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Short-term effects of the allelochemical umbelliferone on *Triticum durum* L. metabolism through GC-MS based untargeted metabolomics

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Plant Science

Short-term effects of the allelochemical umbelliferone on *Triticum durum* L. metabolism through GC-MS based untargeted metabolomics --Manuscript Draft--

Manuscript Number:	PSL-D-19-00155R1
Article Type:	Research Paper
Keywords:	metabolomics; gas chromatography mass-spectrometry; elicitation; polar; time-course; phytotoxicity; allelochemicals
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First Author:	Biswapriya B. Misra
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Abstract:	<p>The present experiment used untargeted metabolomics to investigate the short-term metabolic changes induced in wheat seedlings by the specialized metabolite umbelliferone, an allelochemical. We used 10 day-old wheat seedlings treated with 104 μM umbelliferone over a time course experiment covering 6 time points (0 h, 6 h, 12 h, 24 h, 48 h, and 96 h), and compared the metabolomic changes to control (mock-treated) plants. Using gas chromatography mass spectrometry (GC-MS)-based metabolomics, we obtained quantitative data on 177 metabolites that were derivatized (either derivatized singly or multiple times) or not, representing 139 non-redundant (unique) metabolites. Of these 139 metabolites, 118 were associated with a unique Human Metabolome Database (HMDB) identifier, while 113 were associated with a Kyoto Encyclopedia of Genes and Genomes (KEGG) identifier. Relative quantification of these metabolites across the time-course of umbelliferone treatment revealed 22 compounds (sugars, fatty acids, secondary metabolites, organic acids, and amino acids) that changed significantly (repeated measures ANOVA, P-value < 0.05) over time. Using multivariate partial least squares discriminant analysis (PLS-DA), we showed the grouping of samples based on time-course across the control and umbelliferone-treated plants, whereas the metabolite-metabolite Pearson correlation revealed tightly formed clusters of umbelliferone-derived metabolites, fatty acids, amino acids, and carbohydrates. Also, the time-course umbelliferone treatment revealed that phospho-L-serine, maltose, and dehydroquinic acid were the top three metabolites showing highest importance in discrimination among the time-points. Overall, the biochemical changes converge towards a mechanistic explanation of the plant metabolic responses induced by umbelliferone. In particular, the perturbation of metabolites involved in tryptophan metabolism, as well as the imbalance of the shikimate pathways, which are strictly interconnected, were significantly altered by the treatment, suggesting a possible mechanism of action of this natural compound.</p>
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Response to Reviewers:	

From *Dr. Fabrizio Araniti*

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Regarding: Submission of the revised manuscript ID PSL-D-19-00155 entitled *Short-term effects of the allelochemical umbelliferone on Triticum durum L. metabolism through GC-MS based untargeted metabolomics*

Dear Editor,

With pleasure I would like to submit the revised version of the manuscript (ID: PSL-D-19-00155) entitled “*Evaluation of the short-term effects of the allelochemical umbelliferone on Triticum durum L. metabolism through GC-MS based untargeted metabolomics*” to your journal Plant Science. We thank you for providing the opportunity to resubmit a revised version of the presented study.

We have now taken into account all the comments from the 3 kind reviewers to make amends and additional analysis to the revised manuscript. We have provided point by point rebuttal to all the reviewer comments and have done simultaneous amends to the manuscript.

In addition we have also sent the manuscript for language editing/ polishing for English usage with the help of native English speaking colleagues for clarity in writing style and readability.

We also declare that the study was performed according to the international, national and institutional rules and it has not been submitted elsewhere for publication.

All the listed authors have read and approved the submitted manuscript.

Please, should there be need of more information do not hesitate to contact me.

Thanks in advance

Reggio Calabria, 20th April, 2020

Yours most sincerely,

Dr. Fabrizio Araniti

1 **Research Article**

2 **Evaluation of the ~~s~~Short-term effects of the allelochemical umbelliferone on *Triticum durum***
3 **L. metabolism through GC-MS based untargeted metabolomics**

4
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25
26 **Short title:** *Wheat metabolomics of umbelliferone treatment*

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33

34 **Abstract**

35 ~~In~~ ~~the~~ ~~present~~ ~~experiment~~ ~~used~~ ~~untargeted~~ ~~metabolomics~~ ~~to~~ ~~investigate~~ ~~the~~ ~~short-term~~ ~~metabolic~~
36 ~~changes~~ ~~induced~~ ~~in~~ ~~wheat~~ ~~seedlings~~ ~~by~~ ~~the~~ ~~specialized~~ ~~metabolite~~ ~~umbelliferone~~ ~~(,~~ ~~an~~
37 ~~allelochemical)~~ ~~in~~ ~~wheat~~ ~~seedlings~~ ~~have~~ ~~been~~ ~~deeply~~ ~~investigated~~ ~~using~~ ~~untargeted~~
38 ~~metabolomics~~. Allelopathy is a plant defense mechanism by which they protect themselves from
39 competitive species using specialized biochemicals in the form of secretion or volatiles released to
40 the environment. Though, ~~umbelliferone~~ is a well-known allelochemical, its mechanism of action
41 ~~in a short term treatment is far from established~~. We used ~~~10 days~~ ~~10 d~~ ~~day-old~~ wheat seedlings
42 treated with 104 μM umbelliferone over a time course experiment covering 6 time points, ~~i.e.,~~ (0
43 h, 6_h, 12_h, 24_h, 48_h, and 96_h) and compared the metabolomic changes to control (mock-treated)
44 plants. Using gas chromatography mass-spectrometry (GC-MS)-based metabolomics ~~efforts~~, we
45 ~~collectively~~ obtained quantitative data on 177 metabolites that were derivatized (either derivatized
46 singly or multiple times) or not, representing 139 non-redundant (unique) metabolites. ~~Out~~ ~~of~~ these
47 139 metabolites, 118 were associated with a unique [Human Metabolome Database \(HMDB\)](#)
48 [HMDB](#) identifier, while 113 were associated with a [Kyoto Encyclopedia of Genes and Genomes](#)
49 [\(KEGG\)](#) ~~KEGG~~ identifier. Relative quantification of these metabolites across the time-course of
50 umbelliferone treatment revealed 22 compounds (sugars, fatty acids, secondary metabolites,
51 organic acids, and amino acids) that changed significantly (repeated measures ANOVA, P-value <
52 0.05) ~~with over~~ time. Using multivariate partial least squares discriminant analysis (PLS-DA), we
53 showed the grouping of samples based on time-course across the control and ~~umbelliferone~~
54 ~~umbelliferone~~-treated plants, whereas the metabolite-metabolite Pearson correlation revealed tightly
55 formed clusters of umbelliferone-derived metabolites, fatty acids, amino acids, and carbohydrates.
56 Also, ~~the~~ time-course ~~of~~ umbelliferone treatment revealed, that phospho-L-serine, maltose, and
57 dehydroquinic acid were the ~~top 3~~ ~~top three~~ metabolites showing highest importance in
58 discrimination among the time-points. ~~Overall, the biochemical changes converge towards a~~
59 ~~mechanistic explanation of the plant metabolic responses induced by umbelliferone. In particular,~~
60 ~~the perturbation of metabolites involved in tryptophan metabolism,~~ ~~as well as the~~ ~~un~~ ~~im~~ ~~balance~~ ~~of~~
61 ~~the shikimate pathways, which are strictly interconnected, were significantly altered by the~~
62 ~~treatment, suggesting a possible mechanism of action of this natural compound. The above indicate~~
63 ~~a system wide changes induced by umbelliferone, through dysregulation of primary as well as~~
64 ~~specialized metabolism.~~

65 **Keywords:** metabolomics, gas chromatography mass-spectrometry, elicitation, polar, time-course,
66 phytotoxicity, allelochemicals.

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69 **Introduction**

70 Allelopathy is a complex ecological phenomenon, ~~that and~~ refers to the direct and/or indirect effects
71 of one organism (plants, insects, etc.) on another through the production and release of specialized
72 chemical compounds into the environment [1]. Due to ~~this the~~ complexity ~~in of~~ interpretation and
73 analysis, the elucidation of allelopathy using chemical signatures is a challenge, which requires
74 expertise in diverse scientific fields, and the use of multidisciplinary tools and approaches [2]. In
75 recent years, to unravel the ecological roles of specialized metabolites, rapid advancements ~~were~~
76 ~~made owed to have made~~ use of *-omics* techniques and/or targeted and untargeted metabolic
77 profiling of plant materials [3-6]. Techniques such as transcriptomics, proteomics, and
78 metabolomics allow simultaneous analysis of the total molecular and biochemical constituents of a
79 given sample [7]. In ~~studies involving~~ allelopathy ~~studies~~, the use of metabolomics as an analytical
80 technique allows identification and quantification of both primary and specialized metabolites in
81 complex samples [8, 9]. Moreover, metabolomics is a useful tool in understanding the response ~~of a~~
82 ~~living system~~ to biotic and abiotic stress, for the determination of complex pathways of primary and
83 specialized metabolite biosynthesis, and ~~providing~~ ~~ing~~ a broader understanding of biological activity
84 and mode of action of critical specialized metabolites [6, 10]. In fact, metabolomics as a technique
85 best represents the molecular phenotype, since it directly reflects the underlying biochemical
86 activity and state of cells, tissues, and organism, being closest to ~~the~~ functional phenotype [11].

87 Among noteworthy allelochemicals, coumarins, ~~that which~~ derive from the lactonization of o-
88 hydroxycinnamic acid, is a class of specialized metabolites that are widely distributed in the plant
89 kingdom, and they are synthesized by almost all higher plants [12], playing a pivotal role in both
90 plant communication and defense [13]. ~~Another One~~ coumarin, umbelliferone, ~~so named so due~~
91 ~~to because of~~ its wide occurrence within the Umbelliferae family, is an extremely biologically active
92 compound widely distributed in the plant kingdom (Asteraceae, Rutaceae, Acanthaceae, ~~and~~
93 Hydrangeaceae, among others) [14]. ~~Further, u~~ Umbelliferone accumulates and is released to the
94 environment through volatilization and root exudation [15-17]. The critical ecological role of
95 umbelliferone has been demonstrated in several studies. For example, Minamikawa et al. [18]
96 ~~demonstrated showed~~ that umbelliferone production is induced in response to infection by plant
97 pathogens. Similarly, it was noted, in the medicinal plant *Chamomilla recutita*, that under abiotic
98 and biotic (powdery mildew *Erysiphe cichoracearum*) stress conditions, umbelliferone
99 concentration ~~was extremely increasing~~ ~~increased to an extreme degree~~ [19]. Those results suggest

100 that this specialized metabolite could play a pivotal role in some plants as a first line of defense.
101 This hypothesis was further confirmed by studies from Yang et al. [20], ~~that~~ which highlighted its
102 ability to suppress the *Ralstonia solanacearum*-induced wilting disease process by reducing fungi
103 colonization and proliferation, and by Hamerski et al. [21], ~~which~~ who demonstrated that extract of
104 fungal cell wall acts as elicitor in *Amni majus*, increasing umbelliferone production. Umbelliferone
105 is also involved in plant defense against herbivores ~~since it acts,~~ acting as a repellent interfering
106 with the bitter gustatory receptor neurons of fruit flies [22]. Finally, it has been ~~proven~~ shown that ~~it~~
107 umbelliferone determined the chemotactic movement of *Rhizobium* and *Agrobacterium* across
108 chemical gradients towards lower levels of inhibitors and higher levels of potential nutrients [23].
109 Concerning its phytotoxic potential, several studies have demonstrated that this molecule strongly
110 affects both plant growth and development, inducing reactive oxygen species (ROS) accumulation,
111 chlorophyll degradation, alteration of root morphology, and ROS-induced programmed cell death
112 [24-26]. Moreland and Novitzky [27] found that umbelliferone, at relatively high concentrations,
113 inhibits functions in isolated chloroplasts and mitochondria, whereas Einhellig [28] demonstrated
114 that concentrations of umbelliferone that reduce *Glycine max* seedling growth also decreased leaf
115 water potential, stomatal conductance, and the transpiration ratio.
116 Although several ~~proves~~ evidence regarding umbelliferone phytotoxicity are reported in the
117 bibliography, such information ~~are~~ is quite dated and ~~they~~ does not unveil the metabolic
118 pathways altered by the molecule. Moreover, it is ~~largely~~ widely known that allelochemicals could
119 have a multi-target effect leading to a series of cascade effects ~~which final results consists,~~ finally
120 resulting in the inhibition of plant growth and/or ~~to~~ plant death. Therefore, to identify their mode of
121 action, it is quite important to focus ~~the~~ attention on time-course experiments that evaluate the short
122 term effects of these chemicals. This approach could lead ~~in~~ to identifying the primary metabolic
123 pathways affected. ~~In this perspective,~~ the main purpose of this ~~work~~ study was to evaluate the
124 ~~short~~ short-term effect of umbelliferone on seedlings of durum wheat (*Triticum durum*) seedlings,
125 ~~that seedlings that due to its sensitivity to phytotoxins is~~ — a crop species largely often employed in
126 phytotoxicity experiments due to its sensitivity to phytotoxins [29], — in order to identify the
127 impact of this molecule on plant metabolism.

129 2. Materials and Methods

130 2.1. Chemicals and Reagents

131 Methanol for GC-MS SupraSolv® (1.00837), chloroform for GC-MS SupraSolv® (1.02432), [N-](#)
132 [Methyl-N-\(trimethylsilyl\) trifluoroacetamide \(MSTFA\)](#) ≥98.5% (69479), pyridine ≥99% (270407),
133 methoxyamine hydrochloride 98% (226904), umbelliferone 99% ([Hh24003](#)), ribitol ≥99% (A5502),
134 [and](#) alkanes mixture C₁₀-C₄₀ (68281) were acquired from Sigma Aldrich (Italy).

136 2.2 Plant growth conditions and elicitor treatments

137 Durum wheat ~~seeds~~ (*Triticum durum* L. cv. Opera) ~~seeds~~ were germinated in Petri dishes (9 cm) in
138 a growth chamber at 25°C, 70% humidity, ~~and~~ with a photoperiod of 16/8 (light / dark), and light
139 intensity of 90 mol m⁻² s⁻¹ supplied by a cool white fluorescent lamp (Polylux XL FT8, 55W 8440).
140 Immediately after germination, uniform seedlings were transferred to a 4.5 L hydroponic system
141 and grown in a modified Hoagland solution formulated as follows: KNO₃ (10 mM); MgSO₄ (100
142 μM); CaSO₄ (400 μM); KCl (5 μM); K₂SO₄ (200 μM); K H₂PO₄ (175 μM); H₃BO₃ (2.5 μM);
143 MnSO₄ (0.2 μM); ZnSO₄ (0.2 μM); NaMoO₄ (0.05 μM); CuSO₄ (0.05 μM); Fe-EDTA (200 μM).
144 The solution was changed every other day and continuously oxygenated using an air bubble stone.

146 2.2.1 Dose-response curve

147 After the first true leaf formation (10 d from germination), wheat seedlings ([a pool of 30 seedlings](#)
148 [per replicate and treatment](#)) were selected for uniformity in growth, and were transferred ~~into the~~
149 [continuously oxygenated](#) hydroponic solutions enriched with different concentrations of
150 umbelliferone: 0, 12.5, 25, 50, 100, 200, [and](#) 400 μM. After 10 days of treatment, the whole plants
151 were collected, dried in an oven at 40°C, and weighed to monitor changes in total fresh weight
152 (FW). ~~Umbelliferone was previously first dissolved in ethanol (0.1%, w/v) and then poured into the~~
153 [nutrient solution prepared in deionized water. The same amount of ethanol was added to the mock](#)
154 [treatments \(Control\). The, where and the- experiment was experiment was were replicated five](#)
155 [times \(n = 5\).](#)

157 2.2.2 Short-time-term effect of umbelliferone treatment

158 To study the short-term effects of umbelliferone on ~~the~~ wheat metabolome, seedlings ([a pool of 10](#)
159 [seedlings per replicate, per time point, and treatment](#)) were grown for 10 days and were then
160 treated with 104 μM of umbelliferone (the ED₅₀ concentration [was](#) calculated from a dose-
161 response curve). Plant materials were collected after 0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48
162 h (T4), and 96 h (T5) of umbelliferone treatment, [and a parallel set of control plants \(mock](#)
163 [treated with same volume of ethanol/ water? as previously described §2.2.1\) with the same](#)

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164 ~~time points. In order to avoid metabolic fluctuations due to induced due to by plant circadian~~
165 ~~rhythms, all the treatments were applied in order to allow plant collection at the same hour of~~
166 ~~the day (12:00 am'clock) (Eg.i.e., plants belonging to treatment T1 were treated at 06:00~~
167 ~~am, whereas, T2 at 00:00, etc.and so on). After collection, the plant materials was and were~~
168 ~~immediatelywere immediately~~ snap frozen for metabolomic studies. The experiment was
169 replicated five times (n = 5).

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172 2.3. Metabolite extraction and sample derivatization

173 Plant materials were collected at the middle of the light period, and whole plants were immediately
174 snap frozen in liquid nitrogen to quench the endogenous metabolism. Freshly homogenized (100
175 mg) plant material ~~were was~~ obtained ~~for from~~ each biological sample (plant) and replicates. These
176 were transferred to a ~~2 mL~~ microcentrifuge round bottom screw cap tubes (Eppendorf).
177 Extraction was done by adding 1400 ~~μL~~ of methanol (at -20°C) and vortexing for 10 s after
178 addition of 60 ~~μL~~ ribitol (0.2 mg/~~mL~~ stock in ddH₂O) as an internal quantitative standard for
179 the polar phase. Samples were transferred in a thermomixer at 70°C and were shaken for 10 min
180 (950 rpm) and were then further centrifuged for 10 min at 11000 g. The supernatants were collected
181 and transferred to glass vials where 750 ~~μL~~ CHCl₃ (-20°C) and 1500 ~~μL~~ ddH₂O (4°C) were
182 sequentially added. All the samples were vortexed for 10 s and then centrifuged for another 15 min
183 at 2200 g. Upper polar phase (150 ~~μL~~) for each replicate ~~were was~~ collected, transferred to a 1.5
184 ~~mL~~ tube and ~~were~~ dried in a vacuum concentrator without heating. Before freezing and storing
185 at -80°C, the tubes were filled with argon and placed in a plastic bag with silica beads (~~for~~
186 ~~avoiding to avoid~~ moisture and hydration during short-term storage). Before derivatization, stored
187 samples, ~~were~~ placed in a vacuum concentrator for 30 ~~minutes min~~ to eliminate any trace of
188 humidity. Then, ~~to the dried samples,~~ 40 ~~μL~~ methoxyamine hydrochloride (20 mg/~~mL~~ in
189 pyridine) ~~were was~~ added ~~to the dried samples, and which were then~~ incubated for 2 h in a
190 Thermomixer (950 rpm) at 37°C. Methoxyam~~in~~ated samples were then silylated by adding 70 ~~μL~~
191 of MSTFA to the aliquots. Samples were further shaken for 30 min at 37°C. Derivatized samples
192 (110 ~~μL~~) were then transferred into glass vials suitable for the GC/MS autosampler for analysis.

194 2.4. GC-quadrupole/MS analysis

195 The derivatized extracts were injected into a TG-5MS capillary column (30 m x 0.25 mm x 0.25
196 μm) (Thermo Fisher Scientific, Waltham, MA, USA) using a gas chromatograph apparatus (Trace
197 GC 1310, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a single quadrupole mass
198 spectrometer (ISQ LT, Thermo Fisher Scientific, Waltham, [Massachusetts, MA, USA](#)). Injector and
199 source were set at 250°C and 260°C ~~temperature~~, respectively. One μl of sample was injected in
200 splitless mode with a helium flow of 1 ~~mL~~ mL/min using the following programmed temperature:
201 isothermal 5 min at 70°C followed by a 5°C/ min ramp to 350°C and a final 5 min heating at 330
202 °C. Mass spectra were recorded in electronic impact (EI) mode at 70 eV, scanning at 40-600 m/z
203 range, scan time 0.2 ~~see~~. Mass spectrometric solvent delay was settled as 9 min. Pooled samples
204 that served as quality controls (QCs), n-alkane standards, ~~and~~ blank solvents (pyridine) were
205 injected at scheduled intervals for instrumental performance, tentative identification, and
206 monitoring of shifts in retention indices (RI).

207 2.5 GC/MS Analysis and data acquisition

208 2.5.1 GC/MS data analysis using MS-DIAL

209 Raw data (.RAW) from the single quadrupole instrument was converted to ~~ABF_mzML~~ format
210 with the ~~ABF_converter~~ [MSConvertGUI from ProteoWizard](#). ~~The~~ MS-DIAL, with open source
211 publicly available EI spectra library, ~~were~~ ~~was~~ used for raw peaks extraction, and the data
212 baseline filtering and calibration of the baseline, peak alignment, deconvolution analysis, peak
213 identification, and integration of the peak height ~~were~~ essentially followed as described ~~by~~ [\[insert](#)
214 [authors\]](#) [30]. An average peak width of 20 scans and a minimum peak height of 1000 amplitudes
215 was applied for peak detection, and a sigma window value of 0.5, EI spectra cut-off of 5000
216 amplitudes was implemented for deconvolution. For identification ~~setting~~, the retention time
217 tolerance was 0.2 min, the m/z tolerance was 0.5 Da, the EI similarity cut-off was 60%, and the
218 identification score cut-off was 80%. In the alignment parameters setting process, the retention time
219 tolerance was 0.5 min, and retention time factor was 0.5. For MS-DIAL data annotations, we used
220 publicly available libraries (both positive and ~~negative~~) for ~~Compound~~ ~~compound~~ identification,
221 based on the mass spectral pattern as compared to EI spectral libraries such as NIST Mass Spectral
222 Reference Library (NIST14/2014; National Institute of Standards and Technology, USA; with EI-
223 MS data of 242,466 compounds), the MSRI spectral libraries from Golm Metabolome Database
224 [31] available from Max-Planck-Institute for Plant Physiology, Golm, Germany
225 (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>), MassBank [32], ~~and~~ MoNA (Mass Bank
226 of North America, (<http://mona.fiehnlab.ucdavis.edu/>)).

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~~Once the compounds and features were identified using three of the above described were obtained and annotated, only the shared metabolites were only reported as quantified and confidently identified.~~ For metabolite annotation and assignment of the EI-MS spectra, we followed the metabolomics standards initiative (MSI) guidelines for metabolite identification [33], i.e., Level 2: identification was based on spectral database (match factor >80%) and Level 3: only compound groups were known, e.g. specific ions and RT regions of metabolites.

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2.6 Statistical analyses

For metabolomic experiments, standard statistical analyses (summary statistics) were performed using the statistical software R (Version 3.5.3, <http://www.R-project.org>) [34, 35]. Normalized (internal standard), transformed (log2), imputed, and scaled peak areas representative of relative metabolite amounts were obtained using DeviumWeb [36], and are presented in tables and figures. Values reported in all tables and text are presented as means, and differences were considered significant when $P < 0.05$ (nominal P-values).

~~The FW responses to different doses of umbelliferone were evaluated by a nonlinear regression model using a log-logistic equation, largely employed in phytotoxicity screenings [37], that allowed to estimate the ED50 parameter, the dose required to reduce 50% of the total response. To evaluate the ED₅₀ value (the dose necessary to inhibit the FW by 50%), on the FW dose response curve, raw data were fitted through a non linear regression model using the log logistic equation, largely widely employed in phytotoxicity screenings [37].~~ The ED₅₀ value was then used as the key concentration for the short-term metabolomics experiments.

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2.6.1 Univariate analysis

ANalysis Of VAriance (ANOVA) was performed using R. Hierarchical clustering analysis (HCA) using average linkage clustering was performed on Pearson distances ~~from the metabolite abundance data~~, using PermutMatrix [38] ~~from the metabolite abundance data~~. For heat maps, data were normalized using the z-scores of the intensity counts for each of the metabolites under the peak areas.

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2.6.2 Multivariate analysis

~~Exploratory M~~ultivariate analysis was done using R (version 3.6.1). The sample-sample distance clustering was obtained via package “Pheatmap.” using “Pearson” correlation and default parameters. The exploratory interactive MDS plots were done ~~via~~with the Glimma package. Other various dimension reduction analysis via principal components analysis (PCA) of overall, separate

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261 control, and treated data was performed bywith the FactoMineR and factoextra packages. **Principal**
262 **components analysis (PCA)**PCA was performed using the **package**-DeviumWeb **package** (**Grapov et**
263 **al., 2014)**[39], where the output consisted of score plots to visualize the contrast between different
264 samples and loading plots to explain the cluster separation. Data were scaled with unit variance,
265 without any transformation. Partial least-squares discriminant analysis (PLS-DA) was used to
266 highlight differences between the metabolic phenotypes at **three-six** time points (0 h, 6 h, 12 h, 24 h,
267 48 h, **and** 96 h) **and umbelliferone elicitation in the study**.

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269 **2.7 Time-course analysis of control and umbelliferone-treated metabolomes**

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270 **For short time series metabolomics data analysis, we used the tool, Short Time series Expression**
271 **Miner (STEM) tool** (**Ernst & Bar Joseph, 2006)**[40], originally used for short microarray time series
272 **experiments that are short** (3–8 time points for >~80% of the datasets). The novel STEM clustering
273 **takes advantage of the few time points in a dataset, and it first selects a set of distinct and**
274 **representative temporal expression profiles (i.e., model profiles), where these model profiles are**
275 **independent of data. The clustering algorithm then assigns each feature (i.e., metabolite) passing the**
276 **filtering criteria to the model profile that most closely matches the feature's abundance profile as**
277 **determined by the correlation coefficient, and determines which profiles have a statistically**
278 **significant higher number of features assigned using a permutation test. STEM was used as a Java**
279 **implementation with a graphical user interface, available at <http://www.cs.cmu.edu/~jernst/st/> for**
280 **clustering the metabolite accumulation patterns according to time points. For our analysis, we used**
281 **the following criteria: no additional normalization of the data; 0 added as the starting point;**
282 **number of model profiles = 20; maximum unit change in model profiles between time points = 3.**
283 **To explain the model profiles, we used an expression of -1 for decreased levels of a metabolite, 0**
284 **for unchanged levels of a metabolite, and 1 asfor increased levels of a metabolite to explain the**
285 **model profiles. For instance, a model profile with an expression of -1, -1, 0, 1, 1, 0 represents:**
286 **decreased, decreased, unchanged, increased, increased, and unchanged levels of a given set of**
287 **metabolites for the 6 time points in the given model profile.**

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289 **2.7.8 Pathway enrichment and clustering analysis**

290 Pathway enrichment analysis was performed at MetaboAnalyst (www.Metaboanalyst.ca) (**Xia et al.,**
291 **2009)** [41], and Chemical Translation Service (CTS:
292 <http://cts.fiehnlab.ucdavis.edu/conversion/batch>) was used to convert the common chemical names
293 into their **Kyoto Encyclopedia of Genes and Genomes- (KEGG)**KEGG, **Human Metabolome**

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294 ~~Database (HMDB)~~HMDB, CAS, PubChem [Compound ID \(CID\)](#), LipidMAPS IDs and InChiKeys
295 [values/identifiers](#).

296

297 **2.8-9 Data sharing**

298 The raw datasets and the metadata obtained from the GC-EI-MS platform ~~has~~ have ~~now~~ been
299 deposited at the Metabolomics Workbench (Study ID: **ST001056**) ~~and is available at this link: <http://dx.doi.org/10.21228/M81M4X>~~
300 <http://dx.doi.org/10.21228/M81M4X> ~~to the readers and reviewers.~~

301

302 **3. Results and Discussion**

303

304 **3.1 Dose response curve based on wheat biomass production in response to** 305 ~~Umbelliferone~~[umbelliferone](#)

306 The dose response curve built on the variation of wheat fresh biomass (FW), in response to
307 increasing doses of umbelliferone (0-400 μ M), pointed out a significant dose-dependent phytotoxic
308 effect (**Fig. 1**). The lowest concentration (12.5 μ M) did not affect plant growth. ~~On the contrary,~~
309 ~~at~~ 25 μ M, a 17% ~~reduction~~ [reduction](#) of biomass ~~reduction~~ was observed, and ~~such the~~ reduction reached ~~the~~
310 82-% at the highest concentration (400 μ M). The non-linear regression fit of FW raw data ~~pointed~~
311 ~~out~~ [determined](#) an ED₅₀ value of 104 μ M. ~~Further, i~~ Inhibitory effects of umbelliferone to plants such
312 as *Festuca rubra*, *Medicago sativa* and *Lactuca sativa* have been reported [17, ~~3842~~]. Based on the
313 optimized umbelliferone concentrations, we designed the experiment to investigate the
314 metabolomic changes in seedlings ~~subjected~~ [exposed](#) to 6_h, 12_h, 24_h, 48_h, and 96_h of
315 umbelliferone [treatment](#), as compared ~~to~~ [with](#) the controls (mock treated) (**Figure 2**).

316

317 **3.2 Cataloging the wheat seedling metabolome**

318 Using GC-MS ~~based efforts~~, we ~~collectively~~ obtained quantitative data on 177 metabolites that were
319 derivatized (either derivatized singly or multiple times) or not, representing 139 non-redundant
320 (unique) metabolites. ~~Out~~ of these 139 metabolites, 118 were associated with a unique HMDB
321 identifier, while 113 were associated with a KEGG identifier. The derivatized metabolites included
322 sugars (monosaccharides, disaccharides), sugar alcohols, sugar acids, dipeptides, organic acids,

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323 amino acids, phosphates, polyamines, purines, and pyrimidines, while the non-derivatized
324 metabolites ~~were included~~ fatty acids, among others. We also captured several known secondary /
325 specialized metabolites such as phenolic ~~compounds~~ (polyphenols and flavonoids) ~~compounds~~, i.e.,
326 pyrocatechol, protocatechuic acid, chlorogenic acid, pyrogallol, homovanillate, sinapaldehyde,
327 catechin, caffeine, and myricetin; and others, such as phytol and quinolinic acid. We also captured
328 the modified (metabolized) forms of umbelliferone, i.e., 4-methylumbelliferone and psoralen. These
329 metabolites belonged to 50 different KEGG-based metabolic pathways (**Supplementary Figure 1**),
330 with the top pathways belonging to arginine and proline metabolism, glutathione metabolism,
331 aminoacyl-tRNA biosynthesis (all P-value < 0.05), taurine and hypotaurine metabolism, tryptophan
332 metabolism, beta-alanine metabolism, isoquinoline alkaloid biosynthesis, phenylalanine, tyrosine
333 and tryptophan metabolism, alanine, aspartate and glutamate metabolism (all P-value < 0.1), ~~and~~
334 indole alkaloid biosynthesis, among others (**Supplementary Figure 2**).

335

336 **3.3 Impact of umbelliferone on wheat metabolome**

337 Umbelliferone is an extremely biologically active coumarin widespread in the Umbelliferae family,
338 but also in other genera, in plant families such as Asteraceae, Rutaceae, Acanthaceae, ~~and~~
339 Hydrangeaceae, ~~among others~~ [14]. A huge body of research has clearly demonstrated that
340 application of umbelliferone can lead to phytotoxic effects, thereby affecting both plant growth and
341 development [24, 26-28]. We performed a one-way ANOVA on each compound, to test if at least
342 one level of time ~~has had~~ a mean average significantly different from the rest. ~~As a result,~~ There are
343 22 ~~significant~~ compounds (sugars, fatty acids, secondary metabolites, organic acids, and amino
344 acids) ~~significant~~ with p-value lower than 0.05 (**Table 1**). To control for false positive findings, an
345 False Discovery Rate (FDR) was applied ~~on to~~ the nominal p-values; 7 compounds (sugars—
346 maltose, xylulose, ribose, 6-deoxyglucose) were still significant after the FDR correction.

347

348 **3.4 Time-course profiling of umbelliferone treatment (quantitative)**

349 ~~Firstly,~~ ~~To understand the time-course-dependent changes in metabolite accumulation patterns~~
350 ~~across the treatment groups in this complex study design, we started with a clustering analysis.~~
351 Using ~~a tool called,~~ short time-series expression miner (STEM) analysis, we interrogated the time-
352 course changes of the metabolites for further analysis. The ~~datasets~~ ~~metabolite abundances for 177~~

353 ~~metabolites across the 6 time points~~ were ~~fit-put~~ into 20 ~~possible~~ model clusters, which revealed
354 differential accumulation of metabolites for control and ~~umbelliferone-umbelliferone~~-treated groups
355 of plants, ~~over the as a~~ function of time. In ~~the~~ case of the control plants, the most significant model
356 cluster (number 10, with 18 metabolites, P-val, 2E-3) showed a 0, 1, 0, 1, -1, 1 pattern (where 0 is
357 no change, 1 is increase, and -1 is decrease) ~~for the six time-points in the study [0 h (T0), 6 h (T1),~~
358 ~~12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5)]~~. These ~~18~~ metabolites ~~included-were~~ sugars (fucose,
359 maltose, trehalose, ~~xyloseand xylulose~~), organic acids (isohexonic acid, tranexamic acid,
360 ~~aconiticand aconitic~~ acid), amines (pyridoxamine, tryptamine), ribulose 1, 5-bisphosphate, 3-
361 indoleacetone, etc. (**Figure 3 A, B**). In ~~the~~ case of ~~umbelliferone-umbelliferone~~-treated plants,
362 the most significant model cluster (number 9, with 13 metabolites, P-val, 4E-4) showed a 0, 1, -1, -
363 1, 1, -1 pattern ~~for the six time-points in the study~~. These ~~13~~ metabolites ~~belonged-towere~~ sugars
364 (trehalose, xylulose, melibiose, ~~rhamnoseand rhamnose~~), organic acids (ascorbic acid, pimelic acid,
365 quinolic acid, ~~aconiticand aconitic~~ acid) ~~and~~ polyamines (putrescine and spermidine), etc. (**Figure 3**
366 **C, D**).

367

368 3.5 Multivariate and clustering analysis reveal metabolites

369

370 ~~Secondly, we performed both supervised and unsupervised multivariate analyses- as feature~~
371 ~~extraction strategies, to maximize variance in the data using strongly correlated variables. We first~~
372 ~~performed first-an unsupervised analysis, which did-explained 37-42-% of the variability in data~~
373 ~~using the first 2 PCs, either in all samples clustered together, only control sample groups, or~~
374 ~~umbelliferone groups (Supplementary Figure 8A-C). However, the time points did not cluster~~
375 ~~well-, pointing~~ which points to the non-independent samples which are not well handled by PCA,
376 ~~and~~ the small feature space of 177 metabolites, and too many treatments (6 time points x 2
377 treatments), leading to possible multicollinearity issues, displaying more artefacts than a true
378 biological picture. Following the lack of clustering in the PCA, we performed PLS-DA separately
379 for both control and umbelliferone treatment groups, where time-point based groupings were
380 observed. Using supervised PLS-DA analysis for all the samples (all time points, control and
381 umbelliferone treated plants) and the blanks (B), we showed that the first two components
382 explained ~~of~~ variations from the T0, 6 h, 12 h, 24 h, 48 h, ~~and~~ 96 h time points; components 1 and 2
383 alone explained ~45% of the variation (**Figure 4A**). For the control and ~~umbelliferone~~
384 ~~umbelliferone~~-treated plants, the first two components (1 and 2) helped explain ~14% and ~15% of
385 the variations, respectively (**Figures 4B, C**). ~~Further, †The co-clustering of time points- (i.e., 6 h~~

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386 with 96 h-time point) could be ~~pointing~~point to interesting biological phenomena, such as the
387 appearance of two peaks, one in very short-term defense response and another sustained one later.
388 ~~we have~~These are speculations, and would be very difficult to validate further using
389 metabolomics experiments and the premises of this study.

390
391 Using metabolite-metabolite (Pearson) correlation, we monitored the clusters ~~of groups~~ of
392 metabolites. ~~We found, among~~Among secondary metabolites, ~~we found that~~ 3-indoleacetonitrile
393 (an auxin, from tryptophan metabolism), psoralen and 4-methylumbelliferone (both umbelliferone
394 derivatives) and 2-coumaric acid were highly correlated (Supplementary Figure 3, 4), indicating
395 their possibly coordinated biosynthesis and regulation. ~~For the rest another tight cluster was also~~
396 ~~evident~~. Similarly, tight clusters were observed for fatty acids (Supplementary Figure 5), groups
397 of amino acids (Supplementary Figure 6), and carbohydrates (Supplementary Figure 7). A
398 recent study— that looked at various polyphenols across diverse species ~~have~~observed that
399 ~~quantitatively~~, umbelliferone and kaempferol are ~~quantitatively~~ associated with each other, while
400 ~~there was a~~ positive correlation of epicatechin ~~existed~~ with umbelliferone and kaempferol [3943].

401
402 In order to identify the metabolites responsible for the discrimination among the metabolomic
403 profiles, the VIP scores ~~was were~~ used to select those with the most significant contributions in a
404 PLS-DA model. ~~this is a measure of a variable's importance in the PLS-DA model.~~ VIP scores
405 are a weighted sum of PLS weights for each variable, and measure the contribution of each
406 predictor variable to the model [4044]. ~~Further, the~~The VIP statistic ~~summarizes indicates~~ the
407 importance of the metabolites in differentiating the study groups (~~i.e.~~, umbelliferone treatment
408 times, i.e., 0_h, 6_h, 12_h, 24_h, 48_h, 96_h) in multivariate space. The compounds exhibiting the
409 higher VIP scores are the more influential variables. Our VIP analysis revealed— that the
410 metabolites with high VIPs were phospho-L-serine, maltose, dehydroquinic acid, pyrocatechol,
411 tryptamine, and serotonin, among others (Figure 5). Thus, the biochemical changes induced by
412 umbelliferone treatment may ~~provide support~~ mechanistic explanations of the plant metabolic
413 responses induced by this coumarin compound. In particular, as highlighted by the VIP scores
414 reported (Figure 5), several metabolites involved in both shikimate and tryptophan pathways were
415 significantly altered by the treatment. Among them, fluctuations in dehydroquinic acid abundances
416 during all the treatments ~~in are~~ noteworthy, where the highest values were recorded at 12 h and 96
417 h. Dehydroquinic acid represents the first carbocyclic intermediate of the shikimate pathway, which
418 undergoes five further enzymatic steps in the remainder of the shikimate pathway to yield chorismic
419 acid, a precursor to tyrosine, 3-phenylalanine, tryptophan, and some vitamins [4145]. Interestingly,

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420 pyridoxamine (vitamin B6) ~~showed significant alterations~~was significantly altered by the
421 umbelliferone treatment; ~~it~~ reached highest abundance at 24 h of treatment, dropped after 48 h and
422 ~~again~~increased ~~again~~ at 96 h; ~~it~~ is an essential coenzyme with a high antioxidant potential [4246].
423 Moreover, pyridoxamine in ~~the~~ presence of ATP is converted by the pyridoxal kinase in pyridoxal
424 5'-phosphate, which is strictly connected to the enzyme tryptophan synthetase, an enzyme that
425 catalyses the final two steps in the biosynthesis of tryptophan [4347]. The tryptophan synthetase,
426 typically found as ~~an~~ $\alpha_2\beta_2$ tetramer, catalyses the irreversible condensation of indole and serine to
427 form tryptophan in a pyridoxal 5'-~~phosphate-phosphate~~-dependent reaction [4448]. ~~In addition, the~~
428 ~~conversion of tryptophan to indole~~indole acetic acid leads to the formation of glutamate, which is
429 one of the pyridoxamine precursors [49]. It is therefore conceivable that, as detailed below, the
430 ~~umbelliferone-triggered perturbation of the tryptophan metabolism might be on the basis of the~~
431 ~~observed pyridoxamine accumulation pattern upon~~over time. ~~Also, it is~~It is also possible that ~~the~~
432 ~~fluctuation in pyridoxamine content is attributable to the conversion into their derivatives, namely~~
433 ~~pyridoxal, pyridoxal 5- phosphate, and pyridoxamine [50], involved in many other cellular~~
434 ~~functions, which has not been detected in the present experiment~~were simply not detected /
435 ~~quantified in our metabolomics experiments.~~ Among the metabolites involved in tryptophan
436 biosynthesis, phospho-L-serine [4551] was characterized by ~~the~~ highest VIP score ~~value~~, pointing
437 ~~out to~~ a significant increase in concentration ~~along over~~ time. This molecule ~~play~~ has a pivotal role
438 in plants under environmental stresses; as an upregulation of several genes involved in this
439 pathway were observed during abiotic stresses; such as salinity, cold and flood, indicating its
440 importance in supplying serine under environmental stresses [4652]. Moreover, the phosphorylated
441 pathway might be essential to provide the amino acid; serine for the synthesis of tryptophan, the
442 common precursor for the biosynthesis of indole acetic acid (IAA) [4753]. Interestingly, in our
443 experiments, significant variations in IAA and tryptamine (indole-alkaloid) content, an intermediate
444 in IAA biosynthesis, were observed. In fact, both metabolites were significantly elicited by the
445 umbelliferone treatment. Alteration in IAA biosynthesis and distribution, driven by 4-
446 methylumbelliferone (an umbelliferone derivative), was previously observed by Li et al. [25] in
447 *Arabidopsis* seedlings. In particular, they observed that the exogenous application of 4-
448 methylumbelliferone (125 μ M for 22 days) led to reduced primary root growth, the formation of
449 bulbous root hairs, and an increase in ~~the number of~~ lateral root ~~numbers~~. The authors also ~~unveiled~~
450 ~~uncovered the an~~ accumulation of 4-methylumbelliferyl- β -D-glucoside, derived from UDP-
451 ~~glycosyltransferases~~ glycosyltransferase mediated transformation of ~~umbrelliferone~~umbelliferone in
452 roots ~~as well as the and~~ upregulation of several *UDP-glycosyltransferase* genes, which were
453 supportive for a well-orchestrated mechanism devoted to the detoxification of umbelliferone in

454 plants. During our experiments, the presence of both 4-methylumbelliferone and psoralen,
455 ~~umbelliferone derivatives, were~~ *was* detected in ~~umbelliferone~~ *umbelliferone*-treated plants,
456 suggesting that the umbelliferone ~~supplied~~ was internalized and metabolized by the seedlings.
457 ~~It has been proven~~ *Based on studies from several other species, it has been proven* that both
458 *umbelliferone derivatives can act as phytoalexins themselves; they can protect* ~~ing~~ plants from both
459 *biotic and abiotic stresses, and/or can induce reduction in growth and development [25, 54-56].*
460 *Therefore, it cannot be excluded that the reduction in plant growth observed during the dose*
461 *response curve could be also be due to the accumulation of umbelliferone derivatives. In fact,*
462 ~~Recent studies carried on the species of~~ *Psoralea corylifolia, treated with psoralen elicitors and*
463 *precursors, demonstrated that there is a negative correlation between psoralen accumulation and cell*
464 *growth [57]. Anyway, Furthermore, it should not be forgotten that psoralen accumulation in plants,*
465 *as well as other specialized metabolites, plays a pivotal role in protecting plants from several other*
466 *stresses [57], and probably the observed plant growth reduction could be is probably due to the*
467 *redistribution of plant energies in the activation of (specialized) biosynthetic pathways involved in*
468 *detoxification and/or protection from oxidative stress, instead of the biosynthesis of (primary)*
469 *metabolites fundamental for growth.*
470 ~~Despite the~~ *its* role ~~of~~ *as an* intermediate in auxin biosynthesis, it has been suggested that
471 tryptamine could play an important role during both biotic and abiotic stress. It has been observed,
472 for example, that barley leaves irradiated with UV light were accumulating high ~~contents~~ *levels* of
473 tryptamine. Moreover, its induction was also observed to occur in response to plant pathogenic
474 fungi infection, suggesting that it could act as a plant defense metabolite [4858]. On the other hand,
475 tryptamine accumulation was accompanied by a reduction in serotonin content. It has been ~~largely~~
476 *widely* reported that in graminaceous species the enzyme tryptamine 5-hydroxylase is involved in
477 serotonin biosynthesis, catalyzing the conversion of tryptamine to serotonin [4959, 5060]. Kang et
478 al. [5161] demonstrated that the exogenous application of tryptamine to tissues of rice seedlings
479 induced a dose-dependent increase in serotonin, accompanied by a parallel increase in tryptamine 5-
480 hydroxylase enzyme activity. At the same time, the same tissues grown in ~~the~~ presence of
481 tryptophan did not show any significant increase in serotonin. Therefore, it ~~could~~ *can* be speculated
482 that tryptamine accumulation, followed by the reduction in serotonin content, could be due to an
483 umbelliferone-induced reduction of ~~the~~ tryptamine 5-hydroxylase activity. ~~Further,~~ *Serotonin,*
484 *which plays a pivotal role in plant growth regulation and in plant response to both biotic and abiotic*
485 *stress [5262], and psoralen, are considered to be phytoalexins with antioxidant* ~~activities~~ *properties,*
486 ~~is an important molecule~~ *involved in plant defense [5355].*

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488 *Limitations of the study*

489 ~~Clearly, our study suffers from~~has several limitations. ~~Furthermore,~~Firstly, ~~deconvoluting~~
490 ~~separating~~ sample preparations based on separate ~~analysis of shoots and roots,~~ or leaf analysis,
491 would ~~have provided~~ more spatial information on organ- and ~~plant~~ part-specific metabolic changes,
492 ~~which could have been mixed up in which may have confounded the analysis in this whole plant~~
493 ~~preparations~~seedling analysis approach. Secondly, the overall feature space (i.e., the number of
494 ~~metabolites~~) is also very limited. Our current total metabolites quantified ($p = 177$) is roughly three
495 ~~times the overall sample size~~ ($n = 53$). Hence, the data is limited in dimensionality. These
496 ~~metabolites are also highly correlated both at intra- and inter-group levels, limiting the overall~~
497 ~~variance contributions. High correlations can also often contribute to multicollinearity. All of these~~
498 ~~factors, taken together, can limit the overall results and interpretations of this the current study.~~
499 Lastly, ~~techniques~~ other than mass-~~spectrometry~~-spectrometry-based analysis, i.e., ~~additional~~
500 ~~orthogonal techniques~~ such as liquid chromatography-mass-~~spectrometry~~ (LC-MS) ~~nuclear~~
501 ~~magnetic resonance (NMR) with wider metabolic coverage and less complex sample preparations~~
502 ~~steps (i.e., drying and derivatization), could be~~may have been ~~helpful in the~~ identification and
503 ~~absolute~~-relative quantification of various metabolites ~~belonging to more numbers of pathways, and~~
504 ~~captureing~~ multiple secondary metabolites involved in plant stress metabolic responses.

506 **4. Conclusions**

507 ~~In this study, we clearly showed~~the system-wide metabolomic changes in wheat seedlings in
508 response to ~~the umbelliferone treatment of an elicitor, umbelliferone. Although this molecule has~~
509 ~~been studied largely extensively extensively studied in the past, this is the first time in which that a~~
510 ~~short-term experiments using sub-lethal concentrations has been~~was has been carried out. This
511 ~~untargeted metabolomics approach allowed us to identify the immediate~~system-wide metabolic
512 ~~responses activated by the plants to deal with this phytotoxic compound. Among them, one of the~~
513 ~~first responses activated by plants was the internalization of umbelliferone into its derivative~~
514 ~~psoralen. In addition, umbelliferone induced a system-wide changes through the dysregulation of~~
515 ~~Moreover, the time course analysis revealed that metabolites involved in the shikimate pathways, as~~
516 ~~well as in tryptophan and tryptamine metabolism. confirming metabolism the hypothesis~~
517 ~~previously postulated that this molecule (and its derivatives) could interfere with IAA biosynthesis~~
518 ~~[25]. 22 compounds showed differential abundance during the study, and upon elicitor treatment.~~
519 ~~Further, the umbelliferone treated plants demonstrated a clearly discernible pattern constituting of~~

520 ~~metabolites that belonged to sugars, organic acids, and polyamines.~~ This study provides new
521 insights into the early response of plants to this specialized metabolites, and would represent can be
522 used as giving the a tool references for further studies aimed ~~at~~ at clarifying ~~that will be focused on~~
523 ~~the identification of its mode of action.~~ elicitor-induced metabolic changes in both primary and
524 specialized metabolism-chemical landscape in crop species.

525

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529 **Author Contributions**

530 FA, BBM and MRA conceived and designed the study; FA and ML performed the experiments;
531 BBM, VD, FA analyzed the data; FA and MRA contributed reagents/materials/analysis tools;
532 BBM, FA, MRA and ML wrote the paper.

533

534 **Conflicts of Interest**

535 ~~All~~ VD currently works as a Post-Doctoral Researcher in Novo Nordisk, however he did not
536 receive any funding for this work. ~~All~~ the authors declare that ~~he~~ they had no conflicts of interest.

537

538 **5. References**

- 539 [1] S. Rizvi, H. Haque, V. Singh, V. Rizvi, A discipline called allelopathy, in: Allelopathy,
540 Springer, 1992, pp. 1-10.
- 541 [2] F. Araniti, M. Scognamiglio, A. Chambery, R. Russo, A. Esposito, B. D'Abrosca, A. Fiorentino,
542 A. Lupini, F. Sunseri, M.R. Abenavoli, Highlighting the effects of coumarin on adult plants of
543 *Arabidopsis thaliana* (L.) Heynh. by an integrated-omic approach, *J. Plant Physiol.* 213 (2017) 30-
544 41.
- 545 [3] B. D'Abrosca, M. Scognamiglio, V. Fiumano, A. Esposito, Y.H. Choi, R. Verpoorte, A.
546 Fiorentino, Plant bioassay to assess the effects of allelochemicals on the metabolome of the target
547 species *Aegilops geniculata* by an NMR-based approach, *Phytochem.* 93 (2013) 27-40.

- 548 [4] M. Scognamiglio, B. D'Abrosca, A. Esposito, A. Fiorentino, Metabolomics: an unexplored tool
549 for allelopathy studies, *J. Allelochem. Int.* 1 (2015) 9-21.
- 550 [5] F. Araniti, A. Lupini, F. Sunseri, M.R. Abenavoli, Allelopathic potential of *Dittrichia viscosa*
551 (L.) W. Greuter mediated by VOCs: a physiological and metabolomic approach, *PloS one*, 12
552 (2017) e0170161.
- 553 [6] F. Araniti, A. Lupini, A. Mauceri, A. Zumbo, F. Sunseri, M.R. Abenavoli, The allelochemical
554 trans-cinnamic acid stimulates salicylic acid production and galactose pathway in maize leaves: A
555 potential mechanism of stress tolerance, *Plant Physiol. Biochem.* 128 (2018) 32-40.
- 556 [7] S.O. Duke, J. Bajsa, Z. Pan, Omics methods for probing the mode of action of natural and
557 synthetic phytotoxins, *J. Chem. Ecol.* 39 (2013) 333-347.
- 558 [8] J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A.R. Fernie, Gas chromatography mass
559 spectrometry-based metabolite profiling in plants, *Nat. Protoc.* 1 (2006) 387.
- 560 [9] F. Araniti, T. Gullì, M. Marrelli, G. Statti, A. Gelsomino, M.R. Abenavoli, *Artemisia*
561 *arborescens* L. leaf litter: phytotoxic activity and phytochemical characterization, *Acta Physiol.*
562 *Plant.* 38 (2016) 128.
- 563 [10] U. Roessner, A. Luedemann, D. Brust, O. Fiehn, T. Linke, L. Willmitzer, A.R. Fernie,
564 Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified
565 plant systems, *The Plant Cell.* 13 (2001) 11-29.
- 566 [11] O. Fiehn, Metabolomics—the link between genotypes and phenotypes, in:—_Functional
567 genomics, Springer, 2002, pp. 155-171.
- 568 [12] S. Brown, A. Zobel, Biosynthesis and distribution of coumarins in the plant, in:—_Proceedings
569 of the Conference «Coumarins: Research and Applications», Padua, Italy, 1990, pp. 20-22.
- 570 [13] A. Zobel, S. Brown, Coumarins in the interactions between the plant and its environment,
571 *Allelopathy J.* 2 (1995) 9-20.
- 572 [14] O. Mazimba, Umbelliferone: sources, chemistry and bioactivities review, *Bulletin of Faculty of*
573 *Pharmacy, Cairo University*, 55 (2017) 223-232.
- 574 [15] E. Haggag, I. Mahmoud, E. Abou-Moustafa, T. Mabry, Coumarins, fatty acids, volatile and
575 non-volatile terpenoids from the leaves of *Citrus aurantium* L.(sour orange) and *Citrus sinensis* (L.)
576 Osbeck (sweet orange), *Asian J. Chem.* 11 (1999) 784-789.

- 577 [16] S. Yaoya, H. Kanho, Y. Mikami, T. Itani, K. Umehara, M. Kuroyanagi, Umbelliferone released
578 from hairy root cultures of *Pharbitis nil* treated with copper sulfate and its subsequent glucosylation,
579 *Biosci. Biotech. Biochem.* 68 (2004) 1837-1841.
- 580 [17] H. Guo, H. Cui, H. Jin, Z. Yan, L. Ding, B. Qin, Potential allelochemicals in root zone soils of
581 *Stellera chamaejasme* L. and variations at different geographical growing sites, *Plant Growth*
582 *Regul.* 77 (2015) 335-342.
- 583 [18] T. Minamikawa, T. Akazawa, I. Uritani, Analytical study of umbelliferone and scopoletin
584 synthesis in sweet potato roots infected by *Ceratocystis fimbriata*, *Plant Physiol.* 38 (1963) 493.
- 585 [19] M. Repčák, J. Imrich, M. Franeková, Umbelliferone, a stress metabolite of *Chamomilla*
586 *recutita* (L.) Rauschert, *J. Plant Physiol.* 158 (2001) 1085-1087.
- 587 [20] L. Yang, S. Li, X. Qin, G. Jiang, J. Chen, B. Li, X. Yao, P. Liang, Y. Zhang, W. Ding,
588 Exposure to umbelliferone reduces *Ralstonia solanacearum* biofilm formation, transcription of type
589 III secretion system regulators and effectors and virulence on tobacco, *Front. Microbiol.* 8 (2017)
590 1234.
- 591 [21] D. Hamerski, R.C. Beier, R.E. Kneusel, U. Matern, K. Himmelpacht, Accumulation of
592 coumarins in elicitor-treated cell suspension cultures of *Ammi majus*, *Phytochem.* 29 (1990) 1137-
593 1142.
- 594 [22] L.A. Weiss, A. Dahanukar, J.Y. Kwon, D. Banerjee, J.R. Carlson, The molecular and cellular
595 basis of bitter taste in *Drosophila*, *Neuron.* 69 (2011) 258-272.
- 596 [23] A. Brencic, S.C. Winans, Detection of and response to signals involved in host-microbe
597 interactions by plant-associated bacteria, *Microbiol. Mol. Biol. Rev.* 69 (2005) 155-194.
- 598 [24] E. Kupidowska, M. Kowalec, G. Sulkowski, A. Zobel, The effect of coumarins on root
599 elongation and ultrastructure of meristematic cell protoplast, *Ann. Bot.* 73 (1994) 525-530.
- 600 [25] X. Li, M.Y. Gruber, D.D. Hegedus, D.J. Lydiate, M.-J. Gao, Effects of a coumarin derivative,
601 4-methylumbelliferone, on seed germination and seedling establishment in *Arabidopsis*, *J. Chem.*
602 *Ecol.* 37 (2011) 880.
- 603 [26] L. Pan, X.-z. Li, Z.-q. Yan, H.-r. Guo, B. Qin, Phytotoxicity of umbelliferone and its analogs:
604 Structure–activity relationships and action mechanisms, *Plant Physiol. Biochem.* 97 (2015) 272-
605 277.

606 [27] D.E. Moreland, W.P. Novitzky, Effects of phenolic acids, coumarins, and flavonoids on
607 isolated chloroplasts and mitochondria, *Allelochemicals: role in agriculture and forestry*, 330 (1987)
608 247-261.

609 [28] F. Einhellig, The physiology of allelochemical action: clues and views, *Allelopathy from*
610 *molecules to ecosystems*, (2002) 1-23.

611 [29] F.A. Macías, D. Castellano, J.M. Molinillo, Search for a standard phytotoxic bioassay for
612 allelochemicals. Selection of standard target species, *J. Agricul. Food Chem.* 48 (2000) 2512-2521.

613 [30] H. Tsugawa, T. Cajka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. VanderGheynst,
614 O. Fiehn, M. Arita, MS-DIAL: data-independent MS/MS deconvolution for comprehensive
615 metabolome analysis, *Nat. Meth.* 12 (2015) 523.

616 [31] J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmüller, P. Dörmann, W.
617 Weckwerth, Y. Gibon, M. Stitt, GMD@ CSB. DB: the Golm metabolome database, *Bioinformatics*,
618 21 (2004) 1635-1638.

619 [32] H. Horai, M. Arita, S. Kanaya, Y. Nihei, T. Ikeda, K. Suwa, Y. Ojima, K. Tanaka, S. Tanaka,
620 K. Aoshima, MassBank: a public repository for sharing mass spectral data for life sciences, *J. Mass*
621 *Spectr.* 45 (2010) 703-714.

622 [33] S.-A. Sansone, T. Fan, R. Goodacre, J.L. Griffin, N.W. Hardy, R. Kaddurah-Daouk, B.S.
623 Kristal, J. Lindon, P. Mendes, N. Morrison, The metabolomics standards initiative, *Nat. Biotech.* 25
624 (2007) 846.

625 [34] R.C. Team, R: A language and environment for statistical computing; 2015, in, 2018.

626 [35] F.J. Rohlf, R.R. Sokal, *Statistical tables*, Macmillan, 1995.

627 [36] D. Grapov, DeviumWeb: version 0.3.2. ZENODO doi:10.5281/zenodo.12879,
628 <https://github.com/dgrapov/DeviumWeb>, (2014).

629 [37] R.G. Belz, K. Hurle, S.O. Duke, Dose-response—a challenge for allelopathy?, *Nonlin. Biol.*
630 *Toxic. Med.* 3 (2005) nonlin. 003.

631 [38] G. Caraux, S. Pinloche, [PermutMatrix: a graphical environment to arrange gene expression](#)
632 [profiles in optimal linear order](#), *Bioinformatics* 21(7) (2005) 1280-1281 (2014).

633 [39] D. Grapov, DeviumWeb: Version 0.3.2. ZENODO. <https://doi.org/10.5281/zenodo.12879>,
634 <https://github.com/dgrapov/DeviumWeb>.

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635 [\[40\] J. Ernst, Z. Bar-Joseph, STEM: a tool for the analysis of short time series gene expression data.](#)
636 [BMC bioinformatics 7\(1\) \(2006\) 191.](#)

637 [\[41\] J. Xia, N. Psychogios, N. Young, D.S. Wishart, MetaboAnalyst: a web server for metabolomic](#)
638 [data analysis and interpretation. Nucl. Acids Res. 37\(suppl 2\) \(2009\) W652-W660.](#)

639 [\[3842\]](#) Z. Yan, D. Wang, H. Cui, D. Zhang, Y. Sun, H. Jin, X. Li, X. Yang, H. Guo, X. He,
640 Phytotoxicity mechanisms of two coumarin allelochemicals from *Stellera chamaejasme* in lettuce
641 seedlings, Acta Physiol. Plant. 38 (2016) 248.

642

643 [\[3943\]](#) R. Kumar, S. Mahey, R. Arora, J. Mahajan, V. Kumar, S. Arora, Insights into biological
644 properties of less explored bark of industrially important *Acacia catechu* Willd, Ind. Crops Prod.
645 138 (2019) 111486.

646 [\[4044\]](#) R.C. Smart, E. Hodgson, Molecular and biochemical toxicology, John Wiley & Sons, 2018.

647 [\[4145\]](#) B. Ganem, From glucose to aromatics: recent developments in natural products of the
648 shikimic acid pathway, Tetrahedron, 34 (1978) 3353-3383.

649 [\[4246\]](#) O. Titiz, M. Tambasco- Studart, E. Warzych, K. Apel, N. Amrhein, C. Laloi, T.B.
650 Fitzpatrick, PDX1 is essential for vitamin B6 biosynthesis, development and stress tolerance in
651 Arabidopsis, The Plant J. 48 (2006) 933-946.

652 [\[4347\]](#) M.F. Dunn, D. Nicks, H. Ngo, T.R. Barends, I. Schlichting, Tryptophan synthase: the
653 workings of a channeling nanomachine, Trends Biochem. Sci. 33 (2008) 254-264.

654 [\[4448\]](#) S. Raboni, S. Bettati, A. Mozzarelli, Tryptophan synthase: a mine for enzymologists, Cell.
655 Molecul. Life Sci. 66 (2009) 2391-2403.

656 [\[49\] M. Tambasco-Studart, O. Titiz, T. Raschle, G. Forster, N. Amrhein, T.B. Fitzpatrick, Vitamin](#)
657 [B6 biosynthesis in higher plants. P. Natl. A. Sci. 102\(38\) \(2005\) 13687-13692](#)

658 [\[50\] P. Bilski, M.Y.-Li, M. Ehrenshaft, M.E. Daub, C.F. Chignell, Vitamin B6 \(pyridoxine\) and its](#)
659 [derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. Photochem.](#)
660 [Photobiol. 71\(2\) \(2000\) 129-134](#)

661 [\[4551\]](#) F. Busch, C. Rajendran, O. Mayans, P. Löffler, R. Merkl, R. Sterner, TrpB2 enzymes are O-
662 phospho-L-serine dependent tryptophan synthases, Biochem. 53 (2014) 6078-6083.

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663 [4652] C.-L. Ho, K. Saito, Molecular biology of the plastidic phosphorylated serine biosynthetic
664 pathway in *Arabidopsis thaliana*, *Amino acids*, 20 (2001) 243-259.

665 [4753] C. Won, X. Shen, K. Mashiguchi, Z. Zheng, X. Dai, Y. Cheng, H. Kasahara, Y. Kamiya, J.
666 Chory, Y. Zhao, Conversion of tryptophan to indole-3-acetic acid by tryptophan aminotransferases
667 of *Arabidopsis* and YUCCAs in *Arabidopsis*, *Proceedings of the National Academy of Sciences*,
668 108 (2011) 18518-18523.

669 [54] L. Nebo, R.M. Varela, J.M. Molinillo, O.M. Sampaio, V.G. Severino, C.M. Casal, M.F. das
670 Grac_{as} Fernandes, J.B. Fernandes, Macías, F. A., Phytotoxicity of alkaloids, coumarins and
671 flavonoids isolated from 11 species belonging to the Rutaceae and Meliaceae families. *Phytochem.*
672 *Lett.*, 8 (2014) 226-232.

673 [55] S. Jan, T. Parween, T. Siddiqi, Anti-oxidant modulation in response to gamma radiation
674 induced oxidative stress in developing seedlings of *Psoralea corylifolia* L. *J. Environ. Radioact.*
675 *113* (2012) 142-149.

676 [56] D. Solecka, Role of phenylpropanoid compounds in plant responses to different stress factors.
677 *Acta Physiol. Plantarum* 19(3) (1997) 257-268.

678 [57] G. Hari, K. Vadlapudi, P.D. Vijendra, J. Rajashekar, T. Sannabommaji, G. Basappa, A
679 combination of elicitor and precursor enhances psoralen production in *Psoralea corylifolia* Linn.
680 suspension cultures. *Ind. Crops Prod.* 124 (2018) 685-691.

681 [4858] H. Miyagawa, H. Toda, T. Tsurushima, T. Ueno, J. Shishiyama, Accumulation of tryptamine
682 in barley leaves irradiated with UV light, *Biosci. Biotech. Biochem.* 58 (1994) 1723-1724.

683 [4959] T. Fujiwara, S. Maisonneuve, M. Isshiki, M. Mizutani, L. Chen, H.L. Wong, T. Kawasaki,
684 K. Shimamoto, Sekiguchi lesion gene encodes a cytochrome P450 monooxygenase that catalyzes
685 conversion of tryptamine to serotonin in rice, *J. Biol. Chem.* 285 (2010) 11308-11313.

686 [5060] S. Park, T.N.N. Le, Y. Byeon, Y.S. Kim, K. Back, Transient induction of melatonin
687 biosynthesis in rice (*Oryza sativa* L.) during the reproductive stage, *J. Pineal Res.* 55 (2013) 40-45.

688 [5461] S. Kang, K. Kang, K. Lee, K. Back, Characterization of tryptamine 5-hydroxylase and
689 serotonin synthesis in rice plants, *Plant Cell Rep.* 26 (2007) 2009-2015.

690 [5262] H. Kaur, S. Mukherjee, F. Baluska, S.C. Bhatla, Regulatory roles of serotonin and melatonin
691 in abiotic stress tolerance in plants, *Plant Sign. Behav.* 10 (2015) e1049788.

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692 ~~[5355] S. Jan, T. Parween, T. Siddiqi, Anti oxidant modulation in response to gamma radiation~~
693 ~~induced oxidative stress in developing seedlings of *Psoralea corylifolia* L, J. Environ. Radioact.~~
694 ~~113 (2012) 142-149.~~

696 Figure Captions and Table Legends

698 Figures

699 **Figure 1:** Dose-response curve evaluated on a FW base of *Triticum durum* cv. Opera [seedlings](#)
700 ~~(n=??)~~ treated for 10 days with different doses (0, 12.5, 25, 50, 100, 200, 400 μ M) of
701 ~~Umbelliferone~~[umbelliferone](#). Data were analyzed through one-way ANOVA using LSD as post hoc
702 ($P \leq 0.05$). ED₅₀ (μ M) value was calculated through a log-logistic equation fitting the total FW data
703 gotten from seedlings treated with different doses of the allelochemical. The curve pointed out a
704 significance level of $P < 0.001$. Bars indicate standard deviation. n=5.

706 **Figure 2.** Schematic diagram displaying the experimental design, platform and software tools used
707 for the analysis of metabolomic changes in wheat seedlings subjected to umbelliferone elicitation.

709 **Figure 3. Time course changes in the control and umbelliferone treated wheat seedlings.** (A)
710 Model [profile](#)s displaying the time-sensitive changes in metabolite abundance in control plants; (B)
711 Metabolite abundance profile in model # 10 (statistically significant) in control plants; (C) ~~Models~~
712 [Model profiles](#) displaying the time-sensitive changes in metabolite abundance in umbelliferone-
713 treated plants; (D) Metabolite abundance profile in model # 9 (statistically significant) in
714 umbelliferone-treated plants. [In panels A and C, the number in the upper left on each model profile](#)
715 [designates the model number \(out of total 20 models generated\), and the number in the bottom left](#)
716 [on each model profile is the statistical significance of the model. n=5.](#)

718 **Figure 4. Multivariate (PLS-DA) analysis of the metabolomic changes.** (A) PLS-DA displaying
719 the separation of blank samples (B) from the rest of the samples showing system robustness; (B)
720 PLS-DA showing clusters of various time points in control plants; (C) PLS-DA showing clusters of
721 various time points in umbelliferone-treated plants. [n=5.](#)

723 **Figure 5.** Top 15 metabolites (variables) based on VIP scores from PLS-DA analysis for each
724 umbelliferone treatment time points (0_h, 6_h, 12_h, 24_h, 48_h, 96_h). The x-axis shows the
725 correlation scores whereas the y-axis corresponds to the metabolites identified. Color bars show
726 median intensity of variable in the respective group. [n=5](#).

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731 **Supplementary Materials**

732 **Supplementary Figure 1.** Visual display of the coverage of metabolites quantified using our GC-
733 MS platform for this metabolomics investigation. (KEGG-based metabolite mapped onto the
734 KEGG metabolic pathway map (*blue dots* represent the mapped metabolites quantified in our
735 study).

736

737 **Supplementary Figure 2.** KEGG-based pathway enrichment analysis displaying the wheat
738 seedling metabolome as covered using our GC-MS platform.—_Pathway names: 1-Glutathione
739 metabolism, 2-Arginine and proline metabolism, 3-Amino acyl-tRNA biosynthesis, 4-Taurine and
740 hypotaurine metabolism, 5-Tryptophan metabolism, 6-beta-Alanine metabolism, and 7-Isoquinoline
741 alkaloid biosynthesis.

742

743 **Supplementary Figure 3.** High Pearson (metabolite-metabolite) correlation of umbelliferone-
744 derived metabolites and polyphenol [metabolism-metabolism](#)-derived metabolites.

745

746 **Supplementary Figure 4.** High Pearson (metabolite-metabolite) correlation of umbelliferone-
747 derived metabolites with other quantified metabolites in the study.

748

749 **Supplementary Figure 5.** High Pearson (metabolite-metabolite) correlation of fatty acids.

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751 **Supplementary Figure 6.** High Pearson (metabolite-metabolite) correlation of amino acids.

752

753 **Supplementary Figure 7.** High Pearson (metabolite-metabolite) correlation among carbohydrates.

754

755 **Supplementary Figure 8.** Unsupervised principal component analysis (PCA) displaying the first 2
756 PCs for (A) all samples (control + Umbelliferone treatment) and time points together, (B) Control
757 samples and time points, and (C) Umbelliferone treatment samples and time points.

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Author Responses to Reviewer and Editorial Comments

Editor and Reviewer comments:

Reviewer #1: *The manuscript covers an interesting topic, which is the identification of the metabolites changing after allelochemical treatment. I find the introduction well written and updated, methodology well explained and results interesting and well discussed. I agree also with the authors about the limitations to the study and I find very honest that they state them clearly after the discussion. However, there are some points that should be changed before definitive submission in Plant Science.*

The authors would like to thank the reviewer for reviewing the presented study, kind comments and suggestions to improve the current work. We have now addressed all the comments below, raised by the reviewer to the best of our abilities.

1) *Indicate how many replicates were used for dose-response curve and for sections 2.2.1 and 2.2.2*

Thank you. As requested we have now specified this in the said sections, in the caption of Figures and in the text

2) *Was the solutions oxygenated during the dose-response curve?*

Thank you. Yes we oxygenated the solutions prior to the dose-response curve experiments, and this is now clarified in the text.

3) *Use ISI for symbols and units*

We have now double checked for the symbols and units throughout the entire manuscript text.

4) *Discuss better why do you think that pyridoxine increases, decreases and increases again along the time of treatment? which is the physiological meaning of these changes?*

Thank you for the comments on pyridoxine. Since we don't have any data concerning enzyme activity involved in pyridoxamine biosynthesis and degradation, we have attempted to explain a possible reason of its fluctuation as reported in the manuscript.

5) *Authors say that psoralen acts as phytoalexin, but that is just at low-medium concentrations. However, after umbelliferone treatment, psoralen content strongly increases. Psoralen has been found to act as phytotoxic substance. Please, discuss better whether you think that the effects you find are due to umbelliferone or to some derivative as psoralen.*

Thanks for your suggestions and pointing out the phytotoxicity of psoralen. The presented metabolite abundance data from our metabolomics experiment are relative and not an absolute quantitation of psoralen and other umbelliferone derivatives; thus we cannot not point to the phytotoxicity levels of the magnitude of increase in terms of their relevance to plant metabolism to conclude on its autotoxicity or protective activity. With the available data we are unable to point out psoralen effects on plant metabolism and its effects on cells of the seedlings, as this is a starting point which could be explored in our future studies for looking at specific umbelliferone-derivatives. Nevertheless, your comment is extremely pertinent (psoralen is phytotoxic and in previous works a negative correlation has been observed between plant growth and psoralen accumulation). Therefore we have added few sentences to take into account it.

6) *Please, review the manuscript. There are some little English mistakes, which should be corrected.*

-Thanks for the comments. We have now re-read and re-edited the entire manuscript for correct usage of grammar and language for a smooth reading experience. In addition we have sought the help from a native English speaking colleague of ours for helping us polish the language for clarity, correctness, and flow.

.....

Reviewer #2: *The manuscript is written well. There are few questions that I ask the authors to respond to:*

The authors would like to thank the reviewer for the comments, and we have now addressed all the concerns raised by the reviewer in the newly submitted version of the revised manuscript.

1) *Explain more clearly at which time were the plants collected?*

Thanks, as requested we have now specified this in the manuscript (§ 2.2.1 and 2.2.2).

2) *What was the control for the treatments T1-T5?*

Thanks for your comment. At each time point we have collected both control and treated plants. Since umbelliferone was previously dissolved in ethanol (01%), the same amount of solvent was also added to the control samples. It has been better specified in the manuscript, (§ 2.2.2)

3) *Where used for the first time, please give the full explanation of the abbreviations (HMDB and KEGG)*

Thanks, we have now spelled out the abbreviations whenever used for the first time.

4) *Were the seedlings treated with ED₅₀ dose of umbelliferone?*

Thanks for your comment. Yes, as reported in the text (2.2.2 “.....were grown for 10 days and were then treated with 104 µM umbelliferone (the ED₅₀ concentration calculated from a dose-response curve.....”) and the seedlings were then treated with the [ED₅₀] only.

5) *The first paragraph for chapter 3.5 should be edited.*

-Thanks, we have now amended the said paragraph in section 3.5 to explain the rationale of the multivariate statistical analyses conducted (i.e., PCA, PLS-DA) conducted and the analysis workflow as well as those in 3.4 (for STEM analysis).

6) The percent of explained variation is low (Fig 4b and C)- any explanation for that?

-Thanks, we have now specified this in the manuscript.

The low variances can often be due to multicollinearity contributed by high correlational behavior between predictor variables of use e.g. here metabolites and time-points in use. These can often lead to a smaller number of dimensions for the entire dataset. Such is often not common in gene expression (transcriptomic based) datasets but seen when dimensions are lower in reality where very few numbers of analytes (e.g. metabolites/proteins) are regarded as features over larger arm of observations (e.g. samples). In the current study the projection plots are based on PLS-DA which fits the supervised classification criteria i.e. often used while assessing and classifying metabolites data. It is either used independently or in conjunction by computing unsupervised PC scores upstream. These PC scores are then projected in lower dimension to outline class separation by supervised PLS-DA analysis for selected features. Often fitting higher PCs leads to low variance contributions due to multicollinearity in metabolites and the time-points used in comparison. This can also lead to such low variances for the ones being projected in PLS-DA. This can contribute to the low variances explained in the plots. Another reason that can also contribute to the same is usage of simple PCA and simple PLS-DA rather than fitting functional PCA upstream or sparse orthogonal PLS-DA that are often capable of integrating multiple time-points.

Ref 1: Shuangge Ma, Ying Dai, Principal component analysis based methods in bioinformatics studies, Briefings in Bioinformatics, Volume 12, Issue 6, November 2011, Pages 714–722, <https://doi.org/10.1093/bib/bbq090>

Ref 2: Worley, B., & Powers, R. (2013). Multivariate Analysis in Metabolomics. Current Metabolomics, 1(1), 92–107. <https://doi.org/10.2174/2213235X11301010092>

[Ref 3: Hadi, A., & Ling, R. \(1998\). Some Cautionary Notes on the Use of Principal Components Regression. The American Statistician, 52\(1\), 15-19. doi:10.2307/2685559](#)

7) Summary: what is the take-home message for this study?

Thanks, we have now summarized this more succinctly.

.....

Reviewer #3: *The authors have completed a lot of very interesting and tedious studies to investigate how elicitors like umbelliferone can impact plant growth and metabolism. The methods are clearly defined and include a great number of best practices for metabolomics analyses, which is very encouraging to see. While I feel this has a lot of important information, I feel that the conclusion isn't fully supported by the analysis. Clearing this up would provide a pathway through to publication, in this reviewer's opinion.*

The authors would like to thank the reviewer for the comments, and we have now addressed all the concerns raised by the reviewer in the newly submitted version of the revised manuscript. We have also taken into consideration the commented version of the PDF for incorporating edits to the revised manuscript.

1) p. 9-10, section 3.4: time-course profiling of umbelliferone treatment.

The authors devote a paragraph and a significant figure talking about a time-course modeling of the metabolome data. **However, this analysis isn't mentioned anywhere in the methods, nor are any references provided so readers can educate**

themselves on this process. A lot of information is implied without being explicitly defined, leaving the reader to only guess.

-Thanks, we have now specified this in the manuscript, in a new sub-section and expanded on the STEM analysis for more clarity.

Regarding the question on analytical/ statistical approach in this study:

As the reviewer can see, with 6 time points (which are short and hence biological variable!), and 2 treatments, and GC-MS as analytical platform available to us (limited with 177 metabolites/ features only!); is already a complex design.

To address this we took 2 analytical approaches:

(A) **Firstly we performed a clustering analysis, i.e., STEM analysis** to find patterns in metabolite changes across the 6 time points in C and U treatment;

(B) **Secondly we performed a multivariate analysis** to find metabolites/ Eigen vectors contributing to the discrimination of the time-points and treatments using:

- (i) **Unsupervised PCA analysis:** We performed first unsupervised analysis, which did explain ~40-43 % variability in data using the first 2 PCs, either in all samples taken together, or separately only on control sample groups, or on umbelliferone treatment groups. However, the time points did not cluster well-pointing to the non-independent samples which are not well handled by PCA; and small feature space of ~177 metabolites and too many treatments (6 time points x 2 treatments) leading to possible multicollinearity issues, displaying more artefacts than true biological picture. The PCA analysis score plots are now included as Supplementary Figure 8 for the readers for clarity. Please see below, more explanation on our PCA analysis:

In control (**Supplementary Figure 8B**)-

Max variance is contained in PC1 vs PC2 (Dim1 vs Dim2) ~ 29.3 % vs 13.4%

Overall control samples variance ~42.7%

Most samples or time-specific groups are along the Dim 2(Y-axes)

Dim 1(X-axes) has 2 samples belonging to 48H contributing to variances and separation in Dim1 (could be true biological)

Along Dim2 (Y-axis) 6H, 12H, and 96H are distributed along +ve Y-axis and seem they are more tightly clustered together.

96H is closer to 0H along +ve Y-axes in the cluster of 0H, 6H, 12H, and 96H

24H is having a higher variance than those along Y-axes.

In Treated samples with Umbelliferone (**Supplementary Figure 8C**)-

Max variance is contained in PC1 vs PC2 (Dim1 vs Dim2) ~ 25.2 % vs 15.2%

Overall treated samples variance ~40.4%

Most samples or time-specific groups are along the Dim 2 (Y-axes)

6H+U and 48H+U are in +ve Y-axis clustered closer to 0H

12H+U, 24H+U, and 96H+U are in -ve Y-axis.

(ii) **Supervised PLS-DA analysis:** Following lack of clustering in the PCA, we performed PLS-DA separately for both control and umbelliferone treatment groups, where time-point based groupings were observed. Though subjected to model over fit, the derived VIP scores for metabolites from PLS-DA pointed to biological mechanisms of actions (i.e., changes in simple sugar and organic acid metabolism). Further, the co-clustering of time points, i.e., 6 h with 96 h time point could be pointing to interesting biological phenomena, such as appearance of two peaks, one in very short term defense response and another sustained one later, we have these as speculations, and very difficult to validate further using metabolomics experiments and premises of this study.

In summary we can claim, that this is a complex experimental design with a limited overall feature space contributed by metabolites across time-points, and that there are

NO robust tools available that can handle such time-course omics experimental study design.

The following has been added now for explaining the STEM analysis to the readers:

“2.7 Time-course analysis of control and umbelliferone treated metabolomes

For short time series metabolomics data analysis we used the tool, Short Time series Expression Miner (STEM) (Ernst & Bar-Joseph, 2006), originally used for microarray time series experiments that are short (3–8 time points for >~80% of the datasets). The novel STEM clustering takes advantage of few time points in a dataset, and it first selects a set of distinct and representative temporal expression profiles (i.e., model profiles) where these model profiles are independent of data. The clustering algorithm then assigns each feature (i.e., metabolite) passing the filtering criteria to the model profile that most closely matches the feature's abundance profile as determined by the correlation coefficient and determines which profiles have a statistically significant higher number of features assigned using a permutation test. STEM was used as a Java implementation with a graphical user interface available at <http://www.cs.cmu.edu/~jernst/st/> for clustering the metabolite accumulation patterns according to time points. For our analysis, we used the following criteria: no additional normalization of the data, 0 added as starting point, number of model profiles= 20, maximum unit change in model profiles between time points= 3. We used expression of -1 for decreased levels of a metabolite, 0 for unchanged levels of metabolite, and 1 as increased levels of a metabolite to explain the model profiles. For instance, a model profile with an expression of -1,-1, 0, 1, 1, 0 represents: decrease, decrease, unchanged, increased, increased, and unchanged levels of a given set of metabolites for the 6 time points in the given model profile.”

2) *"The datasets were fit into 20 possible model clusters..." which datasets? How are the model clusters chosen? Why 20? What sort of prediction are the authors looking for? What are they trying to map it to?*

-Thanks, we have now specified this in the manuscript, in a new subsection, 2.7 in the method section of the manuscript, and expanded on the STEM analysis for more clarity. Please see the response to above comment on STEM analysis.

3) *"In case of the control plants, the most significant model cluster (number 10, with 18 metabolites, P-val, 2E-3) showed a 0, 1, 0, 1, -1, 1 pattern (where 0 is no change, 1 is increase and -1 is decrease)." Which 18 metabolites have been chosen? The previous section had identified 22 metabolites as having significant changes after treatment. What does the pattern relate to? Are these the time points used in the study? If so, that is never articulated.*

Thanks, we have now specified this in the manuscript, and have explicitly stated the metabolites in the very following line. To clarify, "the 20 model profiles are generated by the STEM algorithm" (and not chosen by us). Thus, the most significant model profile (#10) fits 18 metabolites with that pattern (based on the criteria used for our STEM analysis with model profiles= 20, and maximum unit change in model profiles between time points= 3) following which the STEM algorithm finds common clusters from the total number of metabolites .

On the other hand, the 22 significant metabolites coming from (repeated measures, time) ANOVA analysis depicts only in comparison of control vs. treatment to show which metabolites were significantly altered across the time-points and does not necessary depict any patterns/ clusters. Moreover, the STEM analysis generated time-series analysis clusters separately for the two treatments to demonstrate as to which metabolites changed over time for each of the two treatments, over time.

Yes, the patterns correspond to the 6 time-points used in the study, i.e., 0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5) for the STEM time-series analysis. We have now added some of the mentioned text here into the manuscript.

4) *"In case of umbelliferone treated plants, the most significant model cluster (number 9, with 13 metabolites, P-val, 4E-4) showed a 0, 1, -1, -1, 1, -1 pattern." Why is this using a*

different number of metabolites? Why is the pattern different? Does that mean something that the pattern is altered? How different should they be?

This section needs to be completely restructured, as it does not really show any bearing on the results, the conclusions, or any part of the study, and is generally incomprehensible to a reader.

-Thanks, we have now specified this in the manuscript discussion. Please see the comments provided to the above questions and it applies to this query as well. We have now added the explanation above to the text for clarity to the readers.

5) p. 10, section 3.5: multivariate and clustering analysis reveals metabolites

Figure 4. In this section, it becomes apparent that the multivariate analysis is not helping the authors with their data presentation. Figure 4A is a jumble of overlapping confidence ellipses from all the time points and treatment time points. It does not show that there is any significant difference between any of the treatments. The only ellipse which is not overlapping is the blank control.

-Thank you for your comments.

In Figure 4 A, essentially the PLS-DA demonstrates that the analysis “blanks (extraction/ dummy blanks) during the analysis shows a good separation from the rest of the samples, indicating the good S/N and system suitability for a successful metabolomic analysis. Of course, we have a huge contribution of the signals from true metabolite sin samples, the sample and blank cluster discrimination is explained up to 45% by the first two components.

However, among the 6 time-points, for both control (Figure 4 B) and umbelliferone treatment (Figure 4 C) scores plot, only about ~14% explanation of the variance points to several possibilities that are difficult to address solely based on this study:

-Six time-points and 5 replicates for each time points clearly outweighs overall features e.g. metabolites. It is a classical case where sample size or observations are not very

small when compared to number of quantified metabolites. In our case we have a sample size that is 1/3rd of the number of metabolites used.

-inability of simple PLS-DA to handle time-course analysis data to an extent. One of the reasons of such low variances in separate plots can be attributed to the fact that we have used default PLS-DA. Such is often contributing to lower variances if not integrating multiple time-point factors either by using functional unsupervised PC upstream or implement sparse orthogonal PLS-DA while projecting reduced dimensions.

-short term changes in metabolism is possibly very quick and the data is highly variable. Such variability is not seamlessly captured by the latent variables unless explicitly modelled using time-components or increasing the number of metabolites.

- Fig 4b and 4c also shows that despite of the trend in time-point shifts, there is intra-variability among the replicates within each time-points. This can contribute to the current nature of the results, and it can be better assessed with high number of metabolites which is currently not feasible in this study scope.

6) Furthermore, separating out the treatment and control time series (Figures 4C and 4B, respectively) does show a trend in terms of the metabolome shifting. There is a strange exception, the 6-hour point, in both series, which is not explained by the authors at all.

-Thank you for the comments. We double checked our data for any potential batch effects, sample queuing or harvesting etc. for 6 h samples, but could not see anything obvious to the 6 h time point samples. We have also figured out the same on the 6 h time point indicating several other possibilities. The co-clustering of time points, i.e., 6 h with 96 h time point could be pointing to interesting biological phenomena, such as appearance of two peaks, one in very short term defense response and another

sustained one later, we have these as speculations, and very difficult to validate further using metabolomics experiments and premises of this study.

7) *The two PLS-DA analyses also have very low variance accounted for in their scores plots (the first 2 PCs only explain <15% of the variance in the model). With such little being explained away, there is potentially something else that is driving the shifting metabolome. This should be discussed.*

-Thank you for the comments. Please see the explanation to the query above. We can claim, that this is a complex experimental design for a small feature space, and that there are NO robust tools currently available that can handle time-course omics experimental studies. Moreover, native PCA and PLS-DA are not the best ones to handle such data, other than simple clustering analysis.

8) *With such great overlap, it is strange the authors go on to use VIP to find the 'metabolites responsible for the discrimination among the metabolomic profiles.' What meaningful data could be pulled from a PLS-DA which has no real trend or discrimination between treatment and control plants? The data analysis needs to be re-thought.*

-Thanks for the comments. Ideally PLS-DA is a supervised class separation projection that is based on upstream PC computed. Based on those PC's one projects the dimensions using PLS-DA and makes the class or group discrimination. Again, we also did not use multiple time-point factors while computing the dimensions. All these can somehow explain the issue.

In Control according to the Fig 4b we see along X-axes the time-trends except for the 6h which seems to be closer to 48H and 96H. In our PCA 6H, 12H and 96H were clustering together in control. In PLS-DA control plot Y-axes seem to be showing the variability among the replicates in each time-point which is also seen in the PCA plots.

In our Treatment, Fig 4b, 0H is highly variable across Y-axes (intra replicate variation) and this is exhibited almost across all time points. The 12H+U simply behaves like the 0H and clusters closely with them.

The X-axes show a time-shift trend but 6H+U clusters again with 48H+U and 96H+U.

In our current study design, metabolites: samples ratio being 3:1, might lead to multicollinearity as predictor variables are highly correlated. Thus, interfering with distinct separation of time-points as well. Reason why we see low variances in Fig 4b and 4c and a faint trend of shift but not explicit large separation. Again, intra- time-point variability is also high which cannot be controlled and may lead to overfitting unless we have a greater number of metabolites.

Note on VIP: VIP scores larger than 1 indicate the most relevant variables, and help focus on the importance of the X-variables on the latent projection (Ref 1). Thus, with our VIP analysis met the criterion for reporting the VIPs, though the scores are not very large than the average cut-off of 1.

[Ref1 : Mendez, K.M., Broadhurst, D.I. and Reinke, S.N., 2020. Migrating from partial least squares discriminant analysis to artificial neural networks: a comparison of functionally equivalent visualisation and feature contribution tools using Jupyter notebooks. *Metabolomics*, 16\(2\), p.17.](#)

9) One side note: the scores plots presented do not match the scheme outlaid in Figure 2 or the graphical abstract. What is that scores plot?

-Thanks you for the comments. We have now modified the Graphical Abstract and the Figure 2 with the correct scores plot from Figure 4 B, C from the PLS-DA analyses of both C and U treatments and have specified the same. The current one was prepared only based on highly differential metabolites (a biased approach) for representation purposes only.

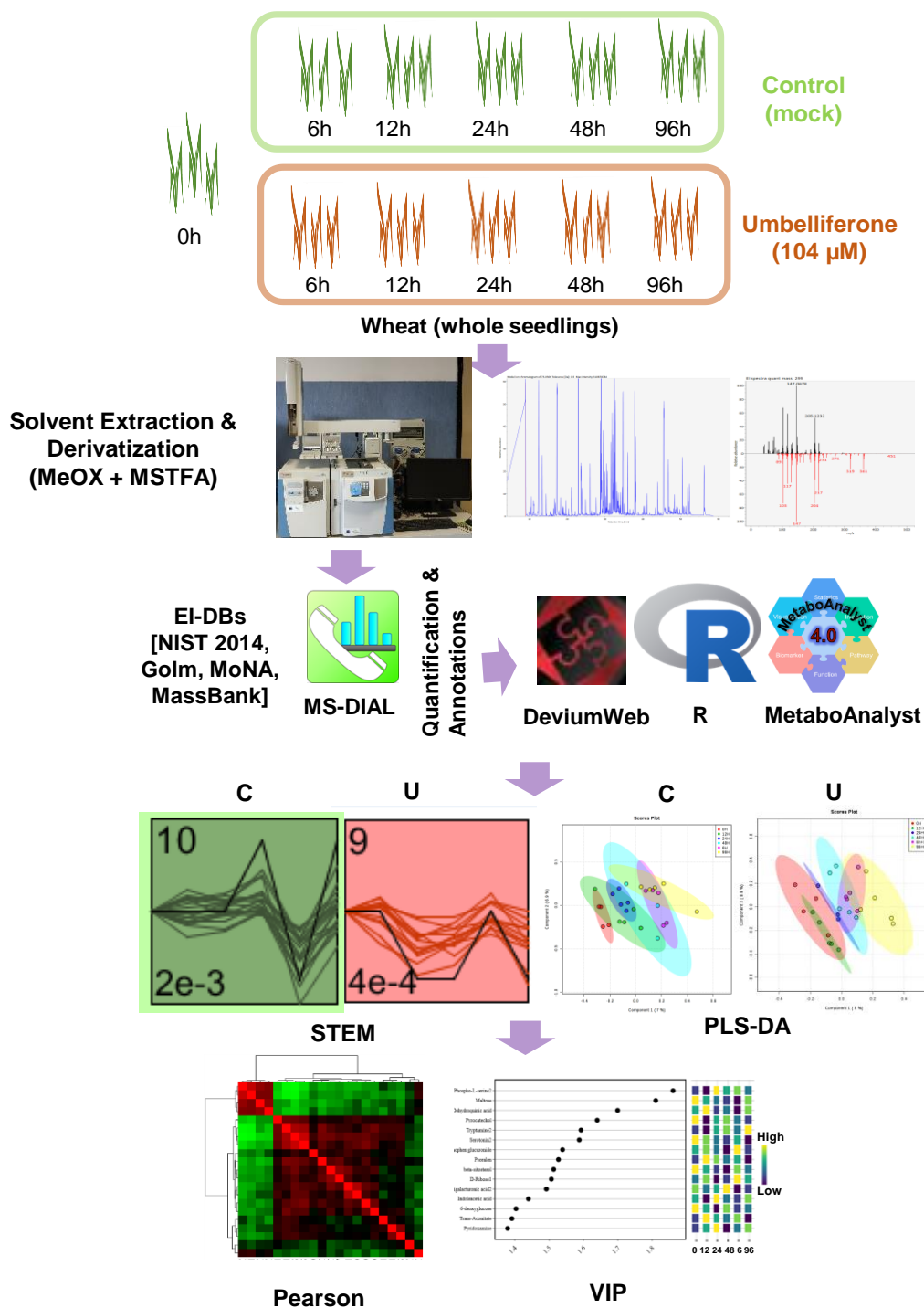
10) p. 13, section 4: **conclusion** - *This is a re-hashing of some basic premises of the study, and doesn't really include a lot of introspection into the study, nor does it speak broadly about the implications and significance of what they've done. It needs a re-write to better articulate the broader themes and future potential of this work.*
-Thank you for the comments. We have now modified the conclusions highlighting the same.

11) **Editing** - *There are also a few grammatical changes that should be addressed, sentence construction and word choice. Some of them are illustrated in the marked up pdf provided, but it should not be treated as a comprehensive close-read for grammar, spelling, and sentence makeup.*
-Thank you for the comments. We have now re-read and edited the entire manuscript for clarity in writing and for the ease of the readers of the journal.

Highlights

- **We studied time-course metabolomics of Umbelliferone (U) treatment in wheat;**
- **U reduced wheat seedling growth by 50% of at a concentration of 104 μM ;**
- **177 metabolites were quantified and 22 of them changed significantly with time;**
- **Short-term changes in metabolite accumulation patters and multivariate analysis reveal temporal changes;**
- **U induced a system-wide change in dysregulating primary and specialized metabolism;**

Graphical Abstract



1 **Research Article**

2 **Short-term effects of the allelochemical umbelliferone on *Triticum durum* L. metabolism**
3 **through GC-MS based untargeted metabolomics**

4

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26 **Short title:** *Wheat metabolomics of umbelliferone treatment*

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34 **Abstract**

35 The present experiment used untargeted metabolomics to investigate the short-term metabolic
36 changes induced in wheat seedlings by the specialized metabolite umbelliferone, an allelochemical.
37 We used 10 day-old wheat seedlings treated with 104 μ M umbelliferone over a time course
38 experiment covering 6 time points (0 h, 6 h, 12 h, 24 h, 48 h, and 96 h), and compared the metabolomic
39 changes to control (mock-treated) plants. Using gas chromatography mass spectrometry (GC-MS)-
40 based metabolomics, we obtained quantitative data on 177 metabolites that were derivatized (either
41 derivatized singly or multiple times) or not, representing 139 non-redundant (unique) metabolites. Of
42 these 139 metabolites, 118 were associated with a unique Human Metabolome Database (HMDB)
43 identifier, while 113 were associated with a Kyoto Encyclopedia of Genes and Genomes (KEGG)
44 identifier. Relative quantification of these metabolites across the time-course of umbelliferone
45 treatment revealed 22 compounds (sugars, fatty acids, secondary metabolites, organic acids, and
46 amino acids) that changed significantly (repeated measures ANOVA, P-value < 0.05) over time.
47 Using multivariate partial least squares discriminant analysis (PLS-DA), we showed the grouping of
48 samples based on time-course across the control and umbelliferone-treated plants, whereas the
49 metabolite-metabolite Pearson correlation revealed tightly formed clusters of umbelliferone-derived
50 metabolites, fatty acids, amino acids, and carbohydrates. Also, the time-course umbelliferone
51 treatment revealed that phospho-L-serine, maltose, and dehydroquinic acid were the top three
52 metabolites showing highest importance in discrimination among the time-points. Overall, the
53 biochemical changes converge towards a mechanistic explanation of the plant metabolic responses
54 induced by umbelliferone. In particular, the perturbation of metabolites involved in tryptophan
55 metabolism, as well as the imbalance of the shikimate pathways, which are strictly interconnected,
56 were significantly altered by the treatment, suggesting a possible mechanism of action of this natural
57 compound.

58 **Keywords:** metabolomics, gas chromatography mass-spectrometry, elicitation, polar, time-course,
59 phytotoxicity, allelochemicals.

60

61

62 **Introduction**

63 Allelopathy is a complex ecological phenomenon, and refers to the direct and/or indirect effects of
64 one organism (plant, insect, etc.) on another through the production and release of specialized

65 chemical compounds into the environment [1]. Due to the complexity of interpretation and analysis,
66 the elucidation of allelopathy using chemical signatures is a challenge which requires expertise in
67 diverse scientific fields, and the use of multidisciplinary tools and approaches [2]. In recent years, to
68 unravel the ecological roles of specialized metabolites, rapid advancements have made use of *-omics*
69 techniques and/or targeted and untargeted metabolic profiling of plant materials [3-6]. Techniques
70 such as transcriptomics, proteomics, and metabolomics allow simultaneous analysis of the total
71 molecular and biochemical constituents of a given sample [7]. In allelopathy studies, the use of
72 metabolomics as an analytical technique allows identification and quantification of both primary and
73 specialized metabolites in complex samples [8, 9]. Moreover, metabolomics is a useful tool in
74 understanding the response to biotic and abiotic stress, for the determination of complex pathways of
75 primary and specialized metabolite biosynthesis, and providing a broader understanding of biological
76 activity and mode of action of critical specialized metabolites [6, 10]. In fact, metabolomics as a
77 technique best represents the molecular phenotype, since it directly reflects the underlying
78 biochemical activity and state of cells, tissues, and organism, being closest to the functional
79 phenotype [11].

80 Among noteworthy allelochemicals, coumarins, which derive from the lactonization of o-
81 hydroxycinnamic acid, is a class of specialized metabolites that are widely distributed in the plant
82 kingdom, and they are synthesized by almost all higher plants [12], playing a pivotal role in both
83 plant communication and defense [13]. One coumarin, umbelliferone, so named because of its wide
84 occurrence within the Umbelliferae family, is an extremely biologically active compound widely
85 distributed in the plant kingdom (Asteraceae, Rutaceae, Acanthaceae, and Hydrangeaceae, among
86 others) [14]. Umbelliferone accumulates and is released to the environment through volatilization and
87 root exudation [15-17]. The critical ecological role of umbelliferone has been demonstrated in several
88 studies. For example, Minamikawa et al. [18] showed that umbelliferone production is induced in
89 response to infection by plant pathogens. Similarly, it was noted, in the medicinal plant *Chamomilla*
90 *recutita*, that under abiotic and biotic (powdery mildew *Erysiphe cichoracearum*) stress conditions,
91 umbelliferone concentration increased to an extreme degree [19]. Those results suggest that this
92 specialized metabolite could play a pivotal role in some plants as a first line of defense. This
93 hypothesis was further confirmed by studies from Yang et al. [20], which highlighted its ability to
94 suppress the *Ralstonia solanacearum*-induced wilting disease process by reducing fungi colonization
95 and proliferation, and by Hamerski et al. [21], who demonstrated that extract of fungal cell wall acts
96 as elicitor in *Amni majus*, increasing umbelliferone production. Umbelliferone is also involved in
97 plant defense against herbivores, acting as a repellent interfering with the bitter gustatory receptor
98 neurons of fruit flies [22]. Finally, it has been shown that umbelliferone determined the chemotactic

99 movement of *Rhizobium* and *Agrobacterium* across chemical gradients towards lower levels of
100 inhibitors and higher levels of potential nutrients [23]. Concerning its phytotoxic potential, several
101 studies have demonstrated that this molecule strongly affects both plant growth and development,
102 inducing reactive oxygen species (ROS) accumulation, chlorophyll degradation, alteration of root
103 morphology, and ROS-induced programmed cell death [24-26]. Moreland and Novitzky [27] found
104 that umbelliferone, at relatively high concentrations, inhibits functions in isolated chloroplasts and
105 mitochondria, whereas Einhellig [28] demonstrated that concentrations of umbelliferone that reduce
106 *Glycine max* seedling growth also decreased leaf water potential, stomatal conductance, and the
107 transpiration ratio.

108 Although several evidence regarding umbelliferone phytotoxicity are reported in the bibliography,
109 such information is quite dated and does not unveil the metabolic pathways altered by the molecule.
110 Moreover, it is widely known that allelochemicals could have a multi-target effect leading to a series
111 of cascade effects, finally resulting in the inhibition of plant growth and/or plant death. Therefore, to
112 identify their mode of action, it is important to focus attention on time-course experiments that
113 evaluate the short term effects of these chemicals. This approach could lead to identifying the primary
114 metabolic pathways affected. The main purpose of this study was to evaluate the short-term effect of
115 umbelliferone on seedlings of durum wheat (*Triticum durum*) – a crop species often employed in
116 phytotoxicity experiments due to its sensitivity to phytotoxins [29] – in order to identify the impact
117 of this molecule on plant metabolism.

118

119 **2. Materials and Methods**

120 **2.1. Chemicals and Reagents**

121 Methanol for GC-MS SupraSolv® (1.00837), chloroform for GC-MS SupraSolv® (1.02432), N-
122 Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) ≥98.5% (69479), pyridine ≥99% (270407),
123 methoxyamine hydrochloride 98% (226904), umbelliferone 99% (H24003), ribitol ≥99% (A5502),
124 and alkanes mixture C₁₀-C₄₀ (68281) were acquired from Sigma Aldrich (Italy).

125

126 **2.2 Plant growth conditions and elicitor treatments**

127 Durum wheat (*Triticum durum* L. cv. Opera) seeds were germinated in Petri dishes (9 cm) in a growth
128 chamber at 25°C, 70% humidity, with a photoperiod of 16 / 8 (light / dark), and light intensity of 90
129 mol m⁻² s⁻¹ supplied by a cool white fluorescent lamp (Polylux XL FT8, 55W 8440). Immediately

130 after germination, uniform seedlings were transferred to a 4.5 L hydroponic system and grown in a
131 modified Hoagland solution formulated as follows: KNO₃ (10 mM); MgSO₄ (100 μM); CaSO₄ (400
132 μM); KCl (5 μM); K₂SO₄ (200 μM); K H₂PO₄ (175 μM); H₃BO₃ (2.5 μM); MnSO₄ (0.2 μM); ZnSO₄
133 (0.2 μM); NaMoO₄ (0.05 μM); CuSO₄ (0.05 μM); Fe-EDTA (200 μM). The solution was changed
134 every other day and continuously oxygenated using an air bubble stone.

135

136 *2.2.1 Dose-response curve*

137 After the first true leaf formation (10 d from germination), wheat seedlings (a pool of 30 seedlings
138 per replicate and treatment) were selected for uniformity in growth, and were transferred into
139 continuously oxygenated hydroponic solutions enriched with different concentrations of
140 umbelliferone: 0, 12.5, 25, 50, 100, 200, and 400 μM. After 10 days of treatment, the whole plants
141 were collected, dried in an oven at 40°C, and weighed to monitor changes in total fresh weight (FW).
142 Umbelliferone was first dissolved in ethanol (0.1%, w/v) and then poured into the nutrient solution
143 prepared in deionized water. The same amount of ethanol was added to the mock treatments (control),
144 and the experiment was replicated five times (n = 5).

145

146 **2.2.2 Short-term effect of umbelliferone treatment**

147 To study the short-term effects of umbelliferone on the wheat metabolome, seedlings (a pool of 10
148 seedlings per replicate, time point, and treatment) were grown for 10 days and were then treated with
149 104 μM of umbelliferone (the ED₅₀ concentration was calculated from a dose-response curve). Plant
150 materials were collected after 0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5) of
151 umbelliferone treatment, and a parallel set of control plants (mock treated with same volume of
152 ethanol as previously described) with the same time points. In order to avoid metabolic fluctuations
153 induced by plant circadian rhythms, all the treatments were applied in order to allow plant collection
154 at the same hour of the day (12:00) (i.e., plants belonging to treatment T1 were treated at 06:00, T2
155 at 00:00, and so on). After collection, the plant materials were immediately snap frozen for
156 metabolomic studies. The experiment was replicated five times (n = 5).

157 **2.3. Metabolite extraction and sample derivatization**

158 Plant materials were collected at the middle of the light period, and whole plants were immediately
159 snap frozen in liquid nitrogen to quench the endogenous metabolism. Freshly homogenized (100 mg)
160 plant material was obtained from each biological sample (plant) and replicates. These were
161 transferred to 2 mL microcentrifuge round bottom screw cap tubes (Eppendorf). Extraction was done
162 by adding 1400 μL of methanol (at -20°C) and vortexing for 10 s after addition of 60 μL ribitol (0.2

163 mg/mL stock in ddH₂O) as an internal quantitative standard for the polar phase. Samples were
164 transferred in a thermomixer at 70°C and were shaken for 10 min (950 rpm) and were then further
165 centrifuged for 10 min at 11000 g. The supernatants were collected and transferred to glass vials
166 where 750 µL CHCl₃ (-20°C) and 1500 µL ddH₂O (4°C) were sequentially added. All the samples
167 were vortexed for 10 s and then centrifuged for another 15 min at 2200 g. Upper polar phase (150
168 µL) for each replicate was collected, transferred to a 1.5 mL tube and dried in a vacuum concentrator
169 without heating. Before freezing and storing at -80°C, the tubes were filled with argon and placed in
170 a plastic bag with silica beads (to avoid moisture and hydration during short-term storage). Before
171 derivatization, stored samples were placed in a vacuum concentrator for 30 min to eliminate any trace
172 of humidity. Then, 40 µL methoxyamine hydrochloride (20 mg/mL in pyridine) was added to the
173 dried samples, which were then incubated for 2 h in a Thermomixer (950 rpm) at 37°C.
174 Methoxyaminated samples were then silylated by adding 70 µL of MSTFA to the aliquots. Samples
175 were further shaken for 30 min at 37°C. Derivatized samples (110 µL) were then transferred into
176 glass vials suitable for the GC/MS autosampler for analysis.

177

178 **2.4. GC-quadrupole/MS analysis**

179 The derivatized extracts were injected into a TG-5MS capillary column (30 m x 0.25 mm x 0.25 µm)
180 (Thermo Fisher Scientific, Waltham, MA, USA) using a gas chromatograph apparatus (Trace GC
181 1310, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a single quadrupole mass
182 spectrometer (ISQ LT, Thermo Fisher Scientific, Waltham, MA, USA). Injector and source were set
183 at 250°C and 260°C, respectively. One µl of sample was injected in splitless mode with a helium flow
184 of 1 mL/min using the following programmed temperature: isothermal 5 min at 70°C followed by a
185 5°C/min ramp to 350°C and a final 5 min heating at 330°C. Mass spectra were recorded in electronic
186 impact (EI) mode at 70 eV, scanning at 40-600 *m/z* range, scan time 0.2 s. Mass spectrometric solvent
187 delay was settled as 9 min. Pooled samples that served as quality controls (QCs), n-alkane standards,
188 and blank solvents (pyridine) were injected at scheduled intervals for instrumental performance,
189 tentative identification, and monitoring of shifts in retention indices (RI).

190 **2.5 GC/MS Analysis and data acquisition**

191 **2.5.1 GC/MS data analysis using MS-DIAL**

192 Raw data (.RAW) from the single quadrupole instrument was converted to .mzML format with the
193 MSConvertGUI from ProteoWizard. MS-DIAL, with open source publicly available EI spectra

194 library, was used for raw peaks extraction, and the data baseline filtering and calibration of the
195 baseline, peak alignment, deconvolution analysis, peak identification, and integration of the peak
196 height were essentially followed as described [30]. An average peak width of 20 scans and a minimum
197 peak height of 1000 amplitudes was applied for peak detection, and a sigma window value of 0.5, EI
198 spectra cut-off of 5000 amplitudes was implemented for deconvolution. For identification, the
199 retention time tolerance was 0.2 min, the m/z tolerance was 0.5 Da, the EI similarity cut-off was 60%,
200 and the identification score cut-off was 80%. In the alignment parameters setting process, the
201 retention time tolerance was 0.5 min, and retention time factor was 0.5. For MS-DIAL data
202 annotations, we used publicly available libraries (both positive and negative) for compound
203 identification, based on the mass spectral pattern as compared to EI spectral libraries such as NIST
204 Mass Spectral Reference Library (NIST14/2014; National Institute of Standards and Technology,
205 USA; with EI- MS data of 242,466 compounds), the MSRI spectral libraries from Golm Metabolome
206 Database [31] available from Max-Planck-Institute for Plant Physiology, Golm, Germany
207 (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>), MassBank [32], and MoNA (Mass Bank
208 of North America, (<http://mona.fiehnlab.ucdavis.edu/>)). For metabolite annotation and assignment of
209 the EI-MS spectra, we followed the metabolomics standards initiative (MSI) guidelines for metabolite
210 identification [33], i.e., Level 2: identification was based on spectral database (match factor >80%)
211 and Level 3: only compound groups were known, e.g. specific ions and RT regions of metabolites.

212

213 **2.6 Statistical analyses**

214 For metabolomic experiments, standard statistical analyses (summary statistics) were performed
215 using the statistical software R (Version 3.5.3, <http://www.R-project.org>) [34, 35]. Normalized
216 (internal standard), transformed (\log_2), imputed, and scaled peak areas representative of relative
217 metabolite amounts were obtained using DeviumWeb [36], and are presented in tables and figures.
218 Values reported in all tables and text are presented as means, and differences were considered
219 significant when $P < 0.05$ (nominal P-values).

220 The FW responses to different doses of umbelliferone were evaluated by a nonlinear regression model
221 using a log-logistic equation, largely employed in phytotoxicity screenings [37] that allowed to
222 estimate the ED₅₀ parameter, the dose required to reduce 50% of the total response. The ED₅₀ value
223 was then used as the key concentration for the short-term metabolomics experiments.

224

225 **2.6.1 Univariate analysis**

226 ANalysis Of VAriance (ANOVA) was performed using R. Hierarchical clustering analysis (HCA)
227 using average linkage clustering was performed on Pearson distances from the metabolite abundance

228 data, using PermutMatrix [38]. For heat maps, data were normalized using the z-scores of the intensity
229 counts for each of the metabolites under the peak areas.

230

231 **2.6.2 Multivariate analysis**

232 Exploratory multivariate analysis was done using R (version 3.6.1). The sample-sample distance
233 clustering was obtained via package Pheatmap, using Pearson correlation and default parameters. The
234 exploratory interactive MDS plots were done with the Glimma package. Other various dimension
235 reduction analysis via principal component analysis (PCA) of overall, separate control, and treated
236 data was performed with the FactoMineR and factoextra packages. PCA and partial least-squares
237 discriminant (PLS-DA) analyses were performed using the DeviumWeb package [39], where the
238 output consisted of score plots to visualize the contrast between different samples and loading plots
239 to explain the cluster separation. Data were scaled with unit variance, without any transformation.
240 Partial least-squares discriminant analysis (PLS-DA) was used to highlight differences between the
241 metabolic phenotypes at six time points (0 h, 6 h, 12 h, 24 h, 48 h, and 96 h).

242

243 **2.7 Time-course analysis of control and umbelliferone-treated metabolomes**

244 For short time series metabolomics data analysis, we used the Short Time series Expression Miner
245 (STEM) tool [40], originally used for short microarray time series experiments (3–8 time points for
246 >~80% of the datasets). The novel STEM clustering takes advantage of the few time points in a
247 dataset, and it first selects a set of distinct and representative temporal expression profiles (i.e., model
248 profiles), where these model profiles are independent of data. The clustering algorithm then assigns
249 each feature (i.e., metabolite) passing the filtering criteria to the model profile that most closely
250 matches the feature's abundance profile as determined by the correlation coefficient, and determines
251 which profiles have a statistically significant higher number of features assigned using a permutation
252 test. STEM was used as a Java implementation with a graphical user interface, available at
253 <http://www.cs.cmu.edu/~jernst/st/> for clustering the metabolite accumulation patterns according to
254 time points. For our analysis, we used the following criteria: no additional normalization of the data;
255 0 added as the starting point; number of model profiles = 20; maximum unit change in model profiles
256 between time points = 3. To explain the model profiles, we used an expression of -1 for decreased
257 levels of a metabolite, 0 for unchanged levels of a metabolite, and 1 for increased levels of a
258 metabolite. For instance, a model profile with an expression of -1, -1, 0, 1, 1, 0 represents decreased,
259 decreased, unchanged, increased, increased, and unchanged levels of a given set of metabolites for
260 the 6 time points in the given model profile.

261

262 **2.8 Pathway enrichment and clustering analysis**

263 Pathway enrichment analysis was performed at MetaboAnalyst (www.Metaboanalyst.ca) [41], and
264 Chemical Translation Service (CTS: <http://cts.fiehnlab.ucdavis.edu/conversion/batch>) was used to
265 convert the common chemical names into their Kyoto Encyclopedia of Genes and Genomes (KEGG),
266 Human Metabolome Database (HMDB), CAS, PubChem Compound ID (CID), LipidMAPS IDs and
267 InChiKeys identifiers.

268

269 **2.9 Data sharing**

270 The raw datasets and the metadata obtained from the GC-EI-MS platform have been deposited at the
271 Metabolomics Workbench (Study ID: **ST001056**, <http://dx.doi.org/10.21228/M81M4X>).

272

273 **3. Results and Discussion**

274

275 **3.1 Dose response curve based on wheat biomass production in response to umbelliferone**

276 The dose response curve built on the variation of wheat fresh biomass (FW), in response to increasing
277 doses of umbelliferone (0-400 μM), pointed out a significant dose-dependent phytotoxic effect (**Fig.**
278 **1**). The lowest concentration (12.5 μM) did not affect plant growth. At 25 μM , a 17% reduction of
279 biomass was observed, and the reduction reached 82% at the highest concentration (400 μM). The
280 non-linear regression fit of FW raw data determined an ED_{50} value of 104 μM . Inhibitory effects of
281 umbelliferone to plants such as *Festuca rubra*, *Medicago sativa* and *Lactuca sativa* have been
282 reported [17, 42]. Based on the optimized umbelliferone concentration, we designed the experiment
283 to investigate the metabolomic changes in seedlings exposed to 6 h, 12 h, 24 h, 48 h, and 96 h of
284 umbelliferone treatment, as compared with the controls (mock treated) (**Figure 2**).

285

286 **3.2 Cataloging the wheat seedling metabolome**

287 Using GC-MS, we obtained quantitative data on 177 metabolites that were derivatized (either
288 derivatized singly or multiple times) or not, representing 139 non-redundant (unique) metabolites. Of
289 these 139 metabolites, 118 were associated with a unique HMDB identifier, while 113 were
290 associated with a KEGG identifier. The derivatized metabolites included sugars (monosaccharides,

291 disaccharides), sugar alcohols, sugar acids, dipeptides, organic acids, amino acids, phosphates,
292 polyamines, purines, and pyrimidines, while the non-derivatized metabolites included fatty acids,
293 among others. We also captured several known secondary / specialized metabolites such as phenolic
294 compounds (polyphenols and flavonoids), i.e., pyrocatechol, protocatechuic acid, chlorogenic acid,
295 pyrogallol, homovanillate, sinapaldehyde, catechin, caffeine, and myricetin; and others, such as
296 phytol and quinolinic acid. We also captured the modified (metabolized) forms of umbelliferone, i.e.,
297 4-methylumbelliferone and psoralen. These metabolites belonged to 50 different KEGG-based
298 metabolic pathways (**Supplementary Figure 1**), with the top pathways belonging to arginine and
299 proline metabolism, glutathione metabolism, aminoacyl-tRNA biosynthesis (all P-value < 0.05),
300 taurine and hypotaurine metabolism, tryptophan metabolism, beta-alanine metabolism, isoquinoline
301 alkaloid biosynthesis, phenylalanine, tyrosine and tryptophan metabolism, alanine, aspartate and
302 glutamate metabolism (all P-value < 0.1), and indole alkaloid biosynthesis, among others
303 (**Supplementary Figure 2**).

304

305 **3.3 Impact of umbelliferone on wheat metabolome**

306 Umbelliferone is an extremely biologically active coumarin widespread in the Umbelliferae family,
307 but also in other genera, in plant families such as Asteraceae, Rutaceae, Acanthaceae, and
308 Hydrangeaceae [14]. A huge body of research has clearly demonstrated that application of
309 umbelliferone can lead to phytotoxic effects, thereby affecting both plant growth and development
310 [24, 26-28]. We performed a one-way ANOVA on each compound, to test if at least one level of time
311 had a mean average significantly different from the rest. There are 22 significant compounds (sugars,
312 fatty acids, secondary metabolites, organic acids, and amino acids) with p-value lower than 0.05
313 (**Table 1**). To control for false positive findings, a False Discovery Rate (FDR) was applied to the
314 nominal p-values; 7 compounds (sugars: maltose, xylulose, ribose, 6-deoxyglucose) were still
315 significant after the FDR correction.

316

317 **3.4 Time-course profiling of umbelliferone treatment (quantitative)**

318 To understand the time-course-dependent changes in metabolite accumulation patterns across the
319 treatment groups in this complex study design, we started with a clustering analysis. Using short time-
320 series expression miner (STEM) analysis, we interrogated the time-course changes of the metabolites

321 for further analysis. The metabolite abundances for 177 metabolites across the 6 time points were put
322 into 20 model clusters, which revealed differential accumulation of metabolites for control and
323 umbelliferone-treated groups of plants, as a function of time. In the case of the control plants, the
324 most significant model cluster (number 10, with 18 metabolites, P-val, 2E-3) showed a 0, 1, 0, 1, -1,
325 1 pattern (where 0 is no change, 1 is increase, and -1 is decrease) for the six time-points in the study
326 [0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5)]. These 18 metabolites were sugars
327 (fucose, maltose, trehalose, and xylulose), organic acids (isohexonic acid, tranexamic acid, and
328 aconitic acid), amines (pyridoxamine, tryptamine), ribulose 1, 5-bisphosphate, 3-indoleacetonitrole,
329 etc. (**Figure 3 A, B**). In the case of umbelliferone-treated plants, the most significant model cluster
330 (number 9, with 13 metabolites, P-val, 4E-4) showed a 0, 1, -1, -1, 1, -1 pattern for the six time-points
331 in the study. These 13 metabolites were sugars (trehalose, xylulose, melibiose, and rhamnose),
332 organic acids (ascorbic acid, pimelic acid, quinolic acid, and aconitic acid) polyamines (putrescine
333 and spermidine), etc. (**Figure 3 C, D**).

334

335 **3.5 Multivariate and clustering analysis reveal metabolites**

336

337 Secondly, we performed both supervised and unsupervised multivariate analyses as feature extraction
338 strategies, to maximize variance in the data using strongly correlated variables. We first performed
339 an unsupervised analysis, which explained ~40–43% of the variability in data using the first 2 PCs,
340 either in all samples grouped together, only control sample groups, or umbelliferone groups
341 (**Supplementary Figure 8A-C**). However, the time points did not cluster well, which points to the
342 non-independent samples which are not well handled by PCA, the small feature space of 177
343 metabolites, and too many treatments (6 time points x 2 treatments), leading to possible
344 multicollinearity issues, displaying more artifacts than a true biological picture. Following the lack
345 of clustering in the PCA, we performed PLS-DA separately for both control and umbelliferone
346 treatment groups, where time-point based groupings were observed. Using supervised PLS-DA
347 analysis for all the samples (all time points, control and umbelliferone treated plants) and the blanks
348 (**B**), we showed that the first two components explained variations from the T0, 6 h, 12 h, 24 h, 48 h,
349 and 96 h time points; components 1 and 2 alone explained ~45% of the variation (**Figure 4A**). For
350 the control and umbelliferone-treated plants, the first two components (1 and 2) helped explain ~14%
351 and ~15% of the variations, respectively (**Figures 4B, C**). The co-clustering of time points (i.e., 6 h
352 with 96 h) could point to interesting biological phenomena, such as the appearance of two peaks, one
353 in very short-term defense response and another sustained one later. These are speculations, and

354 would be very difficult to validate further using metabolomics experiments and the premises of this
355 study.

356

357 Using metabolite-metabolite (Pearson) correlation, we monitored the clusters of metabolites. Among
358 secondary metabolites, we found that 3-indoleacetonitrile (an auxin, from tryptophan metabolism),
359 psoralen and 4-methylumbelliferone (both umbelliferone derivatives), and 2-coumaric acid were
360 highly correlated (**Supplementary Figure 3, 4**), indicating their possibly coordinated biosynthesis
361 and regulation. Similarly, tight clusters were observed for fatty acids (**Supplementary Figure 5**),
362 groups of amino acids (**Supplementary Figure 6**), and carbohydrates (**Supplementary Figure 7**). A
363 recent study that looked at various polyphenols across diverse species observed that umbelliferone
364 and kaempferol are quantitatively associated with each other, while there was a positive correlation
365 of epicatechin with umbelliferone and kaempferol [43].

366

367 In order to identify the metabolites responsible for the discrimination among the metabolomic
368 profiles, the VIP scores were used to select those with the most significant contributions in a PLS-
369 DA model, thus as a measure of a variable's importance in the PLS-DA model. VIP scores are a
370 weighted sum of PLS weights for each variable, and measure the contribution of each predictor
371 variable to the model [44]. The VIP statistic indicates the importance of the metabolites in
372 differentiating the study groups (umbelliferone treatment times, i.e., 0 h, 6 h, 12 h, 24 h, 48 h, 96 h)
373 in multivariate space. The compounds exhibiting the higher VIP scores are the more influential
374 variables. Our VIP analysis revealed that the metabolites with high VIPs were phospho-L-serine,
375 maltose, dehydroquinic acid, pyrocatechol, tryptamine, and serotonin, among others (**Figure 5**).
376 Thus, the biochemical changes induced by umbelliferone treatment may support mechanistic
377 explanations of the plant metabolic responses induced by this coumarin compound. In particular, as
378 highlighted by the VIP scores reported (**Figure 5**), several metabolites involved in both shikimate
379 and tryptophan pathways were significantly altered by the treatment. Among them, fluctuations in
380 dehydroquinic acid abundances during all the treatments are noteworthy, where the highest values
381 were recorded at 12 h and 96 h. Dehydroquinic acid represents the first carbocyclic intermediate of
382 the shikimate pathway, which undergoes five further enzymatic steps in the remainder of the
383 shikimate pathway to yield chorismic acid, a precursor to tyrosine, phenylalanine, tryptophan, and
384 some vitamins [45]. Interestingly, pyridoxamine (vitamin B6) was significantly altered by the
385 umbelliferone treatment; it reached highest abundance at 24 h of treatment, dropped after 48 h, and
386 increased again at 96 h; it is an essential coenzyme with a high antioxidant potential [46]. Moreover,
387 pyridoxamine in the presence of ATP is converted by the pyridoxal kinase in pyridoxal 5'-phosphate,

388 which is strictly connected to the enzyme tryptophan synthetase, an enzyme that catalyses the final
389 two steps in the biosynthesis of tryptophan [47]. The tryptophan synthetase, typically found as a $\alpha 2\beta 2$
390 tetramer, catalyses the irreversible condensation of indole and serine to form tryptophan in a pyridoxal
391 5'-phosphate-dependent reaction [48]. In addition, the conversion of tryptophan to indole acetic acid
392 leads to the formation of glutamate, which is one of the pyridoxamine precursors [49]. It is therefore
393 conceivable that, as detailed below, the umbelliferone-triggered perturbation of the tryptophan
394 metabolism might be on the basis of the observed pyridoxamine accumulation pattern over time. It is
395 also possible that the fluctuation in pyridoxamine content is attributable to the conversion into their
396 derivatives, namely pyridoxal, pyridoxal 5- phosphate, and pyridoxamine [50], involved in many
397 other cellular functions, which were simply not detected / quantified in our metabolomics
398 experiments. Among the metabolites involved in tryptophan biosynthesis, phospho-L-serine [51] was
399 characterized by the highest VIP score, pointing to a significant increase in concentration over time.
400 This molecule has a pivotal role in plants under environmental stresses, as an upregulation of several
401 genes involved in this pathway were observed during abiotic stresses such as salinity, cold, and flood,
402 indicating its importance in supplying serine under environmental stresses [52]. Moreover, the
403 phosphorylated pathway might be essential to provide the amino acid serine for the synthesis of
404 tryptophan, the common precursor for the biosynthesis of indole acetic acid (IAA) [53]. Interestingly,
405 in our experiments, significant variations in IAA and tryptamine (indole-alkaloid) content, an
406 intermediate in IAA biosynthesis, were observed. In fact, both metabolites were significantly elicited
407 by the umbelliferone treatment. Alteration in IAA biosynthesis and distribution, driven by 4-
408 methylumbelliferone (an umbelliferone derivative), was previously observed by Li et al. [25] in
409 *Arabidopsis* seedlings. In particular, they observed that the exogenous application of 4-
410 methylumbelliferone (125 μ M for 22 days) led to reduced primary root growth, the formation of
411 bulbous root hairs, and an increase in the number of lateral roots. The authors also uncovered an
412 accumulation of 4-methylumbelliferyl- β -D-glucoside, derived from UDP-glycosyltransferase
413 mediated transformation of umbelliferone in roots and upregulation of several *UDP-*
414 *glycosyltransferase* genes, which were supportive for a well-orchestrated mechanism devoted to the
415 detoxification of umbelliferone in plants. During our experiments, the presence of both 4-
416 methylumbelliferone and psoralen, umbelliferone derivatives, was detected in umbelliferone-treated
417 plants, suggesting that the umbelliferone was internalized and metabolized by the seedlings.
418 Studies of several other species have proven that both umbelliferone derivatives can act as
419 phytoalexins themselves; they can protect plants from both biotic and abiotic stresses, and/or can
420 induce reduction in growth and development [25, 54-56]. Therefore, it cannot be excluded that the
421 reduction in plant growth observed during the dose response curve could also be due to the

422 accumulation of umbelliferone derivatives. Recent studies of *Psoralea corylifolia*, treated with
423 psoralen elicitors and precursors, demonstrated that there is a negative correlation between psoralen
424 accumulation and cell growth [57]. Furthermore, psoralen accumulation in plants, as well as other
425 specialized metabolites, play a pivotal role in protecting plants from several other stresses [57], and
426 the observed plant growth reduction is probably due to the redistribution of plant energies in the
427 activation of (specialized) biosynthetic pathways involved in detoxification and/or protection from
428 oxidative stress, instead of the biosynthesis of (primary) metabolites fundamental for growth.
429 Despite its role as an intermediate in auxin biosynthesis, it has been suggested that tryptamine could
430 play an important role during both biotic and abiotic stress. It has been observed, for example, that
431 barley leaves irradiated with UV light were accumulating high levels of tryptamine. Moreover, its
432 induction was also observed to occur in response to plant pathogenic fungi infection, suggesting that
433 it could act as a plant defense metabolite [58]. On the other hand, tryptamine accumulation was
434 accompanied by a reduction in serotonin content. It has been widely reported that in graminaceous
435 species the enzyme tryptamine 5-hydroxylase is involved in serotonin biosynthesis, catalyzing the
436 conversion of tryptamine to serotonin [59, 60]. Kang et al. [61] demonstrated that the exogenous
437 application of tryptamine to tissues of rice seedlings induced a dose-dependent increase in serotonin,
438 accompanied by a parallel increase in tryptamine 5-hydroxylase enzyme activity. At the same time,
439 the same tissues grown in the presence of tryptophan did not show any significant increase in
440 serotonin. Therefore, it can be speculated that tryptamine accumulation, followed by the reduction in
441 serotonin content, could be due to an umbelliferone-induced reduction of tryptamine 5-hydroxylase
442 activity. Serotonin, which plays a pivotal role in plant growth regulation and in plant response to both
443 biotic and abiotic stress [62], and psoralen, are considered to be phytoalexins with antioxidant
444 properties involved in plant defense [55].

445

446 *Limitations of the study*

447 Our study has several limitations. First, separating sample preparations based on separate analysis of
448 shoots and roots, or leaf analysis, would have provided more spatial information on organ- and plant
449 part-specific metabolic changes, which may have confounded the analysis in this whole seedling
450 analysis approach. Secondly, the overall feature space (i.e., the number of metabolites) is also very
451 limited. Our current total metabolites quantified ($p = 177$) is roughly three times the overall sample
452 size ($n = 53$). Hence, the data is limited in dimensionality. These metabolites are also highly correlated
453 both at intra- and inter-group levels, limiting the overall variance contributions. High correlations can
454 also contribute to multicollinearity. All of these factors, taken together, limit the overall results and

455 interpretations of the current study. Lastly, techniques other than mass-spectrometry-based analysis,
456 i.e., additional orthogonal technique such as liquid chromatography-mass-spectrometry (LC-MS)
457 with wider metabolic coverage and less complex sample preparations steps (i.e., drying and
458 derivatization), may have been helpful in the identification and relative quantification of various
459 metabolites belonging to more numbers of pathways, and capturing multiple secondary metabolites
460 involved in plant stress metabolic responses.

461

462 **4. Conclusions**

463 This study clearly shows the system-wide metabolomic changes in wheat seedlings in response to
464 umbelliferone treatment. Although this molecule has been studied extensively, this is the first time a
465 short-term experiment using sub-lethal concentrations has been carried out. This untargeted
466 metabolomics approach allowed us to identify the system-wide metabolic responses activated by the
467 plants to deal with this phytotoxic compound. Among them, one of the first responses activated by
468 plants was the internalization of umbelliferone into its derivative psoralen. In addition, umbelliferone
469 induced a system-wide change through the dysregulation of metabolites involved in the shikimate
470 pathways, as well as in tryptophan and tryptamine metabolism. This study provides new insights into
471 the early response of plants to this specialized metabolite. Thus, taken together our work can be used
472 as a reference for further studies aimed at clarifying its mode of action.

473

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477 **Author Contributions**

478 FA, BBM and MRA conceived and designed the study; FA and ML performed the experiments;
479 BBM, VD, FA analyzed the data; FA and MRA contributed reagents/materials/analysis tools; BBM,
480 FA, MRA and ML wrote the paper.

481

482 **Conflicts of Interest**

483 VD currently works as a Post-Doctoral Researcher in Novo Nordisk Research Center Seattle, Inc;
484 however, he did not receive any funding for this work. All authors declare that they had no conflicts
485 of interest.

486

487 **5. References**

488 [1] S. Rizvi, H. Haque, V. Singh, V. Rizvi, A discipline called allelopathy, in: *Allelopathy*, Springer,
489 1992, pp. 1-10.

490 [2] F. Araniti, M. Scognamiglio, A. Chambery, R. Russo, A. Esposito, B. D'Abrosca, A. Fiorentino,
491 A. Lupini, F. Sunseri, M.R. Abenavoli, Highlighting the effects of coumarin on adult plants of
492 *Arabidopsis thaliana* (L.) Heynh. by an integrated-omic approach, *J. Plant Physiol.* 213 (2017) 30-
493 41.

494 [3] B. D'Abrosca, M. Scognamiglio, V. Fiumano, A. Esposito, Y.H. Choi, R. Verpoorte, A.
495 Fiorentino, Plant bioassay to assess the effects of allelochemicals on the metabolome of the target
496 species *Aegilops geniculata* by an NMR-based approach, *Phytochem.* 93 (2013) 27-40.

497 [4] M. Scognamiglio, B. D'Abrosca, A. Esposito, A. Fiorentino, *Metabolomics: an unexplored tool*
498 *for allelopathy studies*, *J. Allelochem. Int.* 1 (2015) 9-21.

499 [5] F. Araniti, A. Lupini, F. Sunseri, M.R. Abenavoli, Allelopathic potential of *Dittrichia viscosa* (L.)
500 W. Greuter mediated by VOCs: a physiological and metabolomic approach, *PloS one*, 12 (2017)
501 e0170161.

502 [6] F. Araniti, A. Lupini, A. Mauceri, A. Zumbo, F. Sunseri, M.R. Abenavoli, The allelochemical
503 trans-cinnamic acid stimulates salicylic acid production and galactose pathway in maize leaves: A
504 potential mechanism of stress tolerance, *Plant Physiol. Biochem.* 128 (2018) 32-40.

505 [7] S.O. Duke, J. Bajsa, Z. Pan, Omics methods for probing the mode of action of natural and synthetic
506 phytotoxins, *J. Chem. Ecol.* 39 (2013) 333-347.

507 [8] J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A.R. Fernie, Gas chromatography mass
508 spectrometry-based metabolite profiling in plants, *Nat. Protoc.* 1 (2006) 387.

509 [9] F. Araniti, T. Gullì, M. Marrelli, G. Statti, A. Gelsomino, M.R. Abenavoli, *Artemisia arborescens*
510 L. leaf litter: phytotoxic activity and phytochemical characterization, *Acta Physiol. Plant.* 38 (2016)
511 128.

- 512 [10] U. Roessner, A. Luedemann, D. Brust, O. Fiehn, T. Linke, L. Willmitzer, A.R. Fernie, Metabolic
513 profiling allows comprehensive phenotyping of genetically or environmentally modified plant
514 systems, *The Plant Cell*. 13 (2001) 11-29.
- 515 [11] O. Fiehn, Metabolomics—the link between genotypes and phenotypes, in: *Functional genomics*,
516 Springer, 2002, pp. 155-171.
- 517 [12] S. Brown, A. Zobel, Biosynthesis and distribution of coumarins in the plant, in: *Proceedings of*
518 *the Conference «Coumarins: Research and Applications»*, Padua, Italy, 1990, pp. 20-22.
- 519 [13] A. Zobel, S. Brown, Coumarins in the interactions between the plant and its environment,
520 *Allelopathy J.* 2 (1995) 9-20.
- 521 [14] O. Mazimba, Umbelliferone: sources, chemistry and bioactivities review, *Bulletin of Faculty of*
522 *Pharmacy, Cairo University*, 55 (2017) 223-232.
- 523 [15] E. Haggag, I. Mahmoud, E. Abou-Moustafa, T. Mabry, Coumarins, fatty acids, volatile and non-
524 volatile terpenoids from the leaves of *Citrus aurantium* L.(sour orange) and *Citrus sinensis* (L.)
525 Osbeck (sweet orange), *Asian J. Chem.* 11 (1999) 784-789.
- 526 [16] S. Yaoya, H. Kanho, Y. Mikami, T. Itani, K. Umehara, M. Kuroyanagi, Umbelliferone released
527 from hairy root cultures of *Pharbitis nil* treated with copper sulfate and its subsequent glucosylation,
528 *Biosci. Biotech. Biochem.* 68 (2004) 1837-1841.
- 529 [17] H. Guo, H. Cui, H. Jin, Z. Yan, L. Ding, B. Qin, Potential allelochemicals in root zone soils of
530 *Stellera chamaejasme* L. and variations at different geographical growing sites, *Plant Growth Regul.*
531 77 (2015) 335-342.
- 532 [18] T. Minamikawa, T. Akazawa, I. Uritani, Analytical study of umbelliferone and scopoletin
533 synthesis in sweet potato roots infected by *Ceratocystis fimbriata*, *Plant Physiol.* 38 (1963) 493.
- 534 [19] M. Repčák, J. Imrich, M. Franeková, Umbelliferone, a stress metabolite of *Chamomilla recutita*
535 (L.) Rauschert, *J. Plant Physiol.* 158 (2001) 1085-1087.
- 536 [20] L. Yang, S. Li, X. Qin, G. Jiang, J. Chen, B. Li, X. Yao, P. Liang, Y. Zhang, W. Ding, Exposure
537 to umbelliferone reduces *Ralstonia solanacearum* biofilm formation, transcription of type III
538 secretion system regulators and effectors and virulence on tobacco, *Front. Microbiol.* 8 (2017) 1234.
- 539 [21] D. Hamerski, R.C. Beier, R.E. Kneusel, U. Matern, K. Himmelpacht, Accumulation of
540 coumarins in elicitor-treated cell suspension cultures of *Ammi majus*, *Phytochem.* 29 (1990) 1137-
541 1142.

- 542 [22] L.A. Weiss, A. Dahanukar, J.Y. Kwon, D. Banerjee, J.R. Carlson, The molecular and cellular
543 basis of bitter taste in *Drosophila*, *Neuron*. 69 (2011) 258-272.
- 544 [23] A. Brenic, S.C. Winans, Detection of and response to signals involved in host-microbe
545 interactions by plant-associated bacteria, *Microbiol. Mol. Biol. Rev.* 69 (2005) 155-194.
- 546 [24] E. Kupidowska, M. Kowalec, G. Sulkowski, A. Zobel, The effect of coumarins on root
547 elongation and ultrastructure of meristematic cell protoplast, *Ann. Bot.* 73 (1994) 525-530.
- 548 [25] X. Li, M.Y. Gruber, D.D. Hegedus, D.J. Lydiate, M.-J. Gao, Effects of a coumarin derivative,
549 4-methylumbelliferone, on seed germination and seedling establishment in *Arabidopsis*, *J. Chem.*
550 *Ecol.* 37 (2011) 880.
- 551 [26] L. Pan, X.-z. Li, Z.-q. Yan, H.-r. Guo, B. Qin, Phytotoxicity of umbelliferone and its analogs:
552 Structure–activity relationships and action mechanisms, *Plant Physiol. Biochem.* 97 (2015) 272-277.
- 553 [27] D.E. Moreland, W.P. Novitzky, Effects of phenolic acids, coumarins, and flavonoids on isolated
554 chloroplasts and mitochondria, *Allelochemicals: role in agriculture and forestry*, 330 (1987) 247-261.
- 555 [28] F. Einhellig, The physiology of allelochemical action: clues and views, *Allelopathy from*
556 *molecules to ecosystems*, (2002) 1-23.
- 557 [29] F.A. Macías, D. Castellano, J.M. Molinillo, Search for a standard phytotoxic bioassay for
558 allelochemicals. Selection of standard target species, *J. Agricul. Food Chem.* 48 (2000) 2512-2521.
- 559 [30] H. Tsugawa, T. Cajka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. VanderGheynst,
560 O. Fiehn, M. Arita, MS-DIAL: data-independent MS/MS deconvolution for comprehensive
561 metabolome analysis, *Nat. Meth.* 12 (2015) 523.
- 562 [31] J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmüller, P. Dörmann, W.
563 Weckwerth, Y. Gibon, M. Stitt, GMD@ CSB. DB: the Golm metabolome database, *Bioinformatics*,
564 21 (2004) 1635-1638.
- 565 [32] H. Horai, M. Arita, S. Kanaya, Y. Nihei, T. Ikeda, K. Suwa, Y. Ojima, K. Tanaka, S. Tanaka, K.
566 Aoshima, MassBank: a public repository for sharing mass spectral data for life sciences, *J. Mass*
567 *Spectr.* 45 (2010) 703-714.
- 568 [33] S.-A. Sansone, T. Fan, R. Goodacre, J.L. Griffin, N.W. Hardy, R. Kaddurah-Daouk, B.S. Kristal,
569 J. Lindon, P. Mendes, N. Morrison, The metabolomics standards initiative, *Nat. Biotech.* 25 (2007)
570 846.

- 571 [34] R.C. Team, R: A language and environment for statistical computing; 2015, in, 2018.
- 572 [35] F.J. Rohlf, R.R. Sokal, Statistical tables, Macmillan, 1995.
- 573 [36] D. Grapov, DeviumWeb: version 0.3.2. ZENODO doi:10.5281/zenodo.12879,
574 <https://github.com/dgrapov/DeviumWeb>, (2014).
- 575 [37] R.G. Belz, K. Hurle, S.O. Duke, Dose-response—a challenge for allelopathy?, *Nonlin. Biol.*
576 *Toxic. Med.* 3 (2005) nonlin. 003.
- 577 [38] G. Caraux, S. Pinloche, PermutMatrix: a graphical environment to arrange gene expression
578 profiles in optimal linear order, *Bioinformatics* 21(7) (2005) 1280-1281 (2014).
- 579 [39] D. Grapov, DeviumWeb: Version 0.3.2. ZENODO. <https://doi.org/10.5281/zenodo.12879>,
580 <https://github.com/dgrapov/DeviumWeb>.
- 581 [40] J. Ernst, Z. Bar-Joseph, STEM: a tool for the analysis of short time series gene expression data.
582 *BMC bioinformatics* 7(1) (2006) 191.
- 583 [41] J. Xia, N. Psychogios, N. Young, D.S. Wishart, MetaboAnalyst: a web server for metabolomic
584 data analysis and interpretation. *Nucl. Acids Res.* 37(suppl_2) (2009) W652-W660.
- 585 [42] Z. Yan, D. Wang, H. Cui, D. Zhang, Y. Sun, H. Jin, X. Li, X. Yang, H. Guo, X. He, Phytotoxicity
586 mechanisms of two coumarin allelochemicals from *Stellera chamaejasme* in lettuce seedlings, *Acta*
587 *Physiol. Plant.* 38 (2016) 248.
- 588 [43] R. Kumar, S. Mahey, R. Arora, J. Mahajan, V. Kumar, S. Arora, Insights into biological
589 properties of less explored bark of industrially important *Acacia catechu* Willd, *Ind. Crops Prod.* 138
590 (2019) 111486.
- 591 [44] R.C. Smart, E. Hodgson, *Molecular and biochemical toxicology*, John Wiley & Sons, 2018.
- 592 [45] B. Ganem, From glucose to aromatics: recent developments in natural products of the shikimic
593 acid pathway, *Tetrahedron*, 34 (1978) 3353-3383.
- 594 [46] O. Titiz, M. Tambasco- Studart, E. Warzych, K. Apel, N. Amrhein, C. Laloi, T.B. Fitzpatrick,
595 PDX1 is essential for vitamin B6 biosynthesis, development and stress tolerance in Arabidopsis, *The*
596 *Plant J.* 48 (2006) 933-946.
- 597 [47] M.F. Dunn, D. Nicks, H. Ngo, T.R. Barends, I. Schlichting, Tryptophan synthase: the workings
598 of a channeling nanomachine, *Trends Biochem. Sci.* 33 (2008) 254-264.

- 599 [48] S. Raboni, S. Bettati, A. Mozzarelli, Tryptophan synthase: a mine for enzymologists, Cell.
600 *Molecul. Life Sci.* 66 (2009) 2391-2403.
- 601 [49] M. Tambasco-Studart, O. Titiz, T. Raschle, G. Forster, N. Amrhein, T.B. Fitzpatrick, Vitamin
602 B6 biosynthesis in higher plants. *P. Natl. A. Sci.* 102(38) (2005) 13687-13692
- 603 [50] P. Bilski, M.Y. Li, M. Ehrenshaft, M.E. Daub, C.F. Chignell, Vitamin B6 (pyridoxine) and its
604 derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. *Photochem.*
605 *Photobiol.* 71(2) (2000) 129-134
- 606 [51] F. Busch, C. Rajendran, O. Mayans, P. Löffler, R. Merkl, R. Sterner, TrpB2 enzymes are O-
607 phospho-L-serine dependent tryptophan synthases, *Biochem.* 53 (2014) 6078-6083.
- 608 [52] C.L. Ho, K. Saito, Molecular biology of the plastidic phosphorylated serine biosynthetic pathway
609 in *Arabidopsis thaliana*, *Amino acids*, 20 (2001) 243-259.
- 610 [53] C. Won, X. Shen, K. Mashiguchi, Z. Zheng, X. Dai, Y. Cheng, H. Kasahara, Y. Kamiya, J.
611 Chory, Y. Zhao, Conversion of tryptophan to indole-3-acetic acid by tryptophan aminotransferases
612 of *Arabidopsis* and YUCCAs in *Arabidopsis*, *Proceedings of the National Academy of Sciences*, 108
613 (2011) 18518-18523.
- 614 [54] L. Nebo, R.M. Varela, J.M. Molinillo, O.M. Sampaio, V.G. Severino, C.M. Casal, M.F. das
615 Grac_{as} Fernandes, J.B. Fernandes, Macías, F. A., Phytotoxicity of alkaloids, coumarins and
616 flavonoids isolated from 11 species belonging to the Rutaceae and Meliaceae families. *Phytochem.*
617 *Lett.* 8 (2014) 226-232.
- 618 [55] S. Jan, T. Parween, T. Siddiqi, Anti-oxidant modulation in response to gamma radiation induced
619 oxidative stress in developing seedlings of *Psoralea corylifolia* L., *J. Environ. Radioact.* 113 (2012)
620 142-149.
- 621 [56] D. Solecka, Role of phenylpropanoid compounds in plant responses to different stress factors.
622 *Acta Physiol. Plantarum* 19(3) (1997) 257-268.
- 623 [57] G. Hari, K. Vadlapudi, P.D. Vijendra, J. Rajashekar, T. Sannabommaji, G. Basappa, A
624 combination of elicitor and precursor enhances psoralen production in *Psoralea corylifolia* Linn.
625 suspension cultures. *Ind. Crops Prod.* 124 (2018) 685-691.
- 626 [58] H. Miyagawa, H. Toda, T. Tsurushima, T. Ueno, J. Shishiyama, Accumulation of tryptamine in
627 barley leaves irradiated with UV light, *Biosci. Biotech. Biochem.* 58 (1994) 1723-1724.

628 [59] T. Fujiwara, S. Maisonneuve, M. Isshiki, M. Mizutani, L. Chen, H.L. Wong, T. Kawasaki, K.
629 Shimamoto, Sekiguchi lesion gene encodes a cytochrome P450 monooxygenase that catalyzes
630 conversion of tryptamine to serotonin in rice, *J. Biol. Chem.* 285 (2010) 11308-11313.

631 [60] S. Park, T.N.N. Le, Y. Byeon, Y.S. Kim, K. Back, Transient induction of melatonin biosynthesis
632 in rice (*Oryza sativa* L.) during the reproductive stage, *J. Pineal Res.* 55 (2013) 40-45.

633 [61] S. Kang, K. Kang, K. Lee, K. Back, Characterization of tryptamine 5-hydroxylase and serotonin
634 synthesis in rice plants, *Plant Cell Rep.* 26 (2007) 2009-2015.

635 [62] H. Kaur, S. Mukherjee, F. Baluska, S.C. Bhatla, Regulatory roles of serotonin and melatonin in
636 abiotic stress tolerance in plants, *Plant Sign. Behav.* 10 (2015) e1049788.

637 [55]

638

639 **Figure Captions and Table Legends**

640

641 **Figures**

642 **Figure 1:** Dose-response curve evaluated on a FW base of *Triticum durum* cv. Opera seedlings treated
643 for 10 days with different doses (0, 12.5, 25, 50, 100, 200, 400 μ M) of umbelliferone. Data were
644 analyzed through one-way ANOVA using LSD as post hoc ($P \leq 0.05$). ED₅₀ (μ M) value was
645 calculated through a log-logistic equation fitting the total FW data gotten from seedlings treated with
646 different doses of the allelochemical. The curve pointed out a significance level of $P < 0.001$. Bars
647 indicate standard deviation. n=5.

648

649 **Figure 2.** Schematic diagram displaying the experimental design, platform and software tools used
650 for the analysis of metabolomic changes in wheat seedlings subjected to umbelliferone elicitation.

651

652 **Figure 3. Time course changes in the control and umbelliferone treated wheat seedlings.** (A)
653 Model profiles displaying the time-sensitive changes in metabolite abundance in control plants; (B)
654 Metabolite abundance profile in model # 10 (statistically significant) in control plants; (C) Model
655 profiles displaying the time-sensitive changes in metabolite abundance in umbelliferone-treated
656 plants; (D) Metabolite abundance profile in model # 9 (statistically significant) in umbelliferone-
657 treated plants. In panels A and C, the number in the upper left on each model profile designates the

658 model number (out of total 20 models generated), and the number in the bottom left on each model
659 profile is the statistical significance of the model. n=5.

660

661 **Figure 4. Multivariate (PLS-DA) analysis of the metabolomic changes.** (A) PLS-DA displaying
662 the separation of blank samples (B) from the rest of the samples showing system robustness; (B) PLS-
663 DA showing clusters of various time points in control plants; (C) PLS-DA showing clusters of various
664 time points in umbelliferone-treated plants. n=5.

665

666 **Figure 5.** Top 15 metabolites (variables) based on VIP scores from PLS-DA analysis for each
667 umbelliferone treatment time points (0 h, 6 h, 12 h, 24 h, 48 h, 96 h). The x-axis shows the correlation
668 scores whereas the y-axis corresponds to the metabolites identified. Color bars show median intensity
669 of variable in the respective group. n=5.

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674 **Supplementary Materials**

675 **Supplementary Figure 1.** Visual display of the coverage of metabolites quantified using our GC-
676 MS platform for this metabolomics investigation. (KEGG-based metabolite mapped onto the KEGG
677 metabolic pathway map (*blue dots* represent the mapped metabolites quantified in our study).

678

679 **Supplementary Figure 2.** KEGG-based pathway enrichment analysis displaying the wheat seedling
680 metabolome as covered using our GC-MS platform. Pathway names: 1-Glutathione metabolism, 2-
681 Arginine and proline metabolism, 3-Amino acyl-tRNA biosynthesis, 4-Taurine and hypotaurine
682 metabolism, 5-Tryptophan metabolism, 6-beta-Alanine metabolism, and 7-Isoquinoline alkaloid
683 biosynthesis.

684

685 **Supplementary Figure 3.** High Pearson (metabolite-metabolite) correlation of umbelliferone-
686 derived metabolites and polyphenol metabolism-derived metabolites.

687

688 **Supplementary Figure 4.** High Pearson (metabolite-metabolite) correlation of umbelliferone-
689 derived metabolites with other quantified metabolites in the study.

690

691 **Supplementary Figure 5.** High Pearson (metabolite-metabolite) correlation of fatty acids.

692

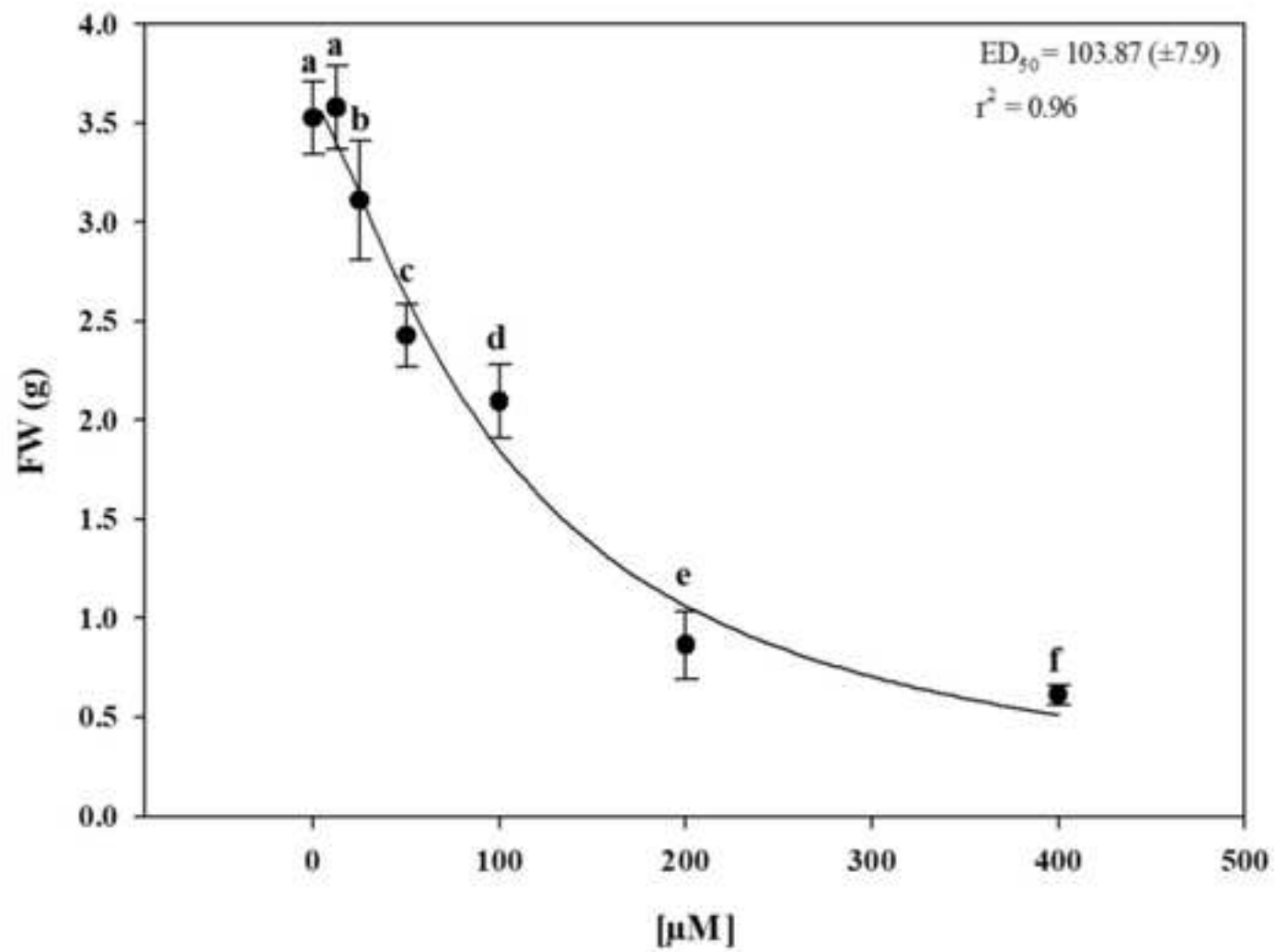
693 **Supplementary Figure 6.** High Pearson (metabolite-metabolite) correlation of amino acids.

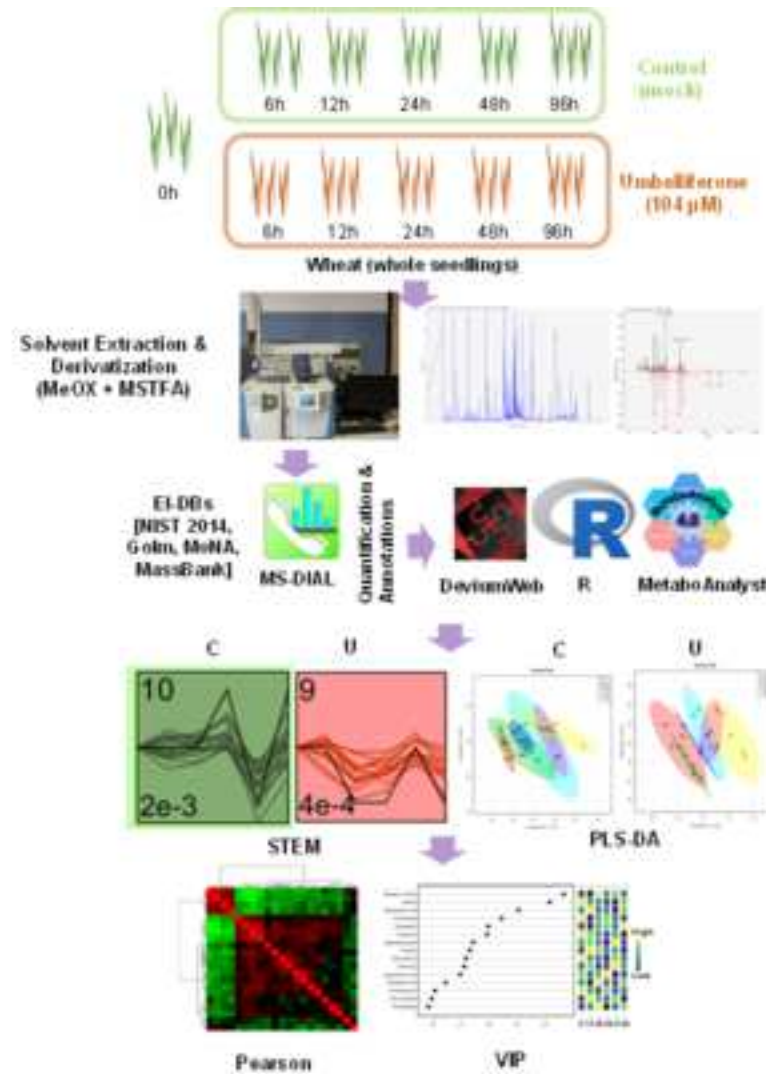
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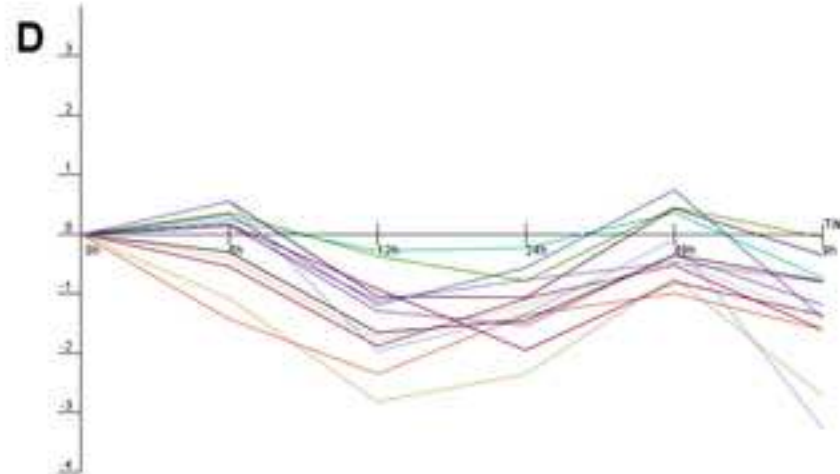
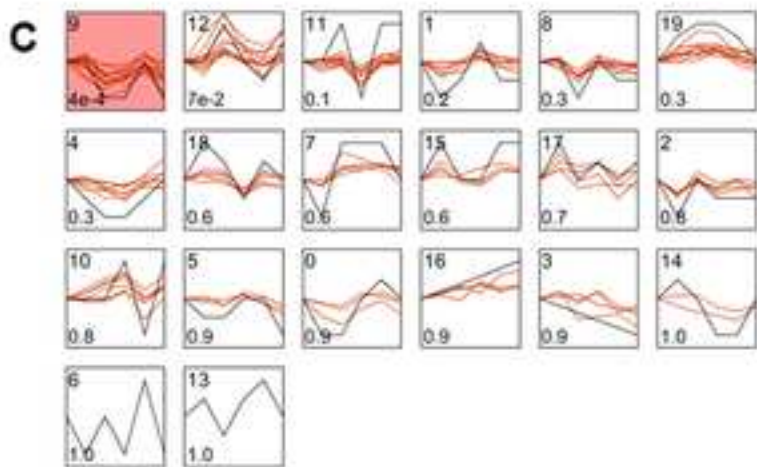
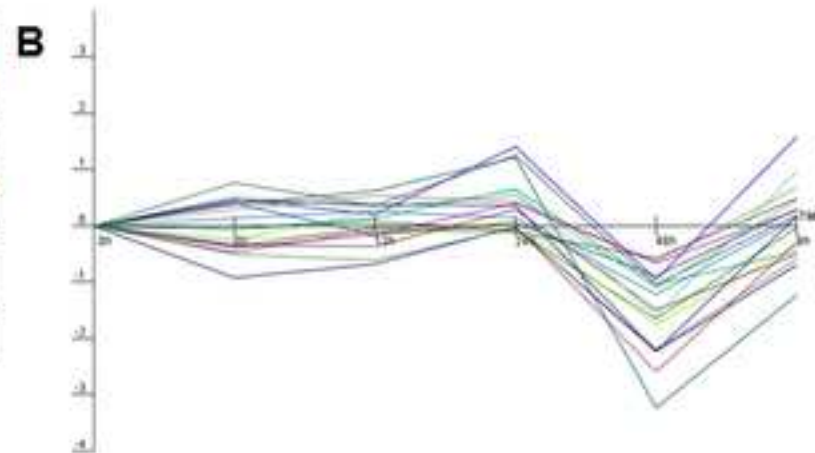
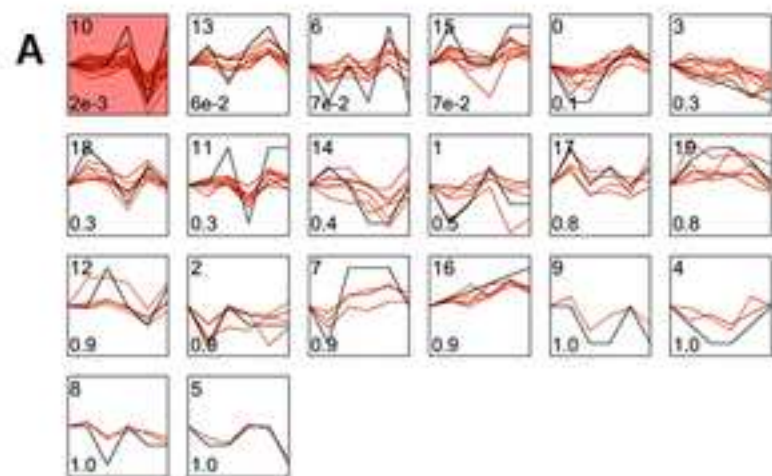
695 **Supplementary Figure 7.** High Pearson (metabolite-metabolite) correlation among carbohydrates.

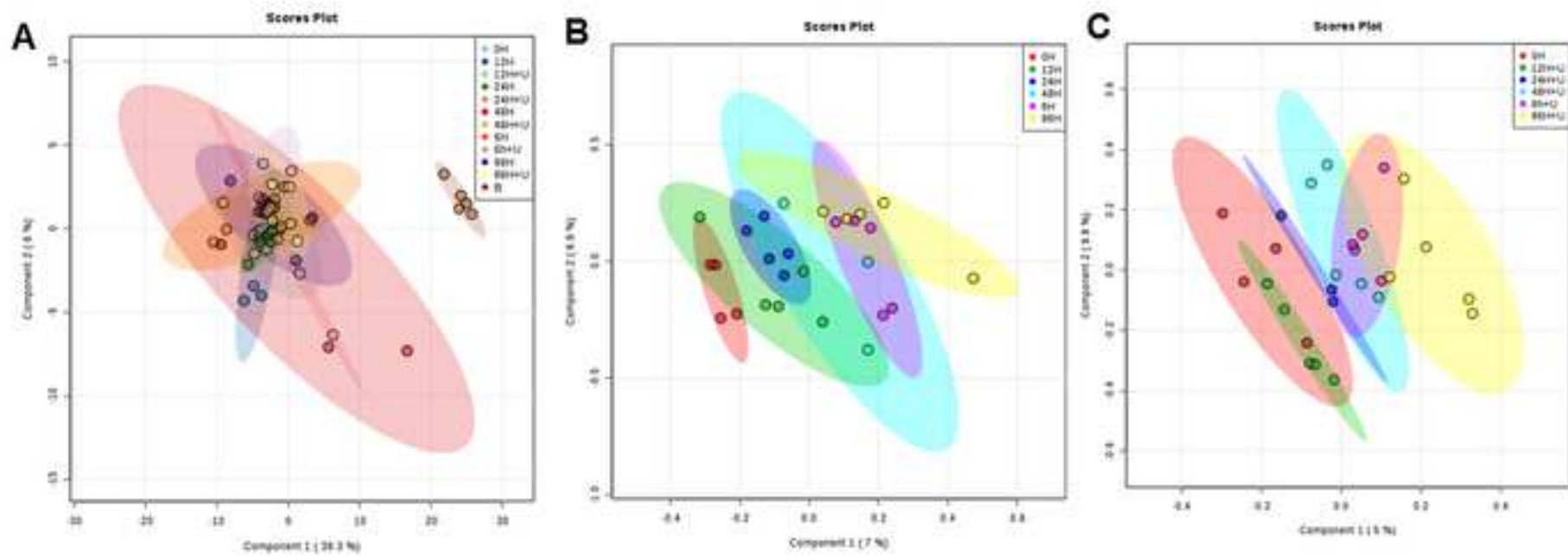
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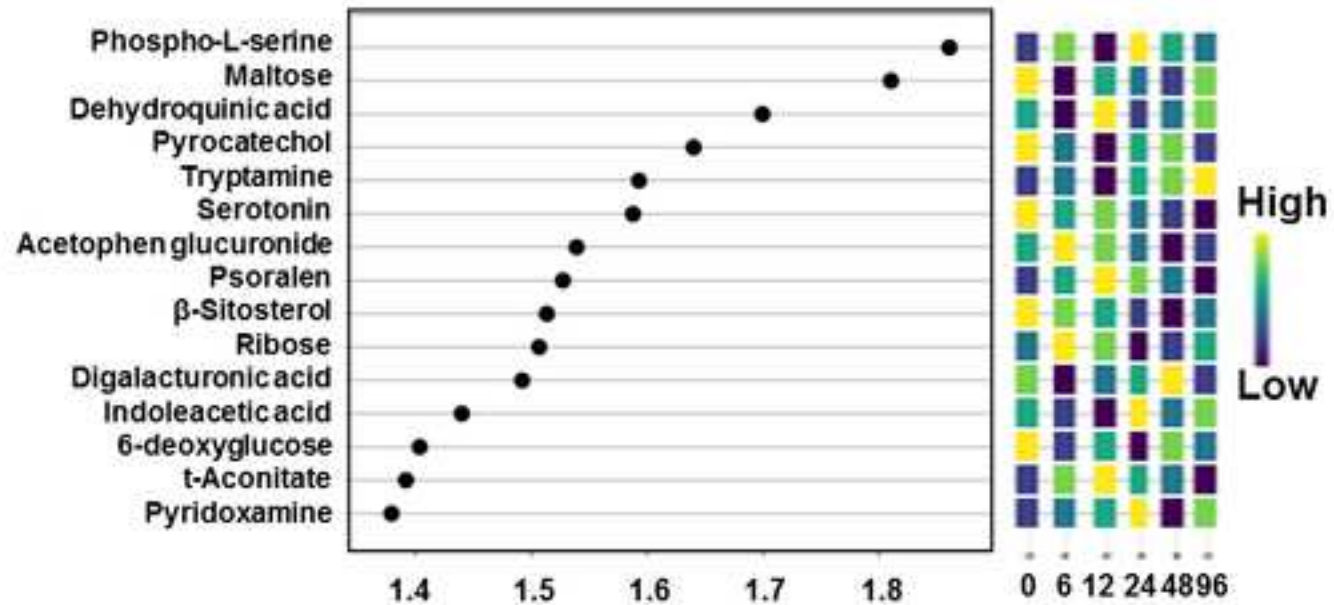
697 **Supplementary Figure 8.** Unsupervised principal component analysis (PCA) displaying the first 2
698 PCs for (A) all samples (control + Umbelliferone treatment) and time points together, (B) Control
699 samples and time points, and (C) Umbelliferone treatment samples and time points.

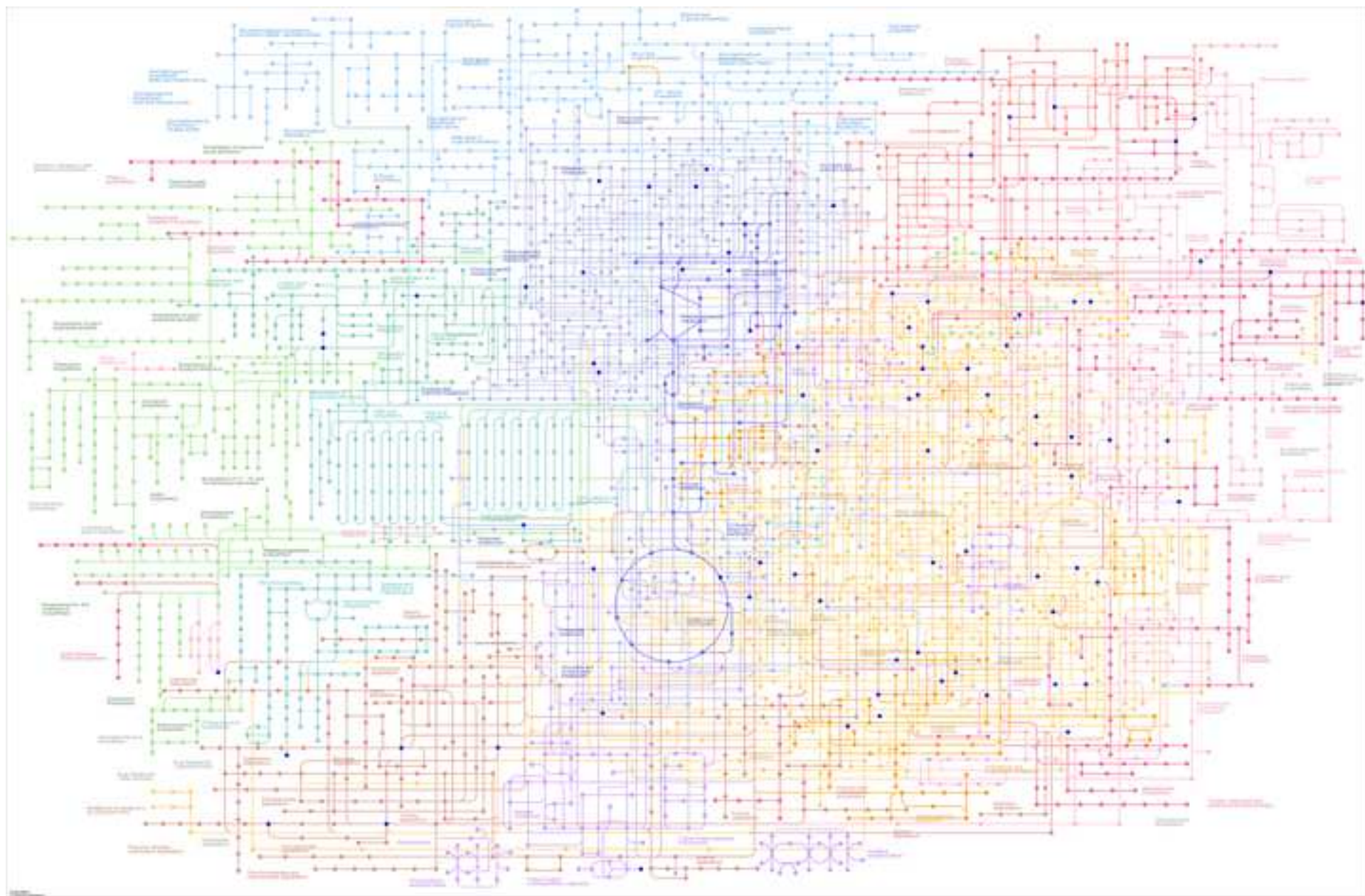


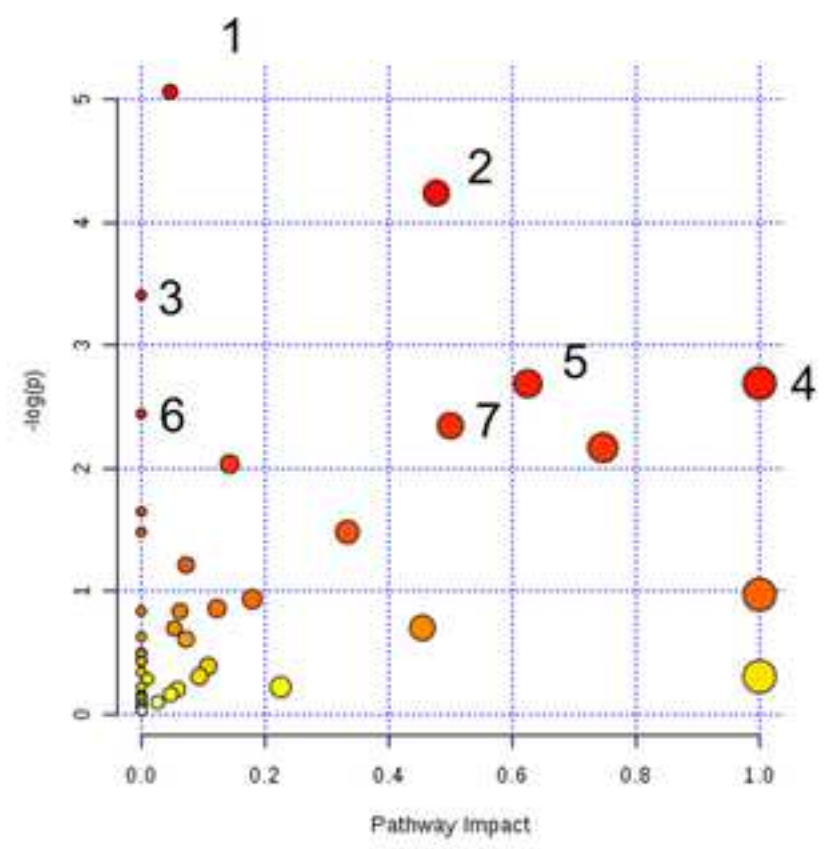


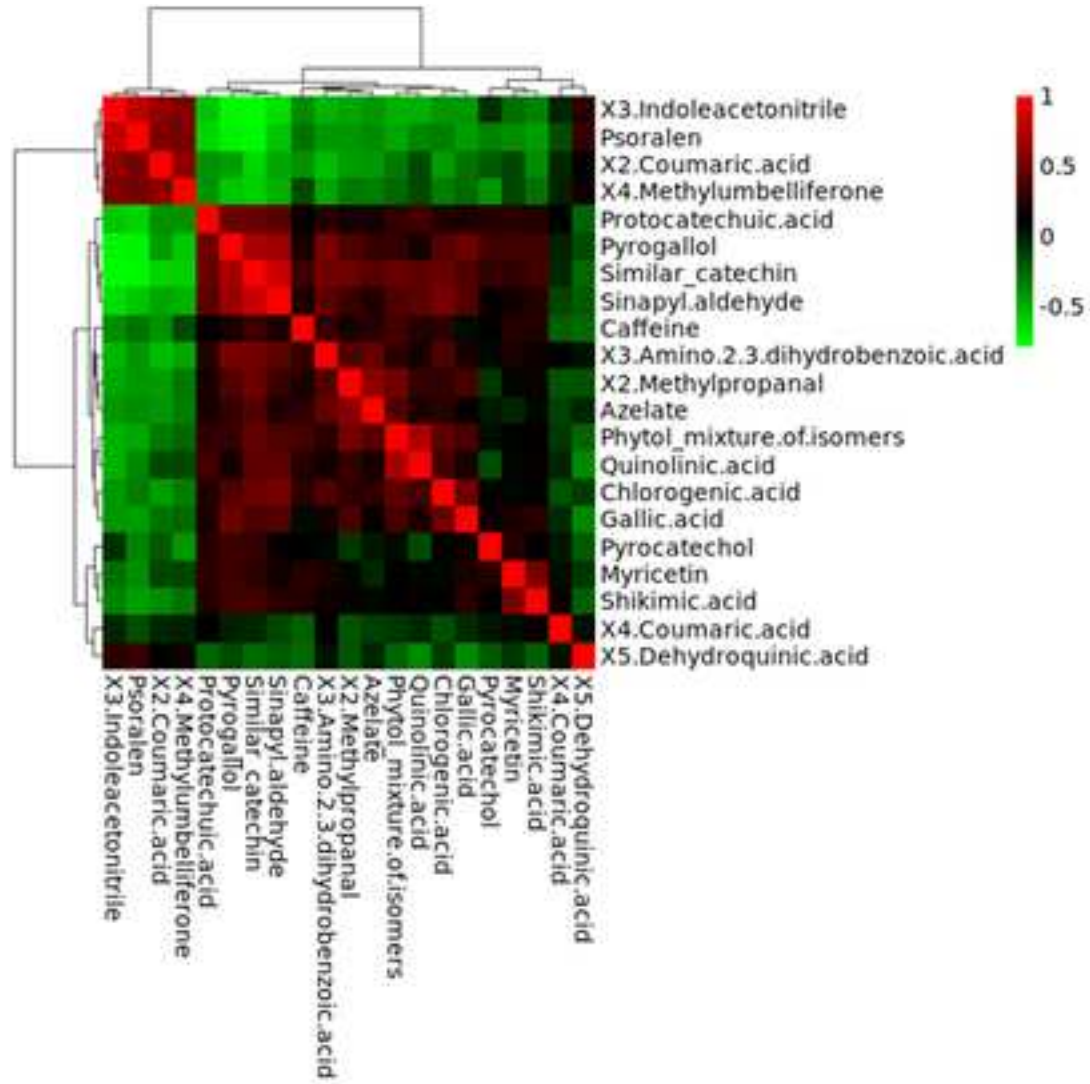


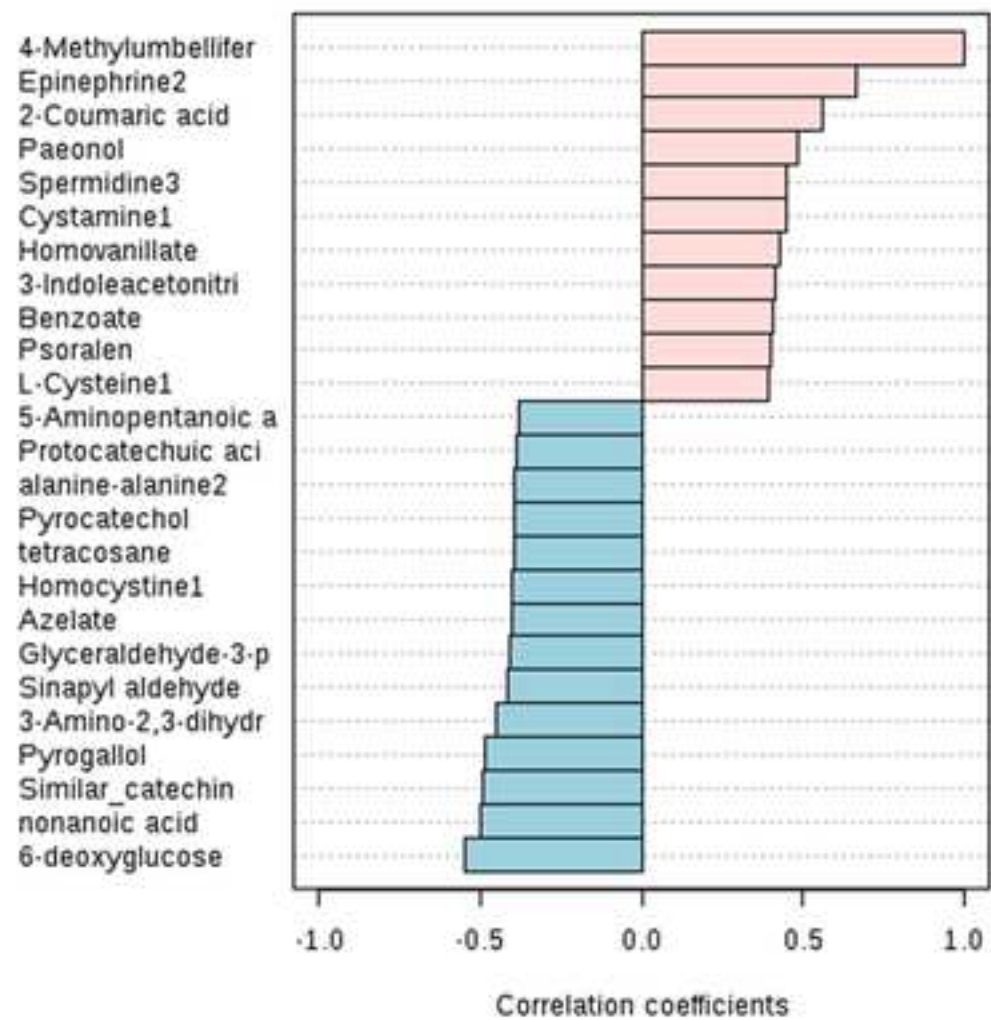


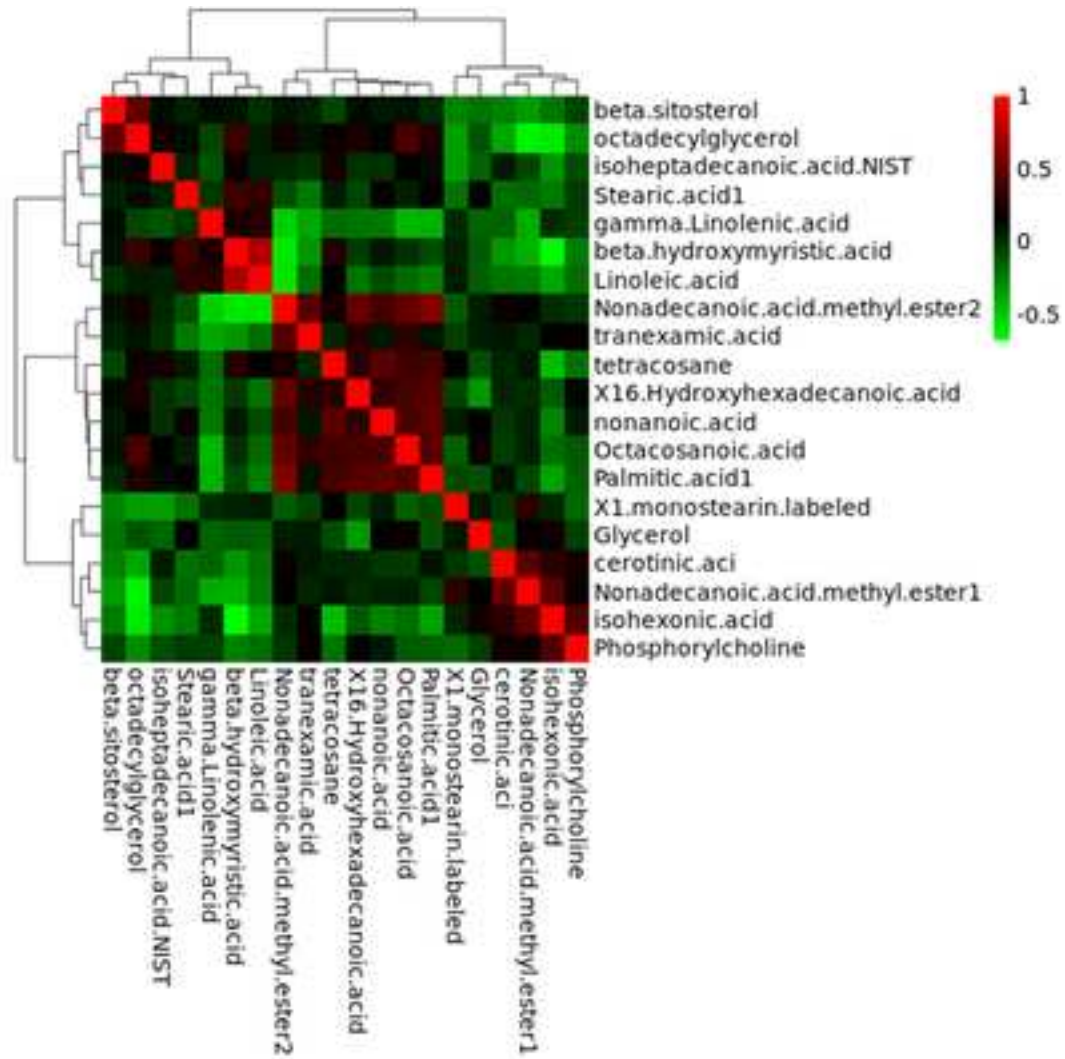


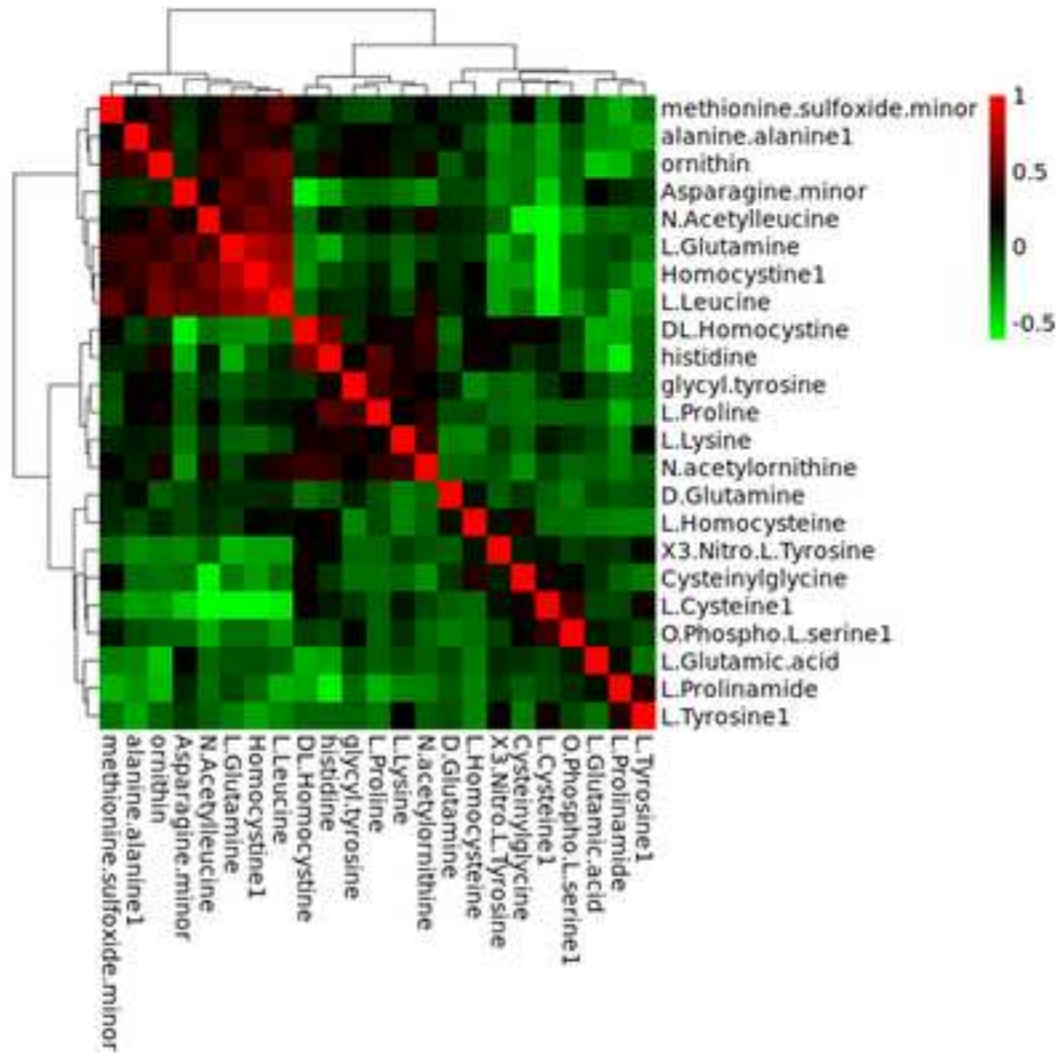


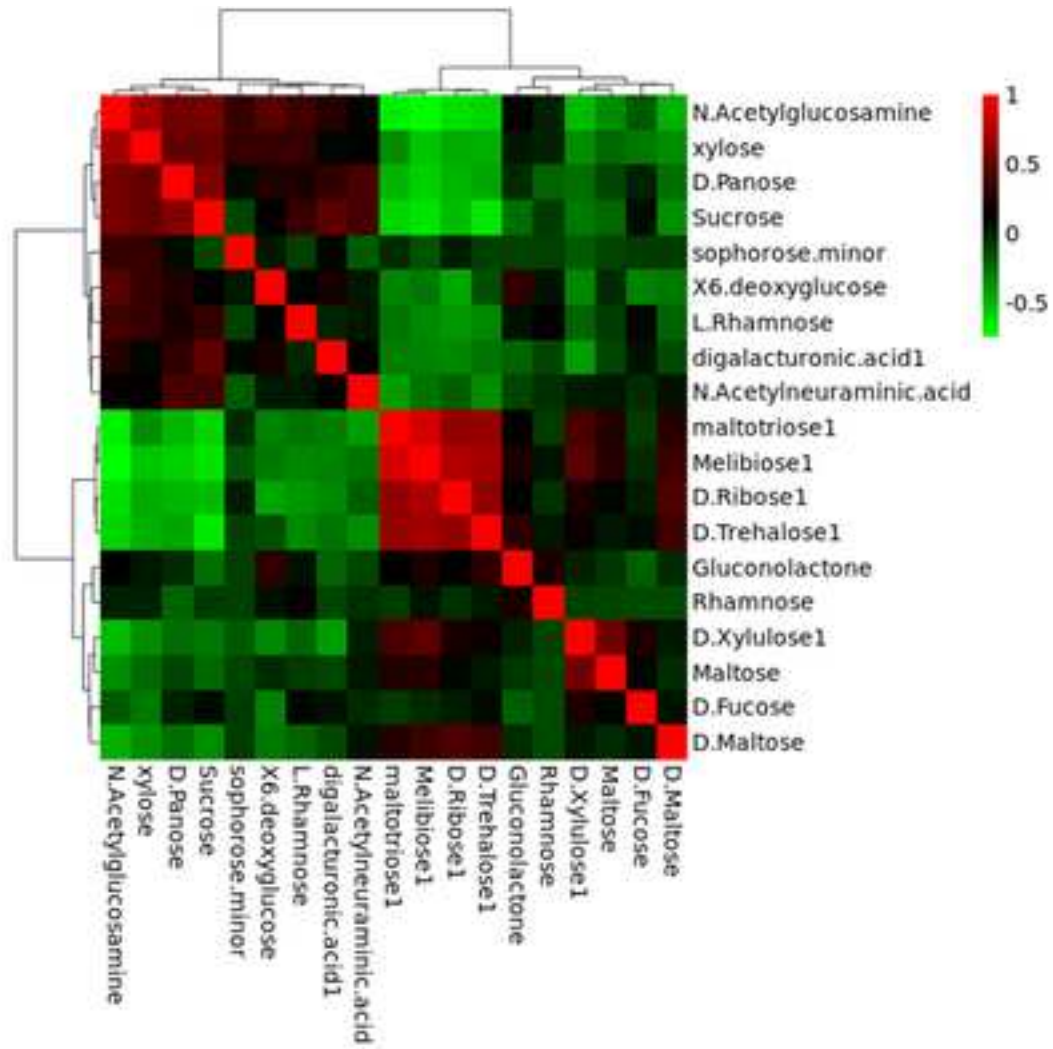


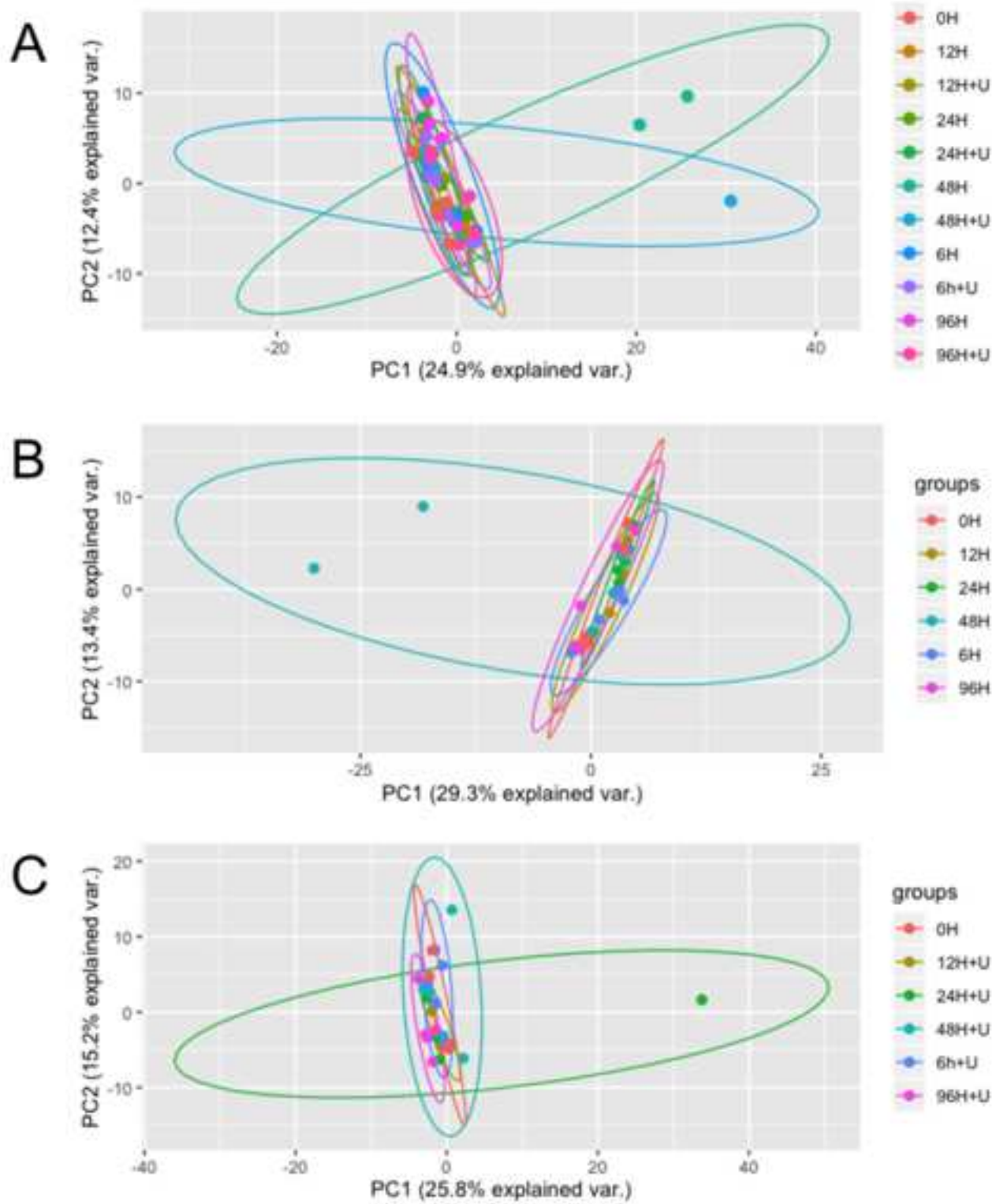














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Table

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: