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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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Abstract:	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.
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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

Research Article

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

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Short title: *Wheat metabolomics of umbelliferone treatment*

Abstract

In tThe present experiment used untargeted metabolomics to investigate the short-term metabolic changes induced in wheat seedlings by the specialized metabolite umbelliferone (, an allelochemical) in wheat seedlings have been deeply investigated using untargeted metabolomics.Allelopathy is a plant defense mechanism by which they protect themselves from competitive species using specialized biochemicals in the form of secretion or volatiles released to the environment. Though, umbelliferone is a well-known allelochemical, its mechanism of action in a short-term treatment is far from established. We used ≈ 10 days10 d day-old wheat seedlings treated with 104 µM umbelliferone over a time course experiment covering 6 times points, i.e., (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 efforts, we collectively obtained quantitative data on 177 metabolites that were derivatized (either derivatized singly or multiple times) or not, representing 139 non-redundant (unique) metabolites. Out of these 139 metabolites, 118 were associated with a unique Human Metabolome Database (HMDB) HMDB identifier, while 113 were associated with a Kyoto Encyclopedia of Genes and Genomes (KEGG) 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) with 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 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 of umbelliferone treatment revealed, that phospho-L-serine, maltose, and dehydroquinic acid were the top3 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 unimbalance of the shikimate pathways, which are strictly interconnected, were significantly altered by the treatment, suggesting a possible mechanism of action of this natural compound.The above indicate a system-wide changes induced by umbelliferone, through dysregulation of primary as well as specialized metabolism.

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

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Introduction

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

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

that this specia~~z~~lized metabolite could play a pivotal role in some plants as a first line of defense. This hypothesis was further confirmed by studies from Yang et al. [20], ~~that~~ which highlighted its ability to suppress the *Ralstonia solanacearum*-induced wilting disease process by reducing fungi colonization and proliferation, and by Hamerski et al. [21], ~~which~~ who demonstrated that extract of fungal cell wall acts as elicitor in *Amni majus*, increasing umbelliferone production. Umbelliferone is also involved in plant defense against herbivores ~~since it acts~~, acting as a repellent interfering with the bitter gustatory receptor neurons of fruit flies [22]. Finally, it has been ~~proven~~ shown that ~~it~~ umbelliferone determined the chemotactic movement of *Rhizobium* and *Agrobacterium* across chemical gradients towards lower levels of inhibitors and higher levels of potential nutrients [23]. Concerning its phytotoxic potential, several studies have demonstrated that this molecule strongly affects both plant growth and development, inducing reactive oxygen species (ROS) accumulation, chlorophyll degradation, alteration of root morphology, and ROS-induced programmed cell death [24-26]. Moreland and Novitzy [27] found that umbelliferone, at relatively high concentrations, inhibits functions in isolated chloroplasts and mitochondria, whereas Einhellig [28] demonstrated that concentrations of umbelliferone that reduce *Glycine max* seedling growth also decreased leaf water potential, stomatal conductance, and the transpiration ratio.

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

2. Materials and Methods

2.1. Chemicals and Reagents

Methanol for GC-MS SupraSolv® (1.00837), chloroform for GC-MS SupraSolv® (1.02432), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) ≥98.5% (69479), pyridine ≥99% (270407), methoxyamine hydrochloride 98% (226904), umbelliferone 99% (Hb24003), ribitol ≥99% (A5502), and alkanes mixture C_{10}-C_{40} (68281) were acquired from Sigma Aldrich (Italy).

2.2 Plant growth conditions and elicitor treatments

Durum wheat seeds (*Triticum durum* L. cv. Opera) seeds were germinated in Petri dishes (9 cm) in a growth chamber at 25°C, 70% humidity, and with a photoperiod of 16/: 8 (light /: dark), and light intensity of 90 mol m^{-2} s^{-1} supplied by a cool white fluorescent lamp (Polylux XL FT8, 55W 8440). Immediately after germination, uniform seedlings were transferred to a 4.5 l L hydroponic system and grown in a modified Hoagland solution formulated as follows: KNO_3 (10 mM); $MgSO_4$ (100 µM); $CaSO_4$ (400 µM); KCl (5 µM); K_2SO_4 (200 µM); K H_2PO_4 (175 µM); H_3BO_3 (2.5 µM); $MnSO_4$ (0.2 µM); $ZnSO_4$ (0.2 µM); $NaMoO_4$ (0.05 µM); $CuSO_4$ (0.05 µM); Fe-EDTA (200 µM). The solution was changed every other day and continuously oxygenated using an air bubble stone.

2.2.1 Dose-response curve

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

2.2.2 Short time-term effect of umbelliferone treatment

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To study the short-term effects of umbelliferone on the wheat metabolome, seedlings (a pool of 10 seedlings per replicate, per time point, and treatment) were grown for 10 days and were then treated with 104 µM of umbelliferone (the ED_{50} concentration was calculated from a dose-response curve). Plant materials were collected after 0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5) of umbelliferone treatment, and a parallel set of control plants (mock treated with same volume of ethanol/ water? as previously described §2.2.1) with the same

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

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2.3. Metabolite extraction and sample derivatization

Plant materials were collected at the middle of the light period, and whole plants were immediately snap frozen in liquid nitrogen to quench the endogenous metabolism. Freshly homogenized (100 mg) plant material ~~were~~ was obtained ~~for~~ from each biological sample (plant) and replicates. These were transferred to ~~a~~ 2 ~~ml~~ mL microcentrifuge round bottom screw cap tubes (Eppendorf). Extraction was done by adding 1400 ~~µl~~ µL of methanol (at -20 °C) and vortexing for 10 s after addition of 60 ~~µl~~ µL ribitol (0.2 mg/~~ml~~ mL stock in ddH_2O) as an internal quantitative standard for the polar phase. Samples were transferred in a thermomixer at 70°C and were shaken for 10 min (950 rpm) and were then further centrifuged for 10 min at 11000 g. The supernatants were collected and transferred to glass vials where 750 ~~µl~~ µL $CHCl_3$ (-20 °C) and 1500 ~~µl~~ µL ddH_2O (4 °C) were sequentially added. All the samples were vortexed for 10 s and then centrifuged for another 15 min at 2200 g. Upper polar phase (150 ~~µl~~ µL) for each replicate ~~were~~ was collected, transferred to a 1.5 ~~ml~~ mL tube and ~~were~~ dried in a vacuum concentrator without heating. Before freezing and storing at -80 °C, the tubes were filled with argon and placed in a plastic bag with silica beads (~~for avoiding~~to avoid moisture and hydration during short-term storage). Before derivatization, stored samples~~,~~ were placed in a vacuum concentrator for 30 ~~minutes~~ min~~.~~ to eliminate any trace of humidity. Then, ~~to the dried samples,~~ 40 ~~µl~~ µL methoxyamine hydrochloride (20 mg/~~ml~~ mL in pyridine) ~~were~~ was added to the dried samples, ~~and~~ which were then incubated for 2 h in a Thermomixer (950 rpm) at 37°C. Methoxyaminated samples were then silylated by adding 70 ~~µl~~ µL of MSTFA to the aliquots. Samples were further shaken for 30 min at 37 °C. Derivatized samples (110 ~~µl~~µL) were then transferred into glass vials suitable for the GC/MS autosampler for analysis.

2.4. GC-quadrupole/MS analysis

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

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2.5 GC/MS Analysis and data acquisition

2.5.1 GC/MS data analysis using MS-DIAL

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

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 ED_{50} 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 ED_{50} value was then used as the key concentration for the short-term metabolomics experiments.

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] ~~(Caraux, S. Pinloche, 2005) 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.

2.6.2 Multivariate analysis

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

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) tool ~~(Ernst & Bar-Joseph, 2006)~~[40], originally used for short microarray time series experiments ~~that are short~~ (3–8 time points for >~80% of the datasets). The novel STEM clustering takes advantage of the 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 the starting point~~,~~; number of model profiles = 20~~,~~; maximum unit change in model profiles between time points = 3. To explain the model profiles, w~~W~~e used an expression of -1 for decreased levels of a metabolite, 0 for unchanged levels of a metabolite, and 1 ~~as~~for 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~~:~~ decreased, decreased, unchanged, increased, increased, and unchanged levels of a given set of metabolites for the 6 time points in the given model profile.

2.~~7~~ 8 Pathway enrichment and clustering analysis

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

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Database (HMDB)~~HMDB~~, CAS, PubChem Compound ID (CID), LipidMAPS IDs and InChiKeys ~~values~~identifiers.

2.~~8~~ 9 Data sharing

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

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3. Results and Discussion

3.1 Dose response curve based on wheat biomass production in response to ~~Umbelliferone~~umbelliferone~~.~~

The dose response curve built on the variation of wheat fresh biomass (FW), in response to increasing doses of umbelliferone (0-400 µM), pointed out a significant dose-dependent phytotoxic effect (**Fig. 1**). The lowest concentration (12.5 µM) did not affect plant growth. ~~On the contrary, a~~At 25 µM, a 17% reduction of biomass ~~reduction~~ was observed, and ~~such~~ the reduction reached ~~the~~ 82~~-~~% at the highest concentration (400 µM). The non-linear regression fit of FW raw data ~~pointed out~~determined an ED_{50} value of 104 µM. ~~Further, i~~Inhibitory effects of umbelliferone to plants such as *Festuca rubra*, *Medicago sativa* and *Lactuca sativa* have been reported [17, ~~38~~42]. Based on the optimized umbelliferone concentration~~s~~, we designed the experiment to investigate the metabolomic changes in seedlings ~~subjected~~ exposed to 6 h, 12 h, 24 h, 48 h, and 96 h of umbelliferone treatment, as compared ~~to~~ with the controls (mock treated) (**Figure 2**).

3.2 **Cataloging the wheat seedling metabolome**

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

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

3.3 Impact of umbelliferone on wheat metabolome

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

3.4 Time-course profiling of umbelliferone treatment (quantitative)

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

metabolites across the 6 time points were ~~fit~~ put into 20 ~~possible~~ model clusters, which revealed differential accumulation of metabolites for control and ~~umbelliferone~~ umbelliferone-treated groups of plants, ~~over the~~as a function of time. In the 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) for the six time-points in the study [0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5)]. These 18 metabolites ~~included~~ were sugars (fucose, maltose, trehalose, ~~xylulose~~and xylulose), organic acids (isohexonic acid, tranexamic acid, ~~aconitic~~and aconitic acid), amines (pyridoxamine, tryptamine), ribulose 1, 5-bisphosphate, 3-indoleacetonitrole, etc. (**Figure 3 A, B**). In the case of ~~umbelliferone~~ 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 for the six time-points in the study. These 13 metabolites ~~belonged to~~were sugars (trehalose, xylulose, melibiose, ~~rhamnose~~and rhamnose), organic acids (ascorbic acid, pimelic acid, quinolic acid, ~~aconitic~~and aconitic acid) ~~and~~ polyamines (putrescine and spermidine), etc. (**Figure 3 C, D**).

3.5 Multivariate and clustering analysis reveal metabolites

Secondly, we performed both supervised and unsupervised multivariate analyses~~,~~ as feature extraction strategies, to maximize variance in the data using strongly correlated variables. We first performed ~~first~~ an unsupervised analysis, which ~~did~~ explained 37~~–~~42~~-~~% of the variability in data using the first 2 PCs, either in all samples ~~clubbed~~grouped together, only control sample groups, or umbelliferone groups (**Supplementary Figure 8A-C**). However, the time points did not cluster well~~-~~, ~~pointing~~which points to the non-independent samples which are not well handled by PCA~~:~~, ~~and~~the small feature space of 177 metabolites, and too many treatments (6 time points x 2 treatments), leading to possible multicollinearity issues, displaying more art~~e~~ifacts than a true biological picture. Following the 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. Using supervised PLS-DA analysis for all the samples (all time points, control and umbelliferone treated plants) and the blanks (B), we showed that the first two components explained ~~of~~ variations from the T0, 6 h, 12 h, 24 h, 48 h, and 96 h time points; components 1 and 2 alone explained ~45% of the variation (**Figure 4A**). For the control and ~~umbelliferone~~ umbelliferone-treated plants, the first two components (1 and 2) helped explain ~14% and ~15% of the variations, respectively (**Figures 4B, C**). ~~Further, t~~The co-clustering of time points~~,~~ (i.e., 6 h

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

Using metabolite-metabolite (Pearson) correlation, we monitored the clusters ~~of groups~~ of metabolites. ~~We found, among~~ Among secondary metabolites, we found that 3-indoleacetonitrile (an auxin, from tryptophan metabolism), psoralen and 4-methylumbelliferone (both umbelliferone derivatives), and 2-coumaric acid were highly correlated (**Supplementary Figure 3, 4)**, indicating their possibly coordinated biosynthesis and regulation. ~~For the rest another tight cluster was also evident.~~ Similarly, tight clusters were observed for fatty acids (**Supplementary Figure 5**), groups of amino acids (**Supplementary Figure 6**), and carbohydrates (**Supplementary Figure 7)**. A recent study~~,~~ ~~–~~ that looked at various polyphenols across diverse species ~~have~~ observed that ~~quantitatively,~~ umbelliferone and kaempferol are quantitatively associated with each other, while there was a positive correlation of epicatechin ~~existed~~ with umbelliferone and kaempferol [~~39~~43].

In order to identify the metabolites responsible for the discrimination among the metabolomic profiles, the VIP scores ~~was~~ were used to select those with the most significant contributions in a PLS-DA model, thus ~~is~~as a measure of a variable's importance in the PLS-DA model.~~.~~ VIP scores are a weighted sum of PLS weights for each variable, and measure the contribution of each predictor variable to the model [~~40~~44]. ~~Further, the~~The VIP statistic ~~summarizes~~ indicates the importance of the metabolites in differentiating the study groups (~~i.e.,~~ umbelliferone treatment times, i.e., 0 h, 6 h, 12 h, 24 h, 48 h, 96 h) in multivariate space. The compounds exhibiting the higher VIP scores are the more influential variables. Our VIP analysis revealed~~,~~ ~~–~~ that the metabolites with high VIPs were phospho-L-serine, maltose, dehydroquinic acid, pyrocatechol, tryptamine, and serotonin, among others **(Figure 5)**. Thus, the biochemical changes induced by umbelliferone treatment may ~~provide~~ support mechanistic explanations of the plant metabolic responses induced by this coumarin compound. In particular, as highlighted by the VIP scores reported (**Figure 5**), several metabolites involved in both shikimate and tryptophan pathways were significantly altered by the treatment. Among them, fluctuations in dehydroquinic acid abundances during all the treatments ~~in~~ are noteworthy, where the highest values were recorded at 12 h and 96 h. Dehydroquinic acid represents the first carbocyclic intermediate of the shikimate pathway, which undergoes five further enzymatic steps in the remainder of the shikimate pathway to yield chorismic acid, a precursor to tyrosine, ~~3~~ phenylalanine, tryptophan, and some vitamins [~~41~~45]. Interestingly,

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pyridoxamine (vitamin B6) ~~showed significant alterations~~was significantly altered by the umbelliferone treatment~~;~~ it reached highest abundance at 24 h of treatment, dropped after 48 h, and ~~again~~ increased again at 96 h~~,~~ ; it is an essential coenzyme with a high antioxidant potential [~~42~~46]. Moreover, pyridoxamine in the presence of ATP is converted by the pyridoxal kinase in pyridoxal 5'-phosphate, which is strictly connected to the enzyme tryptophan synthetase, an enzyme that catalyses the final two steps in the biosynthesis of tryptophan [~~43~~47]. The tryptophan synthetase, typically found as ~~an~~a α2β2 tetramer, catalyses the irreversible condensation of indole and serine to form tryptophan in a pyridoxal 5'-~~phosphate~~ phosphate-dependent reaction [~~44~~48]. In addition, the conversion of tryptophan to ~~indol~~indole acetic acid leads to the formation of glutamate, which is one of the pyridoxamine precursors [49]. It is therefore conceivable that, as detailed below, the umbelliferone-triggered perturbation of the tryptophan metabolism might be on the bas~~e~~is of the observed pyridoxamine accumulation pattern ~~upon~~over time. ~~Also, it is~~ It is also possible that~~-~~ the fluctuation in pyridoxamine content is attributable to the conversion into their derivatives, namely pyridoxal, pyridoxal 5- phosphate, and pyridoxamine [50], involved in many other cellular functions, which ~~has not been detected in the present experiment~~were simply not detected / quantified in our metabolomics experiments. Among the metabolites involved in tryptophan biosynthesis, phospho-L-serine [~~45~~51] was characterized by the highest VIP score ~~value~~, pointing ~~out~~ to a significant increase in concentration ~~along~~ over time. This molecule ~~play~~ has a pivotal role in plants under environmental stresses~~,~~; as an upregulation of several genes involved in this pathway were observed during abiotic stresses~~,~~ such as salinity, cold, and flood, indicating its importance in supplying serine under environmental stresses [~~46~~52]. Moreover, the phosphorylated pathway might be essential to provide the amino acid~~,~~ serine for the synthesis of tryptophan, the common precursor for the biosynthesis of indole acetic acid (IAA) [~~47~~53]. Interestingly, in our experiments, significant variations in IAA and tryptamine (indole-alkaloid) content, an intermediate in IAA biosynthesis, were observed. In fact, both metabolites were significantly elicited by the umbelliferone treatment. Alteration in IAA biosynthesis and distribution, driven by 4-methylumbelliferone (an umbelliferone derivative), was previously observed by Li et al. [25] in *Arabidopsis* seedlings. In particular, they observed that the exogenous application of 4-methylumbelliferone (125 µM for 22 days) led to reduced primary root growth, the formation of bulbous root hairs, and an increase in the number of lateral root ~~numbers~~. The authors also ~~unveiled~~ uncovered ~~the~~ an accumulation of 4-methylumbelliferyl-β-D-glucoside, derived from UDP-~~glycosyltransferases~~ glycosyltransferase mediated transformation of ~~umbrelliferone~~umbelliferone in roots ~~as well as the~~and ~~–~~ upregulation of several *UDP-glycosyltransferase* genes, which were supportive for a well-orchestrated mechanism devoted to the detoxification of umbelliferone in

plants. During our experiments, the presence of both 4-methylumbelliferone and psoralen, umbelliferone derivatives, ~~were~~ was detected in ~~umbelliferone~~ umbelliferone-treated plants, suggesting that the umbelliferone ~~supplied~~ was internalized and metabolized by the seedlings.

~~It has been proven~~Based ~~on s~~Studies ~~from~~of several other species~~, it has~~ have ~~been~~ proven that both umbelliferone derivatives can act as phytoalexins themselves; they can protect~~ing~~ plants from both biotic and abiotic stresses, and/or can induce reduction in growth and development [25, 54-56]. Therefore, it cannot be excluded that the reduction in plant growth observed during the dose response curve could ~~be~~ also be due to the accumulation of umbelliferone derivatives. ~~In fact,~~ ~~r~~Recent studies ~~carried on the speciesin~~ of *Psoralea corylifolia*, treated with psoralen elicitors and precursors, demonstrated that there is a negative correlation between psoralen accumulation and cell growth [57]. ~~Anyway,~~Furthermore, ~~it should not be forgotten that~~ psoralen accumulation in plants, as well as other specialized metabolites, play~~s~~ a pivotal role in protecting plants from several other stresses [57], and ~~probably~~ the observed plant growth reduction ~~could be~~is probably due to the redistribution of plant energies in the activation of (specialized) biosynthetic pathways involved in detoxification and/or protection from oxidative stress, instead of the biosynthesis of (primary) metabolites fundamental for growth.

~~-~~Despite ~~the~~ its role ~~of~~ as an intermediate in auxin biosynthesis, it has been suggested that tryptamine could play an important role during both biotic and abiotic stress. It has been observed, for example, that barley leaves irradiated with UV light were accumulating high ~~contents~~ levels of tryptamine. Moreover, its induction was also observed to occur in response to plant pathogenic fungi infection, suggesting that it could act as a plant defense metabolite [~~48~~58]. On the other hand, tryptamine accumulation was accompanied by a reduction in serotonin content. It has been ~~largely~~ widely reported that in graminaceous species the enzyme tryptamine 5-hydroxylase is involved in serotonin biosynthesis, catalyzing the conversion of tryptamine to serotonin [~~49~~59, ~~50~~60]. Kang et al. [~~51~~61] demonstrated that the exogenous application of tryptamine to tissues of rice seedlings induced a dose-dependent increase in serotonin, accompanied by a parallel increase in tryptamine 5-hydroxylase enzyme activity. At the same time, the same tissues grown in the presence of tryptophan did not show any significant increase in serotonin. Therefore, it ~~could~~ can be speculated that tryptamine accumulation, followed by the reduction in serotonin content, could be due to an umbelliferone-induced reduction of ~~the~~ tryptamine 5-hydroxylase activity. ~~Further, s~~Serotonin, which plays a pivotal role in plant growth regulation and in plant response to both biotic and abiotic stress [~~52~~62], and psoralen, are considered to be ~~–~~ phytoalexins with antioxidant ~~activities~~properties~~, is an important molecule~~ – involved in plant defense [~~53~~55].

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Limitations ~~to~~ of the study

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

4. Conclusions

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

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

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Author Contributions

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

Conflicts of Interest

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

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Figure Captions and Table Legends

Figures

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

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

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

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

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

Supplementary Materials

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

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

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

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

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

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

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

Supplementary Figure 8. Unsupervised principal component analysis (PCA) displaying the first 2 PCs for (A) all samples (control + Umbelliferone treatment) and time points together, (B) Control 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 phytoallexin, 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 ED_{50} 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 ED_{50} concentration calculated from a dose-response curve……") and the seedlings were then treated with the [ED_{50}] 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.

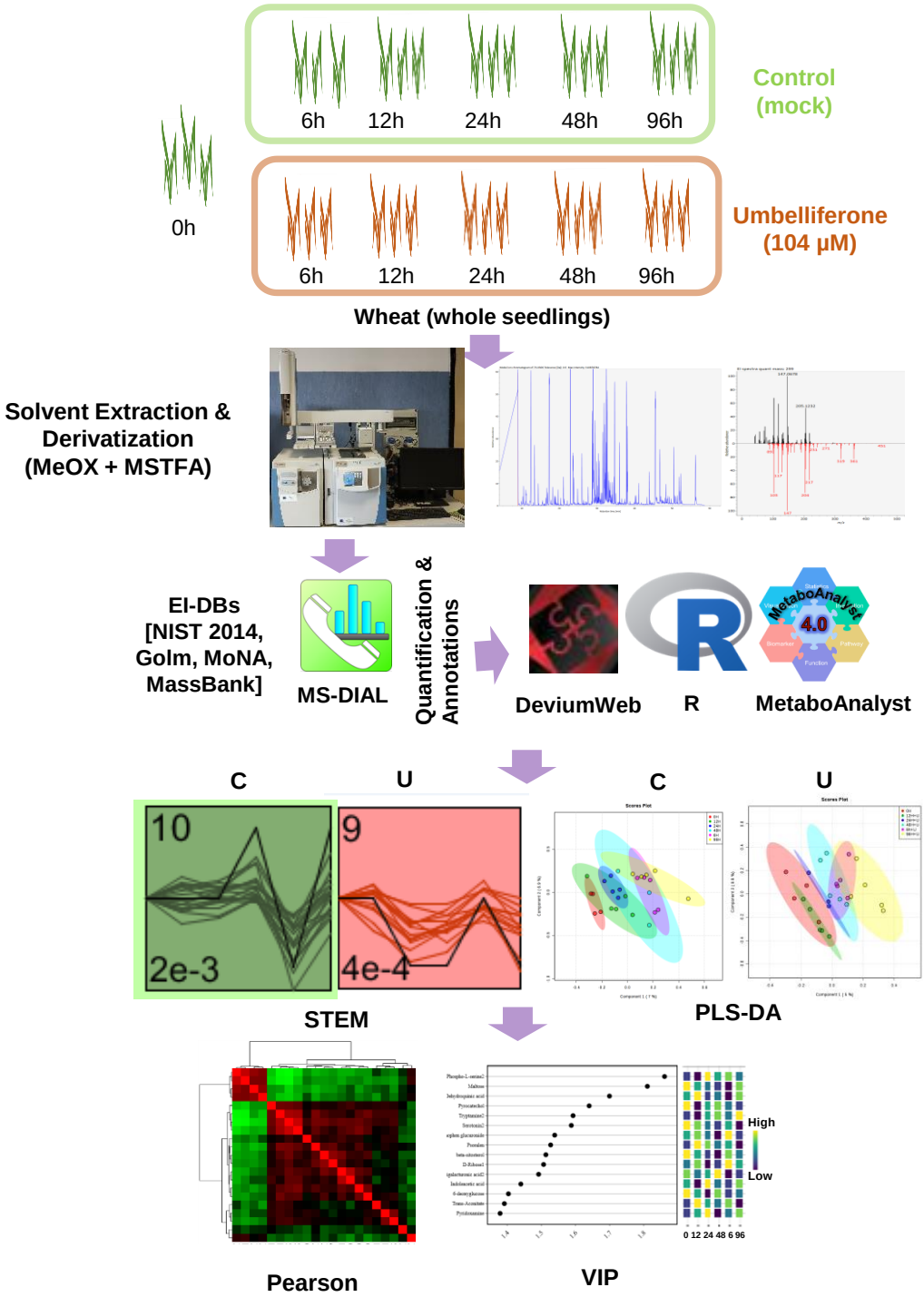
10) p. 13, section 4: **conclusion -** *This is a re-hashing of some basic premises of the study,* ***and doesn't really include a lot of introspection into the study, nor does it speak broadly about the implications and significance of what they've done.*** *It needs a re-write to better articulate 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



Research Article

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

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Short title: *Wheat metabolomics of umbelliferone treatment*

Abstract

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.

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

Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Chemicals and Reagents

Methanol for GC-MS SupraSolv® (1.00837), chloroform for GC-MS SupraSolv® (1.02432), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) ≥98.5% (69479), pyridine ≥99% (270407), methoxyamine hydrochloride 98% (226904), umbelliferone 99% (H24003), ribitol ≥99% (A5502), and alkanes mixture C_{10}-C_{40} (68281) were acquired from Sigma Aldrich (Italy).

2.2 Plant growth conditions and elicitor treatments

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

after germination, uniform seedlings were transferred to a 4.5 L hydroponic system and grown in a modified Hoagland solution formulated as follows: KNO_3 (10 mM); $MgSO_4$ (100 µM); $CaSO_4$ (400 µM); KCl (5 µM); K_2SO_4 (200 µM); K H_2PO_4 (175 µM); H_3BO_3 (2.5 µM); $MnSO_4$ (0.2 µM); $ZnSO_4$ (0.2 µM); $NaMoO_4$ (0.05 µM); $CuSO_4$ (0.05 µM); Fe-EDTA (200 µM). The solution was changed every other day and continuously oxygenated using an air bubble stone.

2.2.1 Dose-response curve

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

2.2.2 Short-term effect of umbelliferone treatment

To study the short-term effects of umbelliferone on the wheat metabolome, seedlings (a pool of 10 seedlings per replicate, time point, and treatment) were grown for 10 days and were then treated with 104 µM of umbelliferone (the ED_{50} concentration was calculated from a dose-response curve). Plant materials were collected after 0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5) of umbelliferone treatment, and a parallel set of control plants (mock treated with same volume of ethanol as previously described) with the same time points. In order to avoid metabolic fluctuations induced by plant circadian rhythms, all the treatments were applied in order to allow plant collection at the same hour of the day (12:00) (i.e., plants belonging to treatment T1 were treated at 06:00, T2 at 00:00, and so on). After collection, the plant materials were immediately snap frozen for metabolomic studies. The experiment was replicated five times (n = 5).

2.3. Metabolite extraction and sample derivatization

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

mg/mL stock in ddH_2O) as an internal quantitative standard for the polar phase. Samples were transferred in a thermomixer at 70°C and were shaken for 10 min (950 rpm) and were then further centrifuged for 10 min at 11000 g. The supernatants were collected and transferred to glass vials where 750 µL $CHCl_3$ (-20°C) and 1500 µL ddH_2O (4°C) were sequentially added. All the samples were vortexed for 10 s and then centrifuged for another 15 min at 2200 g. Upper polar phase (150 µL) for each replicate was collected, transferred to a 1.5 mL tube and dried in a vacuum concentrator without heating. Before freezing and storing at -80°C, the tubes were filled with argon and placed in a plastic bag with silica beads (to avoid moisture and hydration during short-term storage). Before derivatization, stored samples were placed in a vacuum concentrator for 30 min to eliminate any trace of humidity. Then, 40 µL methoxyamine hydrochloride (20 mg/mL in pyridine) was added to the dried samples, which were then incubated for 2 h in a Thermomixer (950 rpm) at 37°C. Methoxyaminated samples were then silylated by adding 70 µL of MSTFA to the aliquots. Samples were further shaken for 30 min at 37°C. Derivatized samples (110 µL) were then transferred into glass vials suitable for the GC/MS autosampler for analysis.

2.4. GC-quadrupole/MS analysis

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

2.5 GC/MS Analysis and data acquisition

2.5.1 GC/MS data analysis using MS-DIAL

Raw data (.RAW) from the single quadrupole instrument was converted to .mzML format with the 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 (log2), 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 ED_{50} 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

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

2.6.2 Multivariate analysis

Exploratory multivariate 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 with the Glimma package. Other various dimension reduction analysis via principal component analysis (PCA) of overall, separate control, and treated data was performed with the FactoMineR and factoextra packages. PCA and partial least-squares discriminant (PLS-DA) analyses were performed using the DeviumWeb package [39], where the output consisted of score plots to visualize the contrast between different samples and loading plots to explain the cluster separation. Data were scaled with unit variance, without any transformation. Partial least-squares discriminant analysis (PLS-DA) was used to highlight differences between the metabolic phenotypes at six time points (0 h, 6 h, 12 h, 24 h, 48 h, and 96 h).

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

For short time series metabolomics data analysis, we used the Short Time series Expression Miner (STEM) tool [40], originally used for short microarray time series experiments (3–8 time points for >~80% of the datasets). The novel STEM clustering takes advantage of the 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 the starting point; number of model profiles = 20; maximum unit change in model profiles between time points = 3. To explain the model profiles, we used an expression of -1 for decreased levels of a metabolite, 0 for unchanged levels of a metabolite, and 1 for increased levels of a metabolite. For instance, a model profile with an expression of -1, -1, 0, 1, 1, 0 represents decreased, decreased, unchanged, increased, increased, and unchanged levels of a given set of metabolites for the 6 time points in the given model profile.

2.8 Pathway enrichment and clustering analysis

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

2.9 Data sharing

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

3. Results and Discussion

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

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

3.2 **Cataloging the wheat seedling metabolome**

Using GC-MS, 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 HMDB identifier, while 113 were associated with a KEGG identifier. The derivatized metabolites included sugars (monosaccharides,

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

3.3 Impact of umbelliferone on wheat metabolome

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

3.4 Time-course profiling of umbelliferone treatment (quantitative)

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

for further analysis. The metabolite abundances for 177 metabolites across the 6 time points were put into 20 model clusters, which revealed differential accumulation of metabolites for control and umbelliferone-treated groups of plants, as a function of time. In the 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) for the six time-points in the study [0 h (T0), 6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), and 96 h (T5)]. These 18 metabolites were sugars (fucose, maltose, trehalose, and xylulose), organic acids (isohexonic acid, tranexamic acid, and aconitic acid), amines (pyridoxamine, tryptamine), ribulose 1, 5-bisphosphate, 3-indoleacetonitrole, etc. (**Figure 3 A, B**). In the 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 for the six time-points in the study. These 13 metabolites were sugars (trehalose, xylulose, melibiose, and rhamnose), organic acids (ascorbic acid, pimelic acid, quinolic acid, and aconitic acid) polyamines (putrescine and spermidine), etc. (**Figure 3 C, D**).

3.5 Multivariate and clustering analysis reveal metabolites

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

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

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

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

which is strictly connected to the enzyme tryptophan synthetase, an enzyme that catalyses the final two steps in the biosynthesis of tryptophan [47]. The tryptophan synthetase, typically found as a α2β2 tetramer, catalyses the irreversible condensation of indole and serine to form tryptophan in a pyridoxal 5'-phosphate-dependent reaction [48]. In addition, the conversion of tryptophan to indole acetic acid leads to the formation of glutamate, which is one of the pyridoxamine precursors [49]. It is therefore conceivable that, as detailed below, the umbelliferone-triggered perturbation of the tryptophan metabolism might be on the basis of the observed pyridoxamine accumulation pattern over time. It is also possible that the fluctuation in pyridoxamine content is attributable to the conversion into their derivatives, namely pyridoxal, pyridoxal 5- phosphate, and pyridoxamine [50], involved in many other cellular functions, which were simply not detected / quantified in our metabolomics experiments. Among the metabolites involved in tryptophan biosynthesis, phospho-L-serine [51] was characterized by the highest VIP score, pointing to a significant increase in concentration over time. This molecule has a pivotal role in plants under environmental stresses, as an upregulation of several genes involved in this pathway were observed during abiotic stresses such as salinity, cold, and flood, indicating its importance in supplying serine under environmental stresses [52]. Moreover, the phosphorylated pathway might be essential to provide the amino acid serine for the synthesis of tryptophan, the common precursor for the biosynthesis of indole acetic acid (IAA) [53]. Interestingly, in our experiments, significant variations in IAA and tryptamine (indole-alkaloid) content, an intermediate in IAA biosynthesis, were observed. In fact, both metabolites were significantly elicited by the umbelliferone treatment. Alteration in IAA biosynthesis and distribution, driven by 4-methylumbelliferone (an umbelliferone derivative), was previously observed by Li et al. [25] in *Arabidopsis* seedlings. In particular, they observed that the exogenous application of 4-methylumbelliferone (125 μM for 22 days) led to reduced primary root growth, the formation of bulbous root hairs, and an increase in the number of lateral roots. The authors also uncovered an accumulation of 4-methylumbelliferyl-β-D-glucoside, derived from UDP-glycosyltransferase mediated transformation of umbelliferone in roots and upregulation of several *UDP-glycosyltransferase* genes, which were supportive for a well-orchestrated mechanism devoted to the detoxification of umbelliferone in plants. During our experiments, the presence of both 4-methylumbelliferone and psoralen, umbelliferone derivatives, was detected in umbelliferone-treated plants, suggesting that the umbelliferone was internalized and metabolized by the seedlings.

Studies of several other species have proven that both umbelliferone derivatives can act as phytoalexins themselves; they can protect plants from both biotic and abiotic stresses, and/or can induce reduction in growth and development [25, 54-56]. Therefore, it cannot be excluded that the reduction in plant growth observed during the dose response curve could also be due to the

accumulation of umbelliferone derivatives. Recent studies of *Psoralea corylifolia*, treated with psoralen elicitors and precursors, demonstrated that there is a negative correlation between psoralen accumulation and cell growth [57]. Furthermore, psoralen accumulation in plants, as well as other specialized metabolites, play a pivotal role in protecting plants from several other stresses [57], and the observed plant growth reduction is probably due to the redistribution of plant energies in the activation of (specialized) biosynthetic pathways involved in detoxification and/or protection from oxidative stress, instead of the biosynthesis of (primary) metabolites fundamental for growth.

Despite its role as an intermediate in auxin biosynthesis, it has been suggested that tryptamine could play an important role during both biotic and abiotic stress. It has been observed, for example, that barley leaves irradiated with UV light were accumulating high levels of tryptamine. Moreover, its induction was also observed to occur in response to plant pathogenic fungi infection, suggesting that it could act as a plant defense metabolite [58]. On the other hand, tryptamine accumulation was accompanied by a reduction in serotonin content. It has been widely reported that in graminaceous species the enzyme tryptamine 5-hydroxylase is involved in serotonin biosynthesis, catalyzing the conversion of tryptamine to serotonin [59, 60]. Kang et al. [61] demonstrated that the exogenous application of tryptamine to tissues of rice seedlings induced a dose-dependent increase in serotonin, accompanied by a parallel increase in tryptamine 5-hydroxylase enzyme activity. At the same time, the same tissues grown in the presence of tryptophan did not show any significant increase in serotonin. Therefore, it can be speculated that tryptamine accumulation, followed by the reduction in serotonin content, could be due to an umbelliferone-induced reduction of tryptamine 5-hydroxylase activity. Serotonin, which plays a pivotal role in plant growth regulation and in plant response to both biotic and abiotic stress [62], and psoralen, are considered to be phytoalexins with antioxidant properties involved in plant defense [55].

Limitations of the study

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

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

4. Conclusions

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

Acknowledgments

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Author Contributions

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

Conflicts of Interest

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

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Figure Captions and Table Legends

Figures

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

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

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

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

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

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

Supplementary Materials

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

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

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

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

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

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

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

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

Figure 1

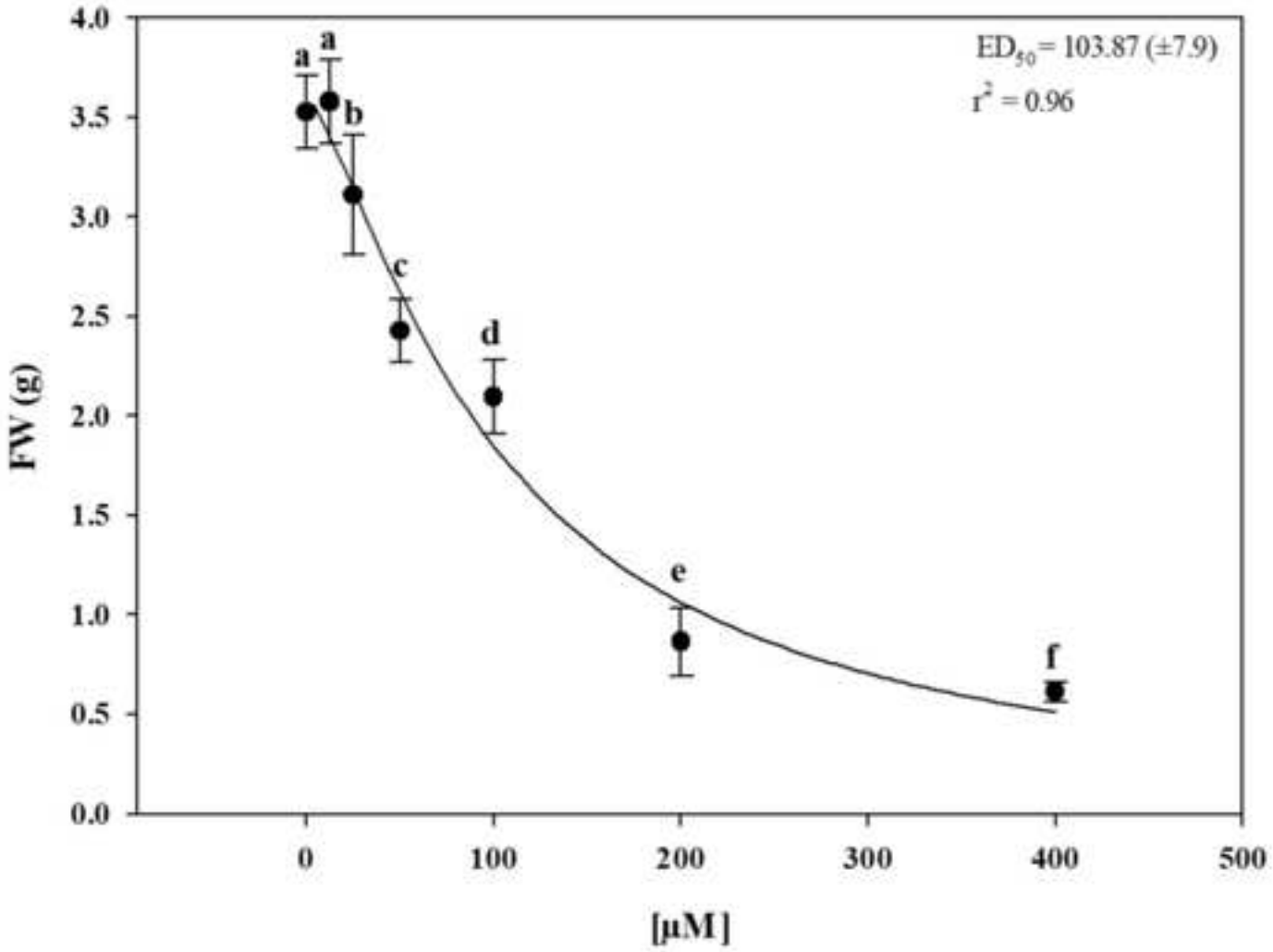

Figure 2

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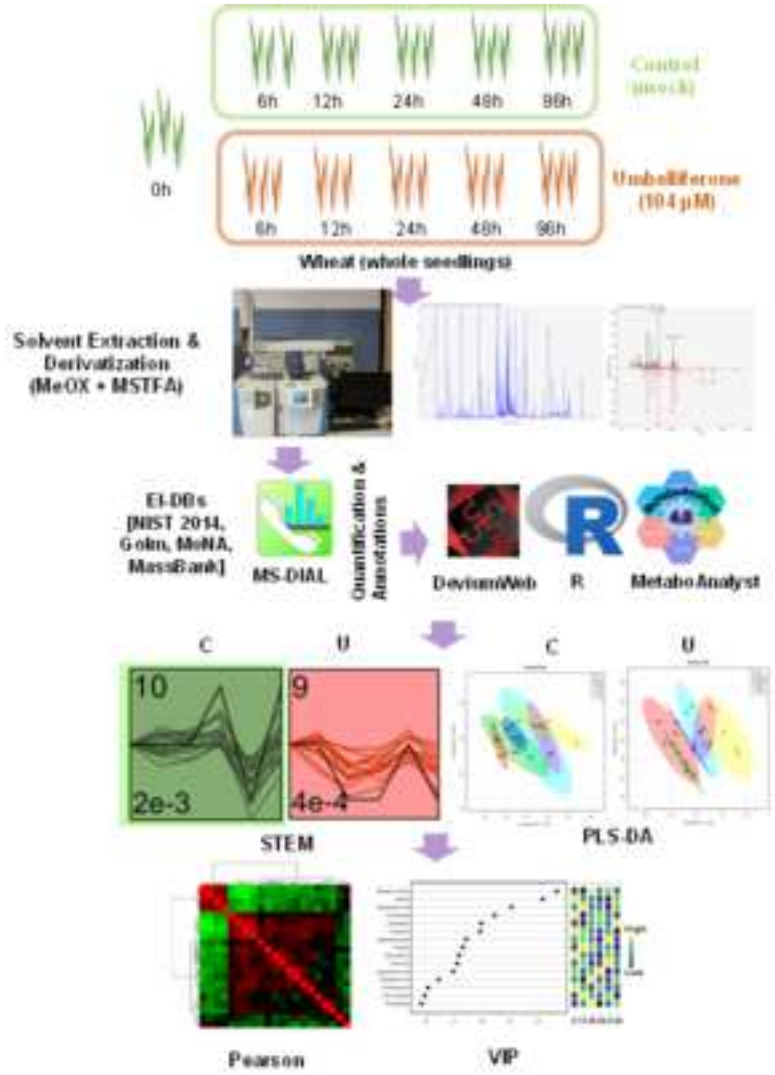

Figure 3

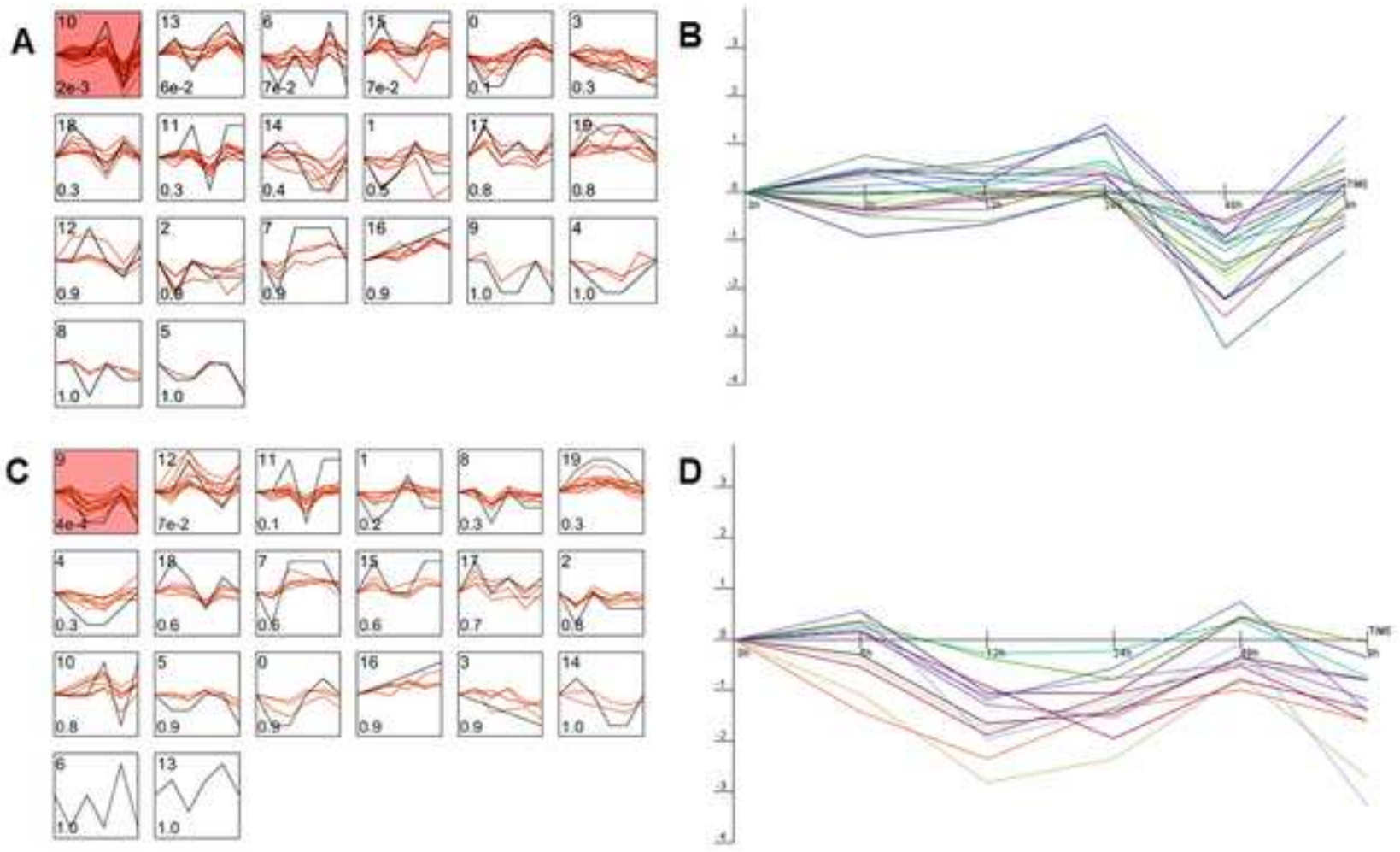

Figure 4

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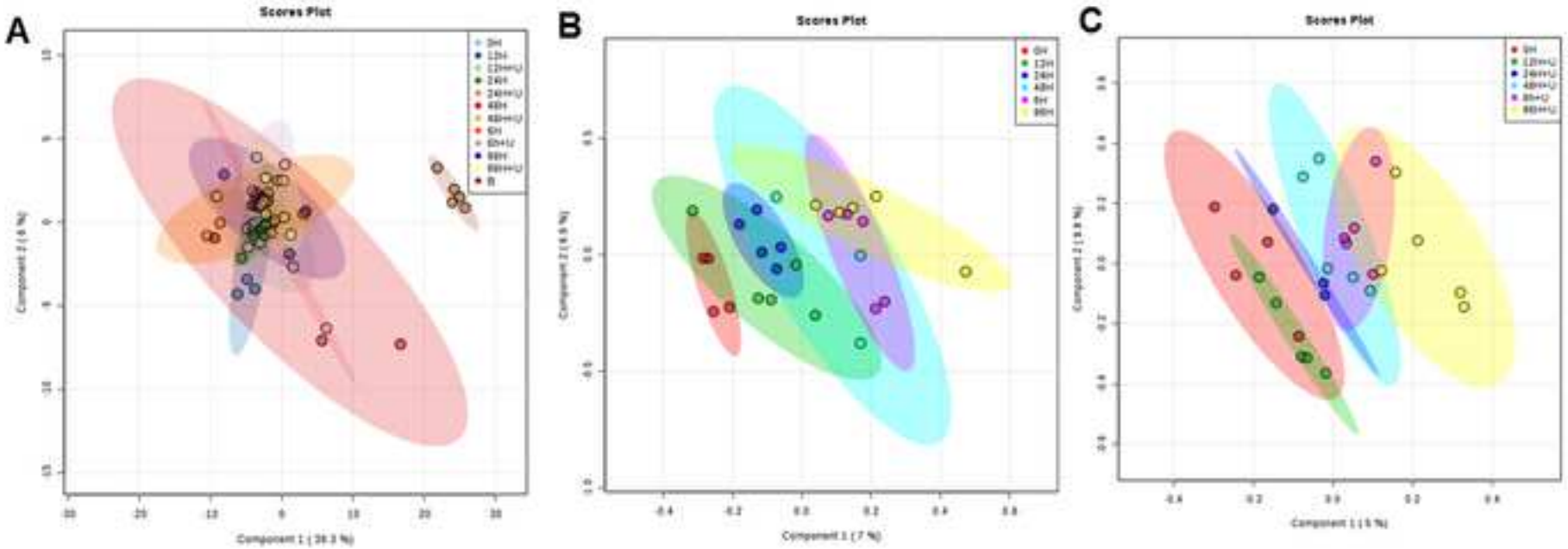
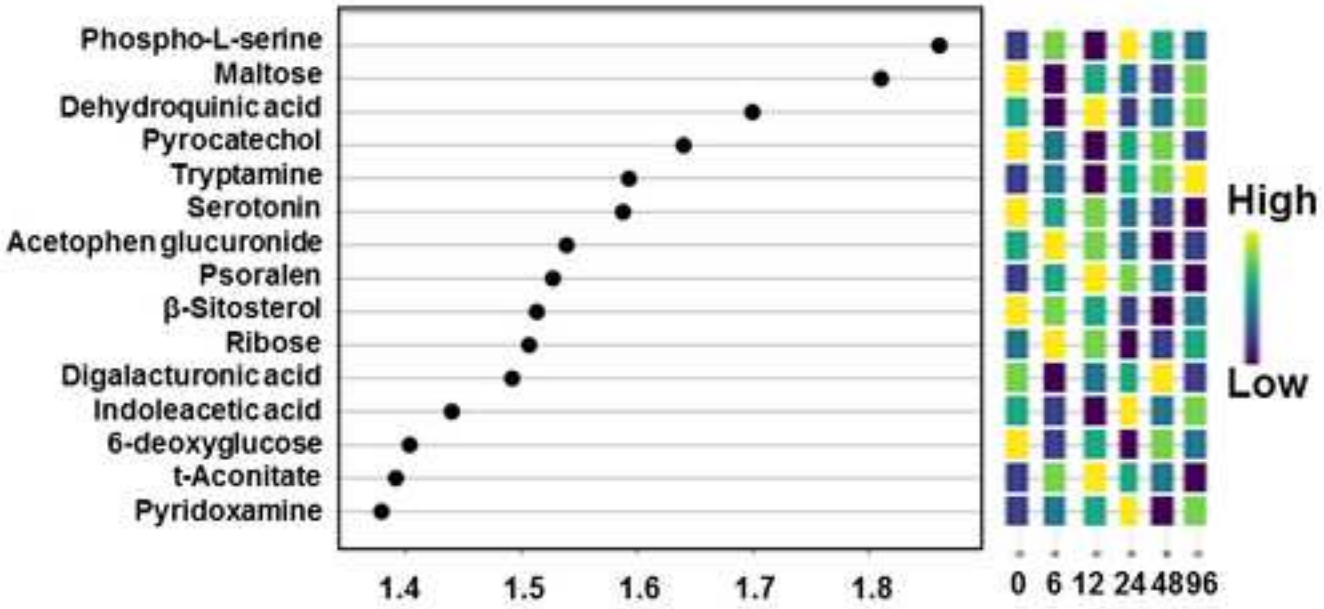


Figure 5



Supplementary Figure 1

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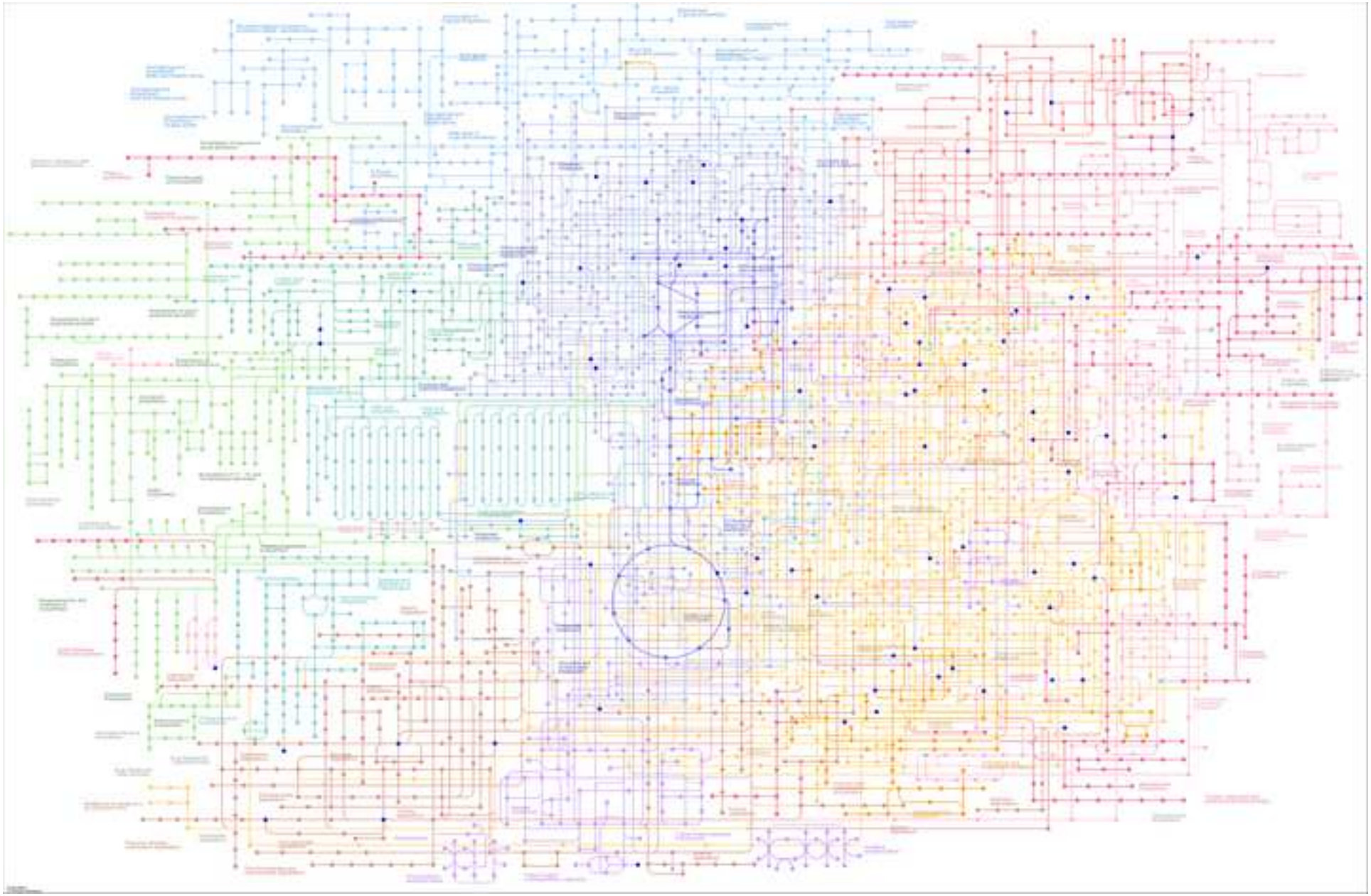

Supplementary Figure 2

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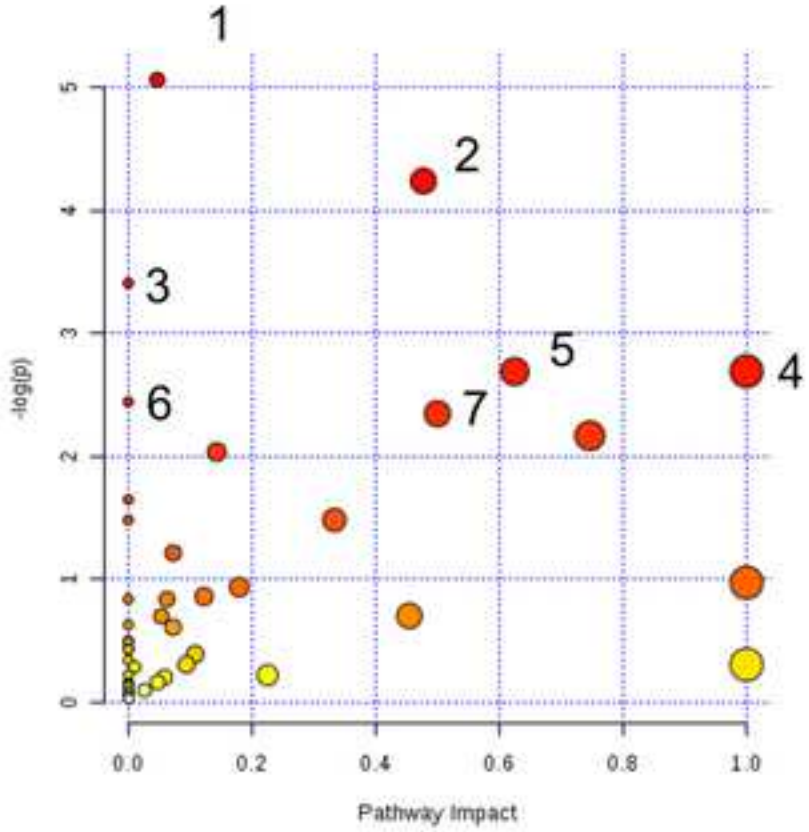

Supplementary Figure 3

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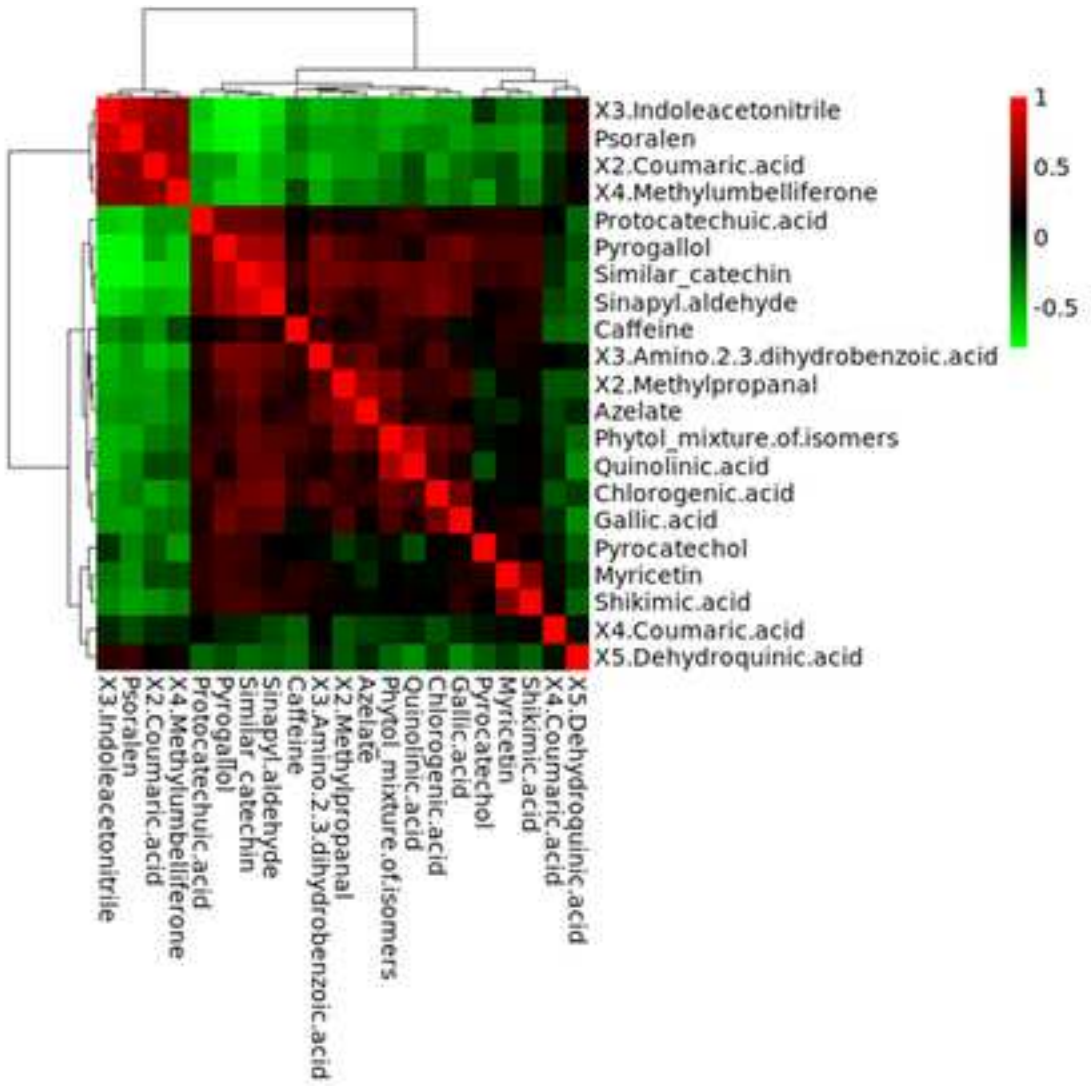

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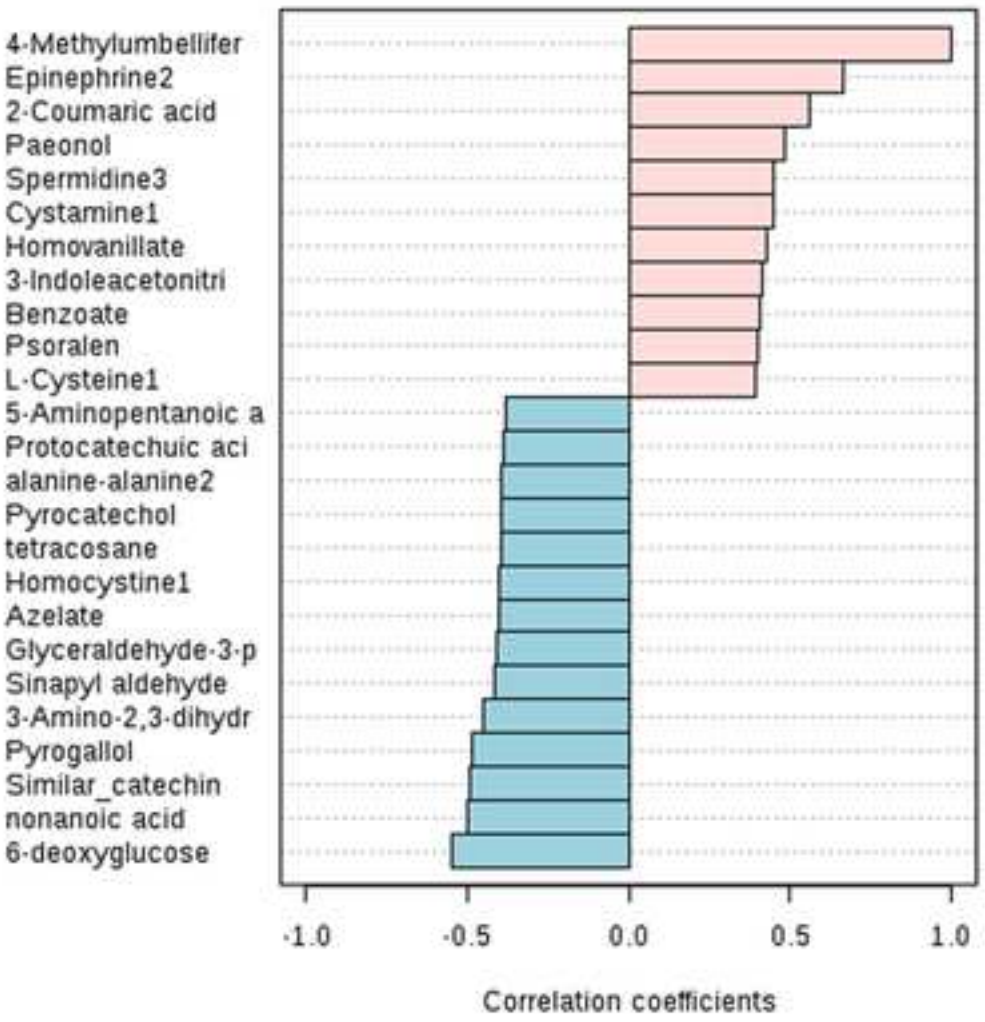

Supplementary Figure 5

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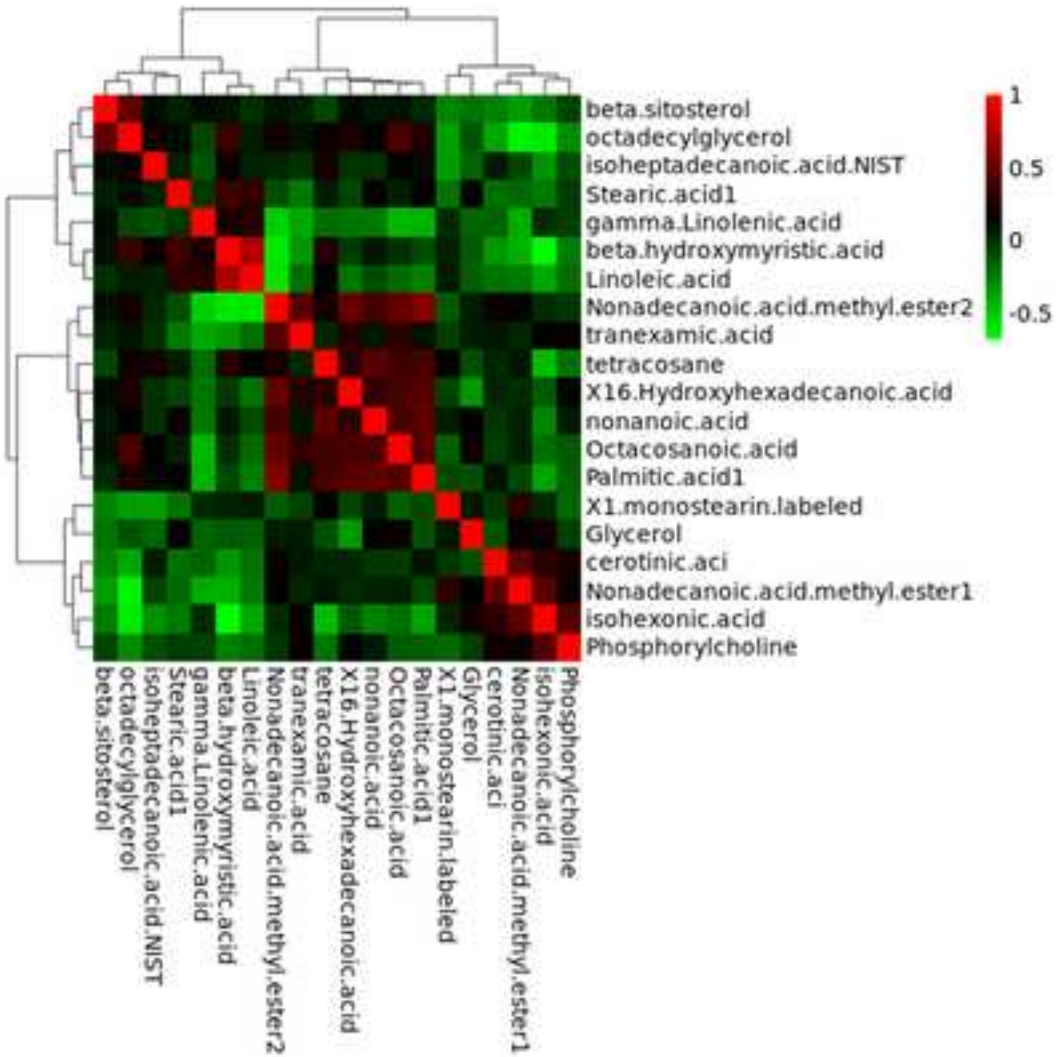

Supplementary Figure 6

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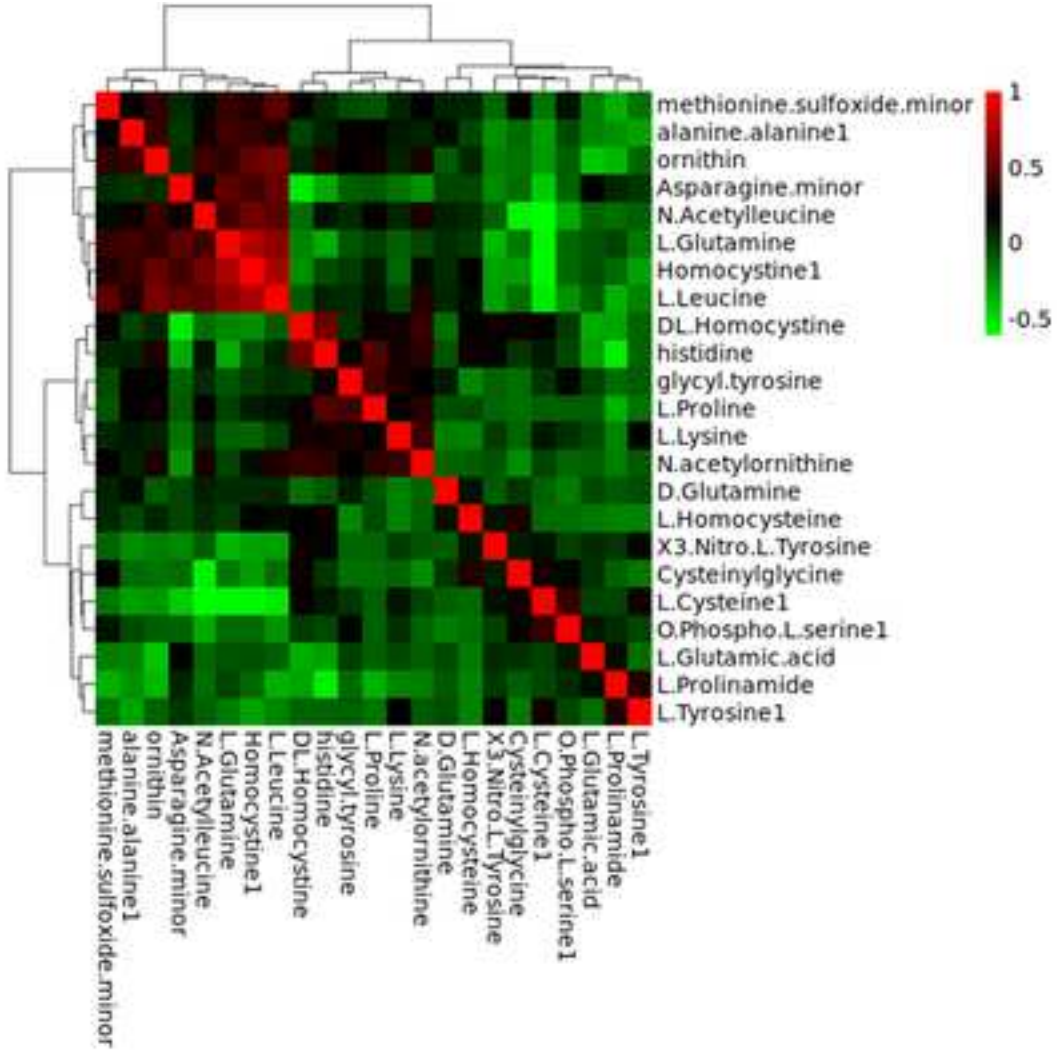

Supplementary Figure 7

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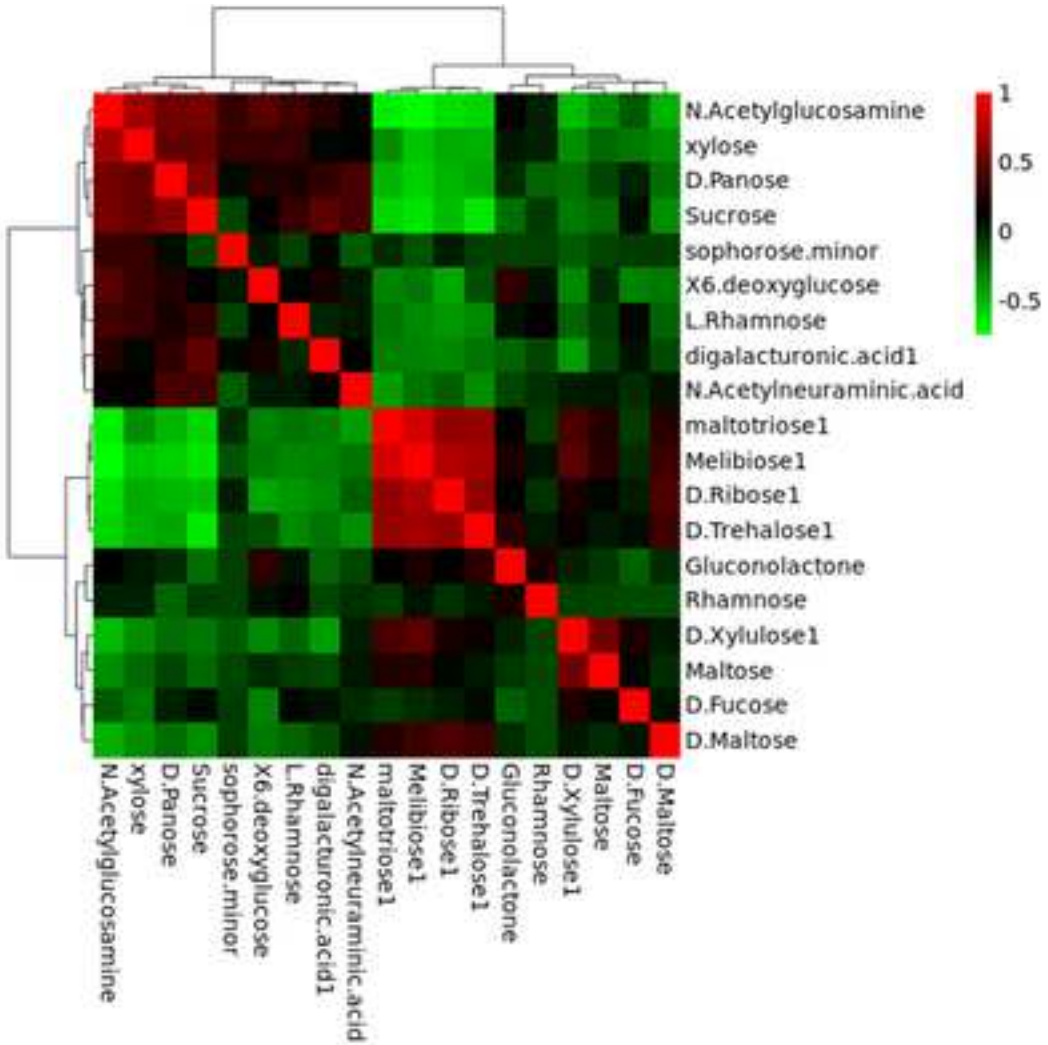

Supplementary Figure 8

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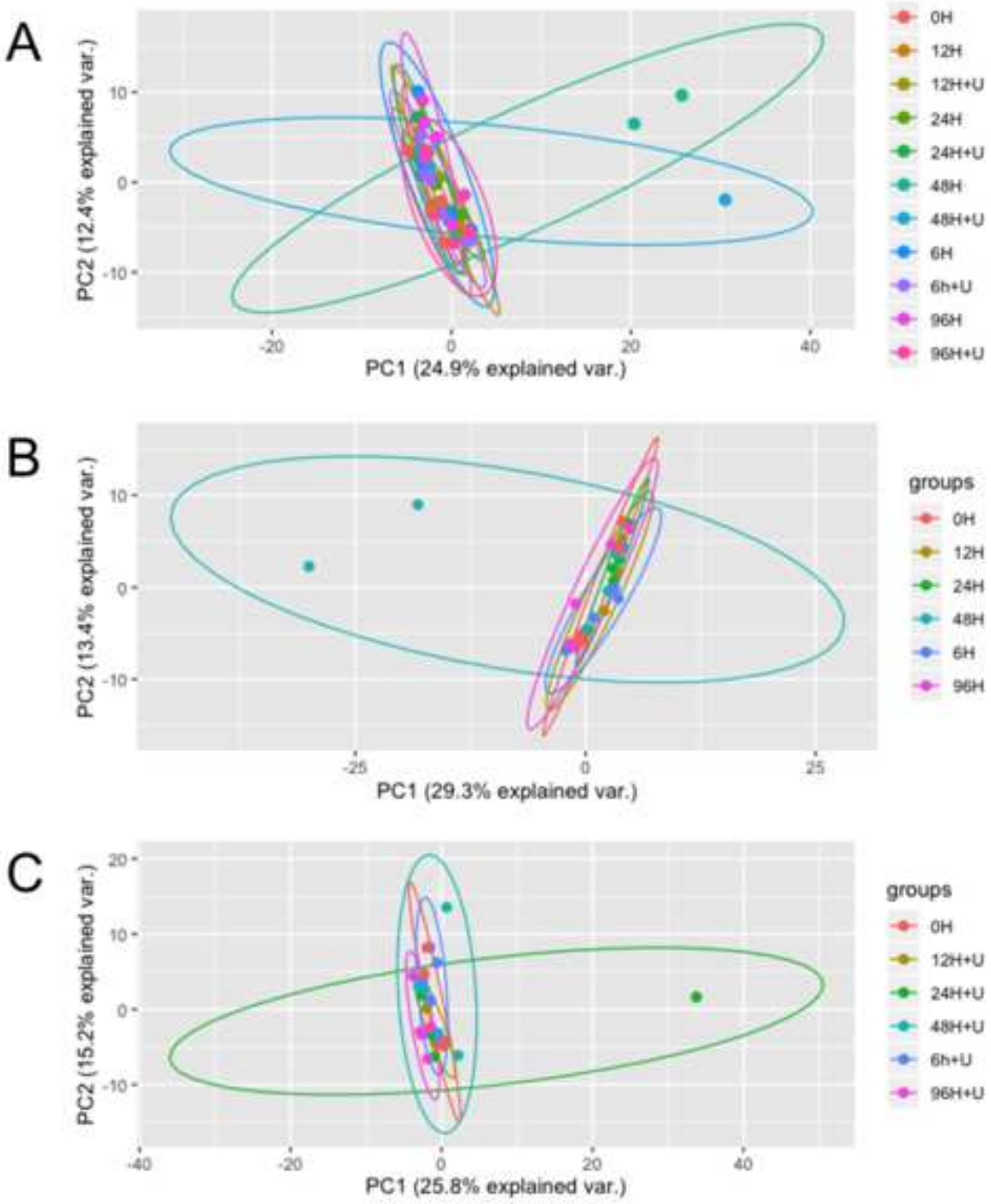

Table 1

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Table

Table 1.docx

Declaration of interests

☒ 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: