant differences in concentrations (after FDR corrections) of

PA signals between control and AMF-colonised treatment groups which was consistent with the lack of discriminating metabolites observed in the above study on the shoot metabolome (Table S4).

DISCUSSION

This study, the first to use a non-targeted metabolomic approach to assess the effect of AMF colonisation on host plant secondary chemistry in both roots and shoots, showed that colonisation by AMF could increase the concentrations of 33 metabolites in roots by between two and many thousand fold. The extent of AMF colonisation was positively correlated with levels of compounds thought to be associated with establishing and maintaining AMF colonisation, specifically seven C_{13} cyclohexenone blumenol structures, including one with a hitherto unidentified structure. In addition, AMF colonisation affected the levels of the main anti-herbivore defences in root tissues, with four PAs significantly increased in concentration. Despite these below-ground changes, no significant changes in the metabolome of above-ground tissues were detected in response to AMF colonisation of roots, although there were clear metabolomic differences between leaf tissues of different ages. This is noteworthy as had changes been found in aboveground tissues then it would indicate the AM fungus had a direct impact upon the plant. However, as differences were only found in the 'mycorrhizal' (literally, 'fungus-root') root tissue, an unknown fraction of the metabolomics response observed here, and in other studies (e.g. Rivero et al. 2015; Saia et al. 2015), may be due directly to the fungal partner. Experimentally distinguishing between responses driven by the AMF, the root and the mycorrhizal root is hampered by the fact that many of these fungal symbionts cannot be grown in the absence of a host plant (Hodge et al. 2010). Nevertheless, our results demonstrate that colonisation of roots by AMF results in clear metabolomics differences compared to the un-colonised condition, a significant finding given that c. two-

thirds of land plants form this type of close symbiotic association (Hodge and Fitter 2013; Smith and Read 2008).

The process of initiating and maintaining the plant-AMF relationship involves a range of metabolic changes within the host (Fester et al. 2011; Schliemann et al. 2008a), and the bumenol metabolites we detected in our non-targeted analysis are of particular significance, as they are thought to be involved in the chemical signalling underpinning the plant-AMF interaction (Walter et al. 2010; Maier et al. 1995). It has been suggested that blumenols and C14 polyene apocarotenoids have a role in maintaining the plant-AMF interaction once formed (Fester et al. 2002). For instance, concentrations of root C14 polyenes and blumenols increase (Peipp et al. 1997; Schliemann et al. 2006; 2008b) as the plant-AMF interaction progresses, particularly in root cells hosting AMF structures (Fester et al. 2002). Mutant plants, where blumenol production is reduced, are associated with an increased amount of dead and degenerating mycorrhizal structures (Floss et al. 2008), providing further evidence that these blumenol C13 cyclohexenones may have a role in the persistence of AMF colonisation (Walter et al. 2010).

In support of this hypothesis, and in accordance with other studies (Maier et al. 2000; Schliemann et al. 2008a), the concentrations of the seven blumenols we identified were all positively related to levels of AMF colonisation. One of these induced blumenols (metabolite 6) was conjugated with a malonyl-sugar and glucuronic acid moiety, a combination that has never been reported before. Species-specific blumenol metabolites have been observed in a range of plant taxa (Fester et al. 2002, 2005; Maier et al. 2000; Peipp et al. 1997; Schliemann et al. 2006, 2008a), and have been suggested as a mechanism of species recognition between the AMF and its host (Strack and Fester 2006). This is a hypothesis we raise for further testing, but current data is sparse. So far, only a few other plant species, such as the legume *Medicago truncatula* colonised by *Glomus intraradices* (Schliemann et al. 2008a), tomato

366 colonised by *Rhizophagus irregularis* and *Funneliformis mossae* (Rivero et al. 2015) and
367 wheat colonised by multiple AM species (Saia et al. 2015) have had the root tissue response
368 to AMF colonisation been examined in an untargeted metabolomic approach. Until a wider
369 variety of plant-AMF species combinations are tested, it is too early to conclude whether any
370 species-specific signalling exists in AMF-plant interactions. It is notable, however, that one
371 recent study (Schweiger et al. 2014) which compared the impacts of the AMF *Rhizophagus*
372 *irregularis* on the leaf metabolome of five different plant species found that species-specific
373 metabolic changes following colonisation far out-numbered more generic cross-species
374 responses. Similarly, Rivero et al. (2015) detected fungus-specific aspects of the AM-
375 associated changes in the tomato metabolome.

376 Despite substantial changes to the root metabolome induced by AMF colonisation,
377 there were no concomitant shifts in the aboveground metabolome. Conceivably, changes in
378 the shoot metabolome may have been too small or inconsistent to have been detected, but this
379 seems unlikely given that our UPLC-QTOFMS methodology clearly discriminated between
380 the metabolomes of old and new leaves (Figure 1b), and that we have used the same
381 technique to successfully detect differences in the metabolomes of above-ground tissues in
382 ragwort previously (Hartley et al. 2012). Previous studies have demonstrated that AMF-
383 mediated changes occur in above-ground plant tissues, with effects on gene expression
384 (Taylor and Harrier 2003) and on herbivore performance (Koricheva et al. 2009), but changes
385 in foliar secondary metabolites in response to colonisation seem more inconsistent. It has
386 been suggested that induction of chemical defences by beneficial microbes are localised
387 rather than systemic (Van Wees et al. 2008), which may explain why the increases in PA
388 concentration in AMF colonised ragwort we observed were confined to the roots. Increases in
389 PA concentrations in the roots may even reduce PA levels in the shoots. Although our study
390 did not detect such a reduction in foliar allocation to PAs, Reidinger et al. (2012) showed that

leaf concentrations of both total PAs and jacoline were negatively correlated with AMF colonisation of ragwort in the field.

The increased PA levels that we found in AMF colonised roots could impact on trophic interactions. AMF colonisation has been shown to have a negative influence on generalist below-ground herbivores such as parasitic nematodes (Li et al. 2006; Rodriguez-Echeverria et al. 2009) and beetle larvae (Gange et al. 1994), but positive effects on the root-feeding larvae of specialist weevils (Currie et al. 2011). Overall, generalist herbivores, whether above or below ground, seem more affected by AMF colonisation of their host plants than specialists (Gange et al. 2012; Koricheva et al. 2009). In ragwort, the highly toxic PAs deter generalist herbivores (Joosten and van Veen 2011; Thoden et al. 2009), whilst specialist herbivores have the capacity to use PAs to locate and select hosts (Cheng et al. 2013), to detoxify them, and even sequester these compounds for their own defence against predators (Beuerle et al. 2007; Narberhaus et al. 2003). Most previous studies have demonstrated these effects on above-ground herbivores (e.g. Cheng et al. 2011b; Wei et al. 2015); far less is known about the impacts of PAs on root-feeding herbivores (Cheng et al. 2011a) and we have found no published studies on how AMF colonisation modifies those effects. It has been shown that root damage increases PA levels (Hol et al. 2004) and that PAs affect root fungi and the composition of the rhizosphere (Hol and van Veen 2002; Kowalchuk et al. 2006), so there is potential for significant ecological consequences deriving from the changes in PAs we observed in AM roots.

In summary, this study has shown that colonisation by the AMF species *R. irregularis* causes numerous and significant changes in the root metabolome of ragwort, but not in the shoot metabolome. In root tissues, the key changes were in groups of chemicals associated with AMF-plant signalling (blumenols) and anti-herbivore defence (PAs). We showed strong correlations between the levels of blumenols and AMF colonisation,

supporting the idea that these metabolites play a key role in the interactions between AMF and their hosts. The discovery that one of these was a novel structure not previously reported raises questions about the significance of structural modifications in species recognition and signalling that would repay further study.

Acknowledgements - For advice and practical assistance with the metabolomic analysis we would like to thank Dr Raghad Al-Salhi and Ms Julia Horwood. Dr Stefan Reidinger and Dr Kate Storer provided mycorrhizal cultures and advice on experimental procedures. We also thank the University of Sussex and the Centre for Ecology and Hydrology (project NEC03605) for co-funding this project.

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TABLE 1 METABOLITES IDENTIFIED IN RAGWORT ROOTS THAT WERE SIGNIFICANTLY INCREASED AFTER COLONISATION BY *RHIZOPHAGUS IRREGULARIS*

Metabolite number[¶]	Observed ion (*m/z*)	UPLC-TOFMS r.t.	Putative formula	Theoretical mass of ion	Putative identity	Fold change [a]	P-value [b]
1	389.2177	6.37	$C_{19}H_{33}O_8$	389.2175	13-hydroxyblumenol C glycoside $[M+H]^+$	1151.2*	6.65×10^{-9}
1	387.2018	6.37	$C_{19}H_{33}O_8$	387.2018	13-hydroxyblumenol C glycoside $[M-H]^-$	746.6*	6.65×10^{-9}
2	373.2228	7.23	$C_{19}H_{33}O_7$	373.2226	Blumenol C glycoside $[M+H]^+$	8065.8	6.65×10^{-9}
3	547.2389	7.30	$C_{25}H_{39}O_{13}$	547.2391	Blumenol C glycosyl-glucuronide $[M-H]^-$	1553.3	6.65×10^{-9}
4	475.2182	7.55	$C_{22}H_{35}O_{11}$	475.2179	13-hydroxyblumenol C malonylglycoside $[M+H]^+$	489.7	2.99×10^{-7}
5	373.2222	8.20	$C_{19}H_{33}O_7$	373.2226	Blumenol C glycoside $[M+H]^+$	5032.0	6.65×10^{-9}
6	635.2551	8.24	$C_{28}H_{43}O_{16}$	635.2551	Blumenol C malonylglycosyl-glucuronide $[M+H]^+$	3101.3*	6.65×10^{-9}
6	633.2396	8.24	$C_{28}H_{41}O_{16}$	633.2395	Blumenol C malonylglycosyl-glucuronide $[M-H]^-$	250.4*	6.65×10^{-9}
7	459.2232	9.38	$C_{22}H_{35}O_{10}$	459.2230	Blumenol C malonylglycoside $[M+H]^+$	1109.2	6.65×10^{-9}
8	494.3247	14.20	$C_{24}H_{49}NO_7P$	494.3247	Hexadecenoyl-glycero-phosphocholine $[M+H]^+$	7.86	2.00×10^{-7}[c]

[¶] Structures given in Figure 2.

r.t.= retention time

[a] Fold change indicates the concentration increase in roots colonised with AMF when compared to the concentrations observed in control plants.

[b] Significance determined using t-tests ([c]) or Mann-Whitney U tests (unmarked) after Bonferroni adjustments.

* Differences in estimation of fold change between $[M+H]^+$ and $[M-H]^-$ signals for the same metabolite were due to presence of a Na adduct competing with the $[M+H]^+$ ion in positive ESI mode.

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TABLE 2 SPEARMAN RANK-ORDER CORRELATIONS (N = 16) BETWEEN THE CONCENTRATION OF THE BLUMENOL METABOLITES MEASURED IN RAGWORT ROOTS AND *RHIZOPHAGUS IRREGULARIS* COLONISATION IN THE AMF-TREATED PLANTS

Metabolite structure (see Figure 3)	Putative identity	Correlation coefficient	P-value¶
1	13-hydroxyblumenol C glycoside	0.852	1.16×10^{-9}
2	Blumenol C glycoside	0.902	4.33×10^{-12}
3*	Blumenol C glycosyl-glucuronide	0.828	9.37×10^{-9}
4	13-hydroxyblumenol C malonylglycoside	0.841	3.21×10^{-9}
5	Blumenol C glycoside	0.844	2.41×10^{-9}
6	Blumenol C malonylglycosyl-glucuronide	0.832	6.39×10^{-9}
7	Blumenol C malonylglycoside	0.855	9.36×10^{-10}

* Result from MS analysis in -ESI mode (required to detect metabolite 3); all other results from +ESI mode

¶All P-values significant after Bonferroni adjustment

TABLE 3 AVERAGE RELATIVE CONCENTRATIONS (±S.E.) OF THE PYRROLIZIDINE ALKALOID (PA) SIGNALS MEASURED IN THE POSITIVE ESI UPLC-TOFMS PROFILES OF CONTROL (N=15) AND *RHIZOPHAGUS IRREGULARIS* COLONISED (N=16) RAGWORT ROOTS

Theoretical mass of ion	Putative formula	UPLC-TOFMS r.t.	Control	*G. intraradices* colonised	P-value[a]	Fold change[b,c]	Putative identity[c]
334.1654	$C_{18}H_{23}NO_5$	6.00	49.7 (±5.3)	59.2 (±5.3)	0.188		
336.1811	$\mathbf{C_{18}H_{25}NO_5}$	**6.86**	**211.7 (±17.7)**	**289.8 (±21.6)**	$\mathbf{3.63 \times 10^{-3}}$	**1.37**	**Senecionine**
350.1604	$C_{18}H_{23}NO_6$	6.34	220.5 (±27.7)	223.2 (±21.6)	0.571		
	$C_{18}H_{23}NO_6$	9.01	24.5 (±3.2)	38.6 (±6.2)	0.054		
352.1760	$C_{18}H_{25}NO_6$	4.89	23.7 (±8.7)	52.0 (±14.2)	0.033		
	$C_{18}H_{25}NO_6$	5.82	76.1 (±11.3)	55.3 (±5.4)	0.216		
	$C_{18}H_{25}NO_6$	6.13	327.9 (±23.9)	303.8 (±17.9)	0.711		
	$C_{18}H_{25}NO_6$	6.99	48.8 (±2.5)	53.0 (±2.4)	0.029		
	$C_{18}H_{25}NO_6$	7.16	108.8 (±4.2)	113.0 (±3.5)	0.110		
366.1553	$C_{18}H_{23}NO_7$	4.29	193.2 (±46.1)	174.4 (±31.4)	0.598		
	$C_{18}H_{23}NO_7$	4.98	126.0 (±33.8)	141.9 (±39.5)	0.891		
368.1709	$\mathbf{C_{18}H_{25}NO_7}$	**5.24**	**45.9 (±6.7)**	**65.2 (±10.2)**	$\mathbf{4.81 \times 10^{-3}}$	**1.42**	**Usaramine N-oxide**
	$C_{18}H_{25}NO_7$	5.62	93.8 (±20.2)	119.3 (±18.8)	0.231		
	$C_{18}H_{25}NO_7$	6.39	550.5 (±54.4)	530.8 (±38.6)	0.953		
370.1866	$C_{18}H_{27}NO_7$	5.94	24.0 (±2.2)	25.8 (±2.8)	0.379		
376.1760	$C_{20}H_{25}NO_6$	9.55	118.5 (±12.1)	118.4 (±14.3)	1.000		
386.1815	$\mathbf{C_{18}H_{27}NO_8}$	**3.68**	**34.6 (±9.9)**	**105.6 (±16.0)**	$\mathbf{2.01 \times 10^{-3}}$	**3.05**	**Jacoline N-oxide**
392.1709	$C_{20}H_{25}NO_7$	9.77	196.5 (±26.17)	194.0 (±31.4	0.740		
404.1476	$\mathbf{C_{18}H_{26}NO_7Cl}$	**5.22**	**39.3 (±9.73)**	**122.2 (±13.5)**	$\mathbf{5.87 \times 10^{-5}}$	**3.11**	**Jaconine N-oxide**

Relative concentrations were quantified as the ratio of the analyte signal: internal standard signal per 0.3 mg plant mass.

[a] Results that were significant after Benjamini and Hochberg corrections (Benjamini et al. 2001) are displayed in bold.

[b] Fold change indicates the concentration increase in roots colonised with AMF when compared to the concentrations observed in control plants.

[c] Only given for those PAs that differed significantly between treatment groups.

FIGURE LEGENDS

FIG. 1 a) Orthogonal partial least square-discriminate analysis (OPLS-DA) scores plot of the chemical profiles of root extracts from control and *Rhizophagus irregularis* treated ragwort, b) Partial least square-discriminate analysis (PLS-DA) scores plot of the chemical profiles of leaves from control and *Rhizophagus irregularis* treated ragwort. Open and closed circles represent control and *R. irregularis* treated ragwort plants, respectively. In b), blue symbols represent samples from new leaves and orange symbols those from old leaves and the percentages of explained variation (R^2Y) modelled by the first two latent variables are displayed on the axes. Both plots show datasets from samples profiled in positive ESI MS mode; data from negative ESI mode revealed similar model characteristics (Figure S1).

FIG. 2 Schematic structure of the identified blumenol apocarotenoids associated with *Rhizophagus irregularis* colonisation of ragwort roots.

Figure

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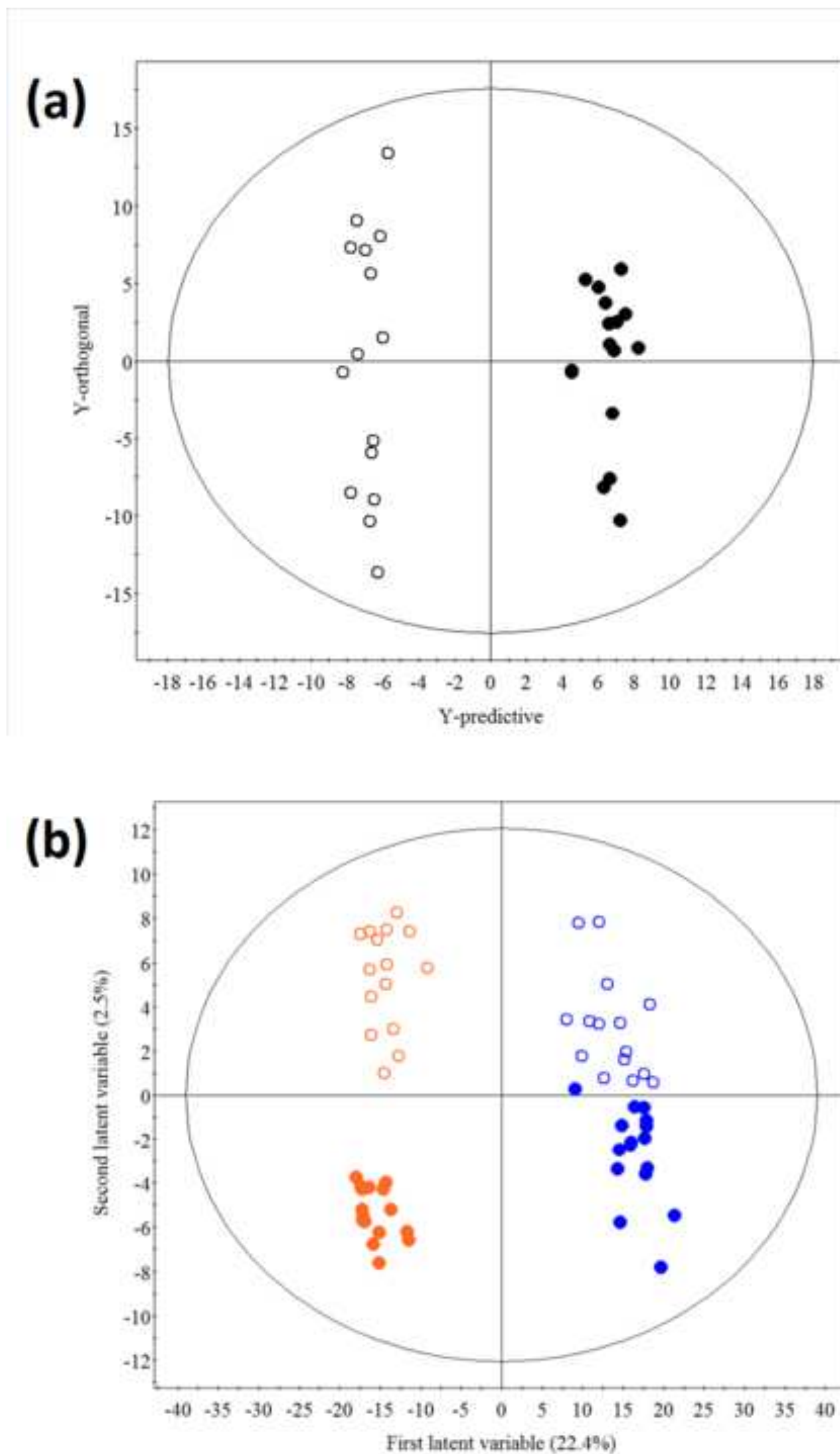

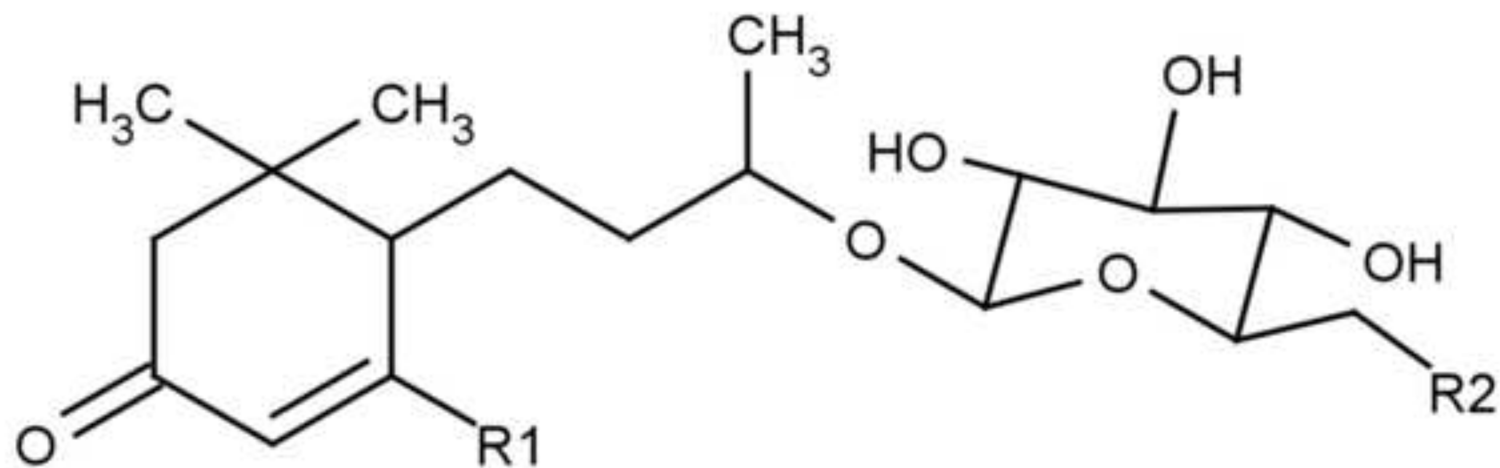

Metabolite	R1	R2
1	$-CH_2OH$	-OH
2	$-CH_3$	-OH
3	$-CH_3$	$-C_6H_9O_7$
4	$-CH_2OH$	$-OCOCH_2CO_2H$
5	$-CH_3$	-OH
6	$-CH_3$	$-OCOCH_2CO-C_6H_9O_7$
7	$-CH_3$	$-OCOCH_2CO_2H$

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