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METHOD ARTICLE

REVISED Unraveling the secrets of plant roots: Simplified method for large scale root exudate sampling and analysis in *Arabidopsis thaliana*

[version 3; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background

Plants exude a plethora of compounds to communicate with their environment. Although much is known about above-ground plant communication, we are only beginning to fathom the complexities of below-ground chemical communication channels. Studying root-exuded compounds and their role in plant communication has been difficult due to the lack of standardized methodologies. Here, we develop an interdisciplinary workflow to explore the natural variation in root exudate chemical composition of the model plant *Arabidopsis thaliana*. We highlight key challenges associated with sampling strategies and develop a framework for analyzing both narrow- and broad-scale patterns of root exudate composition in a large set of natural *A. thaliana* accessions.

Methods

Our method involves cultivating individual seedlings *in vitro* inside a plastic mesh, followed by a short hydroponic sampling period in small quantities of ultrapure water. The mesh makes it easy to handle plants of different sizes and allows for large-scale characterization of individual plant root exudates under axenic conditions. This setup can

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also be easily extended for prolonged temporal exudate collection experiments. Furthermore, the short sampling time minimizes the duration of the experiment while still providing sufficient signal even with small volume of the sampling solution. We used ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) for untargeted metabolic profiling, followed by tentative compound identification using MZmine3 and SIRIUS 5 software, to capture a broad overview of root exudate composition in *A. thaliana* accessions.

Results

Based on 28 replicates of the Columbia genotype (Col-0) compared with 10 random controls, MZmine3 annotated 354 metabolites to be present only in Col-0 by negative ionization. Of these, 254 compounds could be annotated by SIRIUS 5 software.

Conclusions

The methodology developed in this study can be used to broadly investigate the role of root exudates as chemical signals in plant belowground interactions.

Plain language summary
Plants exude many compounds to communicate with their surroundings. For decades, our understanding of this chemical communication was limited to studying the aboveground parts of plants, as roots are hidden within the soil, which makes them difficult to study. We are only beginning to comprehend the complexities and importance of below ground communication channels (including plant-microbes, plant-insects, and plant-plants). Identifying the chemical compounds plant exude belowground (called root exudates) is important for us to fully comprehend their potential roles in a plant´s life. Here, we developed a simplified and easy-to-manage setup for collecting and analyzing root exudates from individual *Arabidopsis thaliana* plants.

Keywords
root exudates, natural variation, Arabidopsis thaliana, interdisciplinary, chemical analysis



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REVISED **Amendments from Version 2**

We express our gratitude to Reviewer 1 for their constructive comments, which have led to a more comprehensive manuscript. One of Reviewer 1's pivotal observations was the need for a detailed time frame for each step of our methodology. In response, we have enriched the Methods section to include the total estimated time for sample processing, starting from collection to vial transfer. This detail not only enhances the feasibility assessment for larger experiments but also ensures that all 28 replicates are completed within a period that minimizes the influence of diurnal variations. Reviewer 1 also noted the importance of terminology in the context of metabolite identifications. To this end, we amended the text to replace the word 'identification' with 'annotation,' a term that more accurately describes the putative nature of our findings in the absence of validation with authentic standards.- The reviewer's inquiry into the confidence scoring system used for metabolite annotations has led us to clarify this aspect in the Results section. We now specify that the scores are raw values sourced directly from the NPC, ClassyFire, and SIRIUS databases and provide a context by referencing the 'Confidence Level 3' classification as outlined in the literature. Lastly, the presence of fatty acids in our samples has prompted a thoughtful query. We clarify that the Waters Acquity UHPLC HSS T3 column employed in our study is designed to separate a wide range of compound polarities, including fatty acids.

Through these revisions, we have made efforts to address the concerns and questions raised by Reviewer 1, thereby elevating the rigor and clarity of our work.

Any further responses from the reviewers can be found at the end of the article

Introduction

Plants have fascinating abilities to detect and respond to numerous chemical signals, both above and below ground, to interact with their environments[1,2]. While much is known about these above-ground communication channels, we are only beginning to understand the complexities of below-ground plant-environment interactions, which are mediated by root exudates[3].

Plant-secreted secondary metabolites are a particularly intriguing fraction of root exudates, which have several hypothesized and demonstrated important functions in belowground signaling mechanisms[1,4,5]. Belowground signaling has been shown to trigger a series of response mechanisms in roots, including neighbor detection, recognition and response strategies for chemical defense and/or root behavioral shifts[4,6–10]. Importantly, these interactions can also directly affect the aboveground plant performance, directly altering plant fitness[6,11].

Surprisingly, despite their demonstrated importance in mediating plant-environment interactions, we still have very little information on the identity, patterns, and levels of variation of the chemical compounds involved in belowground signaling[2,3]. This is partly due to the experimental and analytical challenges associated with trapping and identifying root-exuded chemicals, which are essential for conducting experiments of this nature. In particular, there is no consensus on the best possible conditions and methods for isolating root exudates, exacerbated by the difficulties of standardizing methodologies for their analysis[12]. These challenges are highlighted by multiple recent studies[1,12–17].

Besides, to make any inferences on belowground chemical communication patterns, particularly in the context of natural neighbor interactions, it is important to first gauge the individual composition and variation patterns of root exuded compounds among natural plant populations. This extensive sampling approach, which has not yet been fully implemented in plant-environment interaction studies[3], requires a comprehensive methodology that ensures both thoroughness and time efficiency. Additionally, most studies aimed at root exudates often follow a targeted approach where a compound of interest is tracked for its role in driving plant behaviour using a small set of sample accessions. To date, only a few studies have aimed at identifying any broad-scale patterns in root exudates (reviewed in 1,3).

Depending on the research question, the strategy of trapping exudates and analysis has varied[12,13,18]. However, to avoid major alterations to the exudate profile, which may occur due to the sorption of compounds in soil matrix or decomposition by microbes, hydroponic sterile systems are usually preferred[12,13]. Hydroponic collections of exudates not only minimize root damage, thereby encapsulating almost all rhizodeposits, but also require fewer manipulations in downstream analysis[12,18].

Here, we developed a multidisciplinary method for large-scale sampling of root exudates in a hydroponic system. The method was applied for collecting root exudates from natural accessions of *Arabidopsis thaliana*, where the Columbia (Col-0) accession (CS1092) was placed as a phytometer. The method was optimized for this collection, and we demonstrate its validity by discussing the results obtained from these phytometers. The remaining data will be processed for further investigations. We made several adjustments to previous methods to refine a strategy for broad-scale pattern detection of root exudate chemical composition in natural accessions of *A. thaliana*. The modifications are mainly with respect to sampling method, collection period as well as chemical analysis, and are discussed in the following section.

(1) Sampling method: Previous studies have some well-described hydroponic methods for root exudate sampling including setups where germinated seedlings are cultivated in liquid Murashige and Skoog´s (MS) media for weeks with continuous shaking for aeration and regular changing of nutrient solution to prevent microbial contamination[19,20]. Following this, hydroponic sampling was carried out as in 19,21,22. Some studies employed mixed strategies where soil-grown plants were transferred briefly to a sample solution[23]. Other elegant *in vitro* hydroponic setups have also been developed where sampling was carried out using Magenta boxes[24,25] or

96 well PCR plates[22], which can be easily transferred for sampling and collecting large volumes of root exudates. However, in most cases, the exudates from multiple plants were pooled and analyzed as one sample. This is due to the notion that individual roots often do not yield enough exudates, thus requiring additional steps of pooling samples from many plants and/or pre-concentrating the samples before analysis.

Furthermore, sampling solutions in hydroponic setups often use pure distilled/demineralized water without nutrients for its compatibility with all up-concentration techniques and further analytical approaches. However, it is important to note that such sampling may cause stress to plants and damage root cell membranes irreversibly due to differences in pH and/or salt concentration between growth and sampling solutions. To reduce these shock effects and capture any altered exudate patterns, it has been recommended to briefly submerge the roots in a separate water solution before transfer into the final sampling solution[12].

Finally, older plants might grow large enough to penetrate the agar layer in petri plates and form thick roots, making it difficult to remove them from the media. On the other hand, when dealing with biological/natural variation, plants of all shapes and sizes that have germinated and have developed roots large enough to exude chemical compounds, must be accounted for. Therefore, it was necessary to find a way that allows easy manipulation and handling of plants and helps act as a floater for individuals when being sampled in a hydroponic system.

This method was fine-tuned for large scaling sampling of natural genotypes, where, we grew individual *Arabidopsis thaliana* genotypes in an *in vitro* system under controlled sterile conditions. Each plant was grown inside a plastic float with a small pore size (4mm x 4mm; made of polypropylene); a material that can be easily found in laboratories, gardens or homes for garden fences and plant protection. This material can also be easily autoclaved. The mesh permits easy removal of the plant from media with minimal damage. Before sampling, the plant roots were submerged in MilliQ water for two minutes to absorb any extraneous exudation pattern caused by stress. After this step, sampling was carried out in microquantities of ultrapure MilliQ water. This method thus allowed sampling of a large number of individual plants and eliminated the need for concentrating samples for further analysis.

(2) Sampling period: The root exudate chemical profile of plants has been demonstrated to vary with many physiological factors including the type of nutrient media, plant age, experimental setups (including abiotic and biotic components of the experiment), and even the time of day when sampling is carried out[12,13]. Moreover, it has also been shown that this profile can be altered with the sampling duration. Long periods of stagnant solution sampling may lead to nutrient re-uptake of some compounds, and lack of proper aeration for extended times may stress the plants leading to altered profiles[12,13]. In the case of *Arabidopsis thaliana,* previous studies have included sampling of the exudates between 1 and 6 weeks post germination[19,26,27] when the plants have reached peak vegetative state but have not yet switched to reproductive state. Some temporal exudate collection experiments also included keeping the plants in an orbital shaker with continual sample collection periods ranging between 1 and 7 days[19,22,24,26,28].

However, for large-scale sampling of multitudes of natural accessions, streamlining the time of collection can be tricky. This is due to their varying germination times, which may directly or indirectly impact their size. Moreover, different natural accessions may also differ in the time required to complete their life cycles, thus affecting their chemical profiles[3]. Hence, it might be difficult to capture their natural variation of root chemical profiles throughout their life. Therefore, a setup must be imagined where continual sampling of individuals is possible.

In this method, to capture a snapshot of the chemical composition of root exudates, we sampled plants six weeks after germination and collected the root exudates for two hours in MilliQ water. Despite the potential additional stress imposed during such sampling, ultrapure water sampling solutions are endorsed for capturing semi polar fractions of metabolites with minimal to no root damage[18] and minimizes the manipulation steps required for exudate analysis procedures[18].

The two-hour sampling duration was chosen to minimize the sampling time and potential underestimation of total organic carbon[29]. Additionally, this duration is less likely to be affected by metabolite re-uptake[30]. Our preliminary experiments with time exposure (unpublished results) also supported the suitability of a two-hour collection time for root exudates in *Lotus japonicus* genotypes. Moreover, the exudate collection under sterile/anaerobic conditions for this short duration has been validated in recent studies involving different plant species[29–32]. Reducing the sampling period proved highly advantageous when handling hundreds of natural genotypes while still producing a sufficient signal in downstream analysis.

Additionally, as found in previous experiments, we could also apply this setup for prolonged temporal root exudate collection experiments (unpublished results). Upon removal from the agar media, plants in the mesh could be easily transferred to a hydroponic system in suitable amount of liquid media with recommended orbital shaking for long-term sampling experiments (similar to 19,22,24,26,33).

(3) Sample analysis: The variety of root exudates and their varying chemical properties make it challenging to analyze them. For analysing the secondary fraction of root metabolome, liquid chromatography, in particular high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) has been broadly applied[22,34,35].

However, to date, most studies on root exudates have focused on a targeted approach in which a known compound of interest is investigated across samples using analytical methods such as spectrophotometry, ion chromatography (IC), HPLC, gas or

liquid chromatography coupled with tandem mass spectrometry (GC–MS/MS, LC-MS/MS) etc.[12]. In targeted analysis, specific compound(s) or classes of compound(s) are detected and quantified using authentic standards. This type of analysis sensitively measures even minute quantities of interesting compounds and has been highly useful for investigating plant specific exudate patterns across different environments[23] and ages[36]. However, taking a targeted approach limits the ability to capture any broader patterns in exudate chemical profiles or identify any novel compounds that might serve important signaling and/or response functions for a plant[1].

Non-targeted approaches are becoming increasingly popular in root exudate chemical analysis[19,22,27] because they allow comparisons of metabolic patterns at different conditions across samples and can be used to draw inferential hypotheses about putative biological functions of the compound (s) tentatively characterized. Since the majority of compounds involved in plant communication remain to be discovered[1], an untargeted approach for characterizing the large-scale variation of exudate chemistry in natural accessions represents a first step. These data can be informative for drawing conclusions about broad-scale variation patterns, which can then be used to derive hypotheses followed by multidisciplinary investigations. Furthermore, with this information, a targeted approach can also be implemented, where interesting compound(s) can be identified using authentic standards and quantified in biological samples[37].

To enable the identification, quantification, and comprehensive analysis of metabolites in untargeted analysis of a large dataset encompassing multiple plants, we used a synergistic approach employing two robust open-access software tools: MZmine 3 and SIRIUS 5. This combination of tools provides powerful capabilities for processing and analyzing the LCMS data, ensuring an in-depth exploration of the metabolomic landscape[38].

Methods

Plant root exudate collection

The Col-0 accessions (CS1092) were obtained from the lab of Associate Prof. Simona Radutoiu (Dept. of Plant Molecular Biology, Aarhus University). The methodology was designed to refine the process of root exudate collection from over 100 natural accessions, with Col-0 used as the phytometer. To ensure experimental control, plants were divided into seven batches with one Col-0 and one control (a mesh without plant) per batch. Controls were treated identically to plants throughout the experiment, thus serving as baseline references. The experiment was conducted in five replicates, with each replicate consisting of seven batches, mounting to 35 Col-0 phytometers. With synchronized procedures within each batch, we reduced the effects of confounding factors. Sample collection occurred during a specified period to minimize diurnal variations.

Seed sterilization. The vacuum sterilization procedure described below was adjusted for dealing with many different genotypes. Accessions were surface sterilized using 30 mL of sodium hypochlorite (bleach) and 3 mL of 37% HCl. The reaction between bleach and hydrochloric acid form chlorine gas, which is used to sterilize the seeds in vacuum.

The steps followed in this protocol are described briefly: (1) Open Eppendorf tubes containing seeds were placed in the rack and this setup was transferred to the desiccator. (2) After connecting the vacuum tube, the hypochlorite reaction was started, and the desiccator was closed. A glove can be kept along this setup inside the desiccator to indicate vacuum levels inside the setup. (3) After the vacuum is started, the air inside the desiccator is removed reducing the pressure, thus making the glove indicator expand[39]. Once the glove expands the desiccator valve was closed to shut down the vacuum while the chlorine gas generated in the reaction sterilized the seeds. (4) After 10–15 minutes, desiccator valve was slowly opened to relieve air pressure after which seed vials could be removed. The seeds were taken back to the sterile benches and kept with open lids for 10 minutes to remove any extra sterilization gas.

Plant growth setup. For *in vitro* cultivation of *Arabidopsis thaliana* accessions, we used half-strength Murashige and Skoog´s medium with Gamborg´s Vitamins (Sigma Aldrich) and 0.8% plant agar (Sigma Aldrich). This nutrient solution was prepared and poured into 90mm Petri dishes containing two compartments (Fisher Scientific).

For ease of handling individual plants of varying sizes due to biological variation, and for creating a “floater” allowing easy root access when sampling in liquid media, an autoclaved plastic mesh was placed in each unique position within the Petri plate. Using a sterile plastic toothpick, two sterilized seeds were placed at the center of each mesh, with one plate having 4-5 unique individual replicates/genotypes (Step 1, Figure 1). After marking the positions of genotypes on the plates, the plates were secured with micropore tape. All these steps were performed under sterile conditions. For vernalization (Step 2, Figure 1), Petri plates were covered in aluminum foil and kept in a cold room (at 4°C) for four days after which they were transferred to growth chambers at 21°C with 16H day. The plates were checked daily, and individual germination dates were noted. Within 2–3 days post germination, the plants were thinned so that only one plant remained within each mesh. The location of the plates was shifted every 2–3 days to minimize position effects. Phenotypic measures including rosette diameter, the number of leaves and extent of chlorosis (Figure 1), could be scored directly on the plates.

Sample collection. Six-week-old plants were gently removed from agar media using sterilized tweezers (Step 3, Figure 1). The transfer of plants from the plates to the sampling solution took approximately 5–6 minutes per plant, depending on the plant sample size. All plants from a plate were treated at a time. Since the plants in our study were small and easy to remove from the agar and clean, this step was relatively quick and straightforward. Handling the mesh instead of the plant minimizes the damage caused to the plant during the transfer. The agar sticking to the root was gently removed with

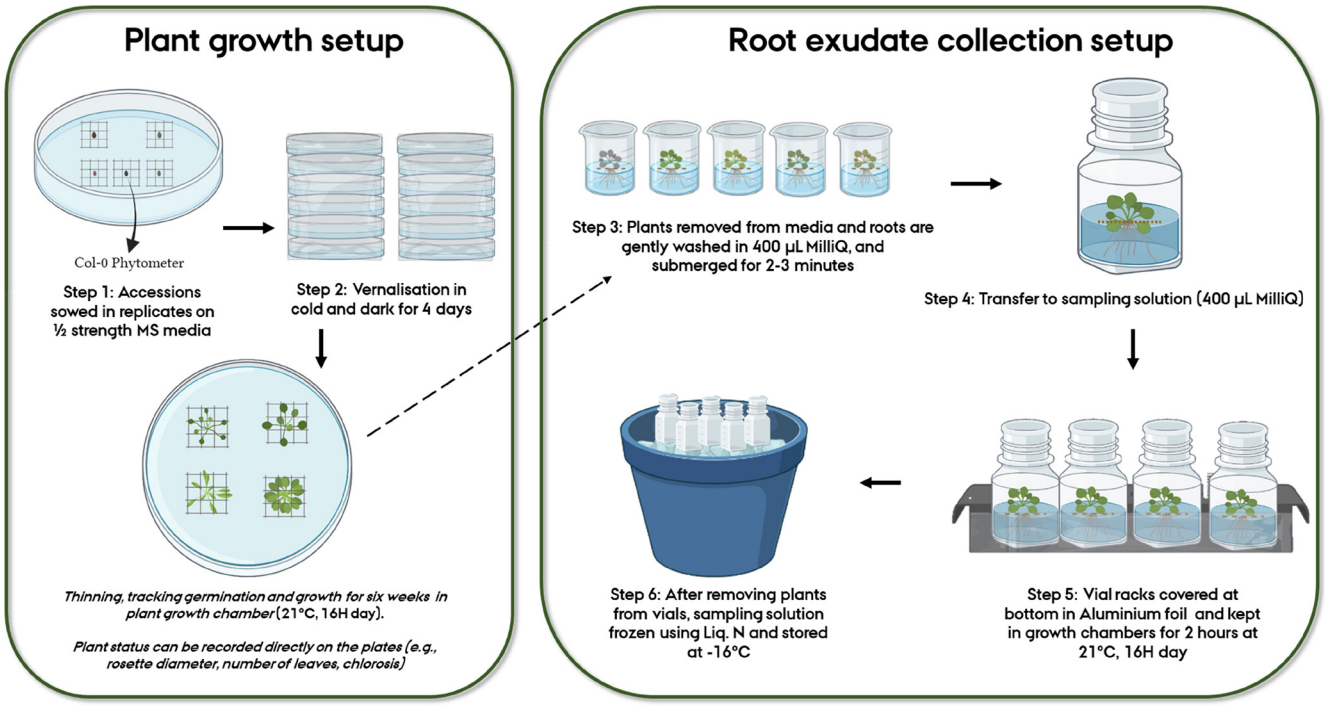


Figure 1. Workflow representing the various steps followed for root exudate collection of *Arabidopsis thaliana* accessions.

a sterilized paint brush. This step took approximately 2–3 minutes per plant (depending on the size of the plant). Following this, the plants were transferred to individual beakers with a cleaning solution of 400 µL MilliQ water- for about two minutes (Step 3, Figure 1). This step is recommended for capturing any impertinent exudates due to the initial shock/stress of transferring to liquid media and is also useful in further removal of agar residues from the roots. The plants were then transferred to already prepared sampling vials of 400 µL MilliQ water in 20 mL cylindrical glass vials (27.5 mm diameter x 57 mm height), where the roots were submerged in the solution and the above ground part remained floating, and placed in racks (Step 4, Figure 1). For the sake of sterility, the vial lids were closed, and aeration was prevented. Control treatments were similarly sampled. All these steps were performed inside a flow bench (LaboGene ScanLaf Model Mars). The racks were removed from the flow bench, and the bottom half of the racks was covered in aluminum foil to mimic the dark conditions roots experience in nature. The setup was then transferred to a growth chamber (21°C) for two hours (Step 5, Figure 1). After accounting for each step, the entire process from collection to vial storage takes approximately 133–136 minutes per plant. This includes the two-hour period (120 minutes) for exudate sampling in the growth chamber. During the experiment, up to 60 samples could be handled in one sitting. The growth chamber facility was conveniently located in the same building as the laminar flow chambers, facilitating the transfer of plants between the two environments without prolonged exposure to external environmental conditions. After this, the setup was brought back to the flow benches where the plants were gently removed from the sampling vials (1–2 minutes per vial). The vials were then immediately frozen in liquid nitrogen and subsequently stored at -18°C (Step 6, Figure 1).

Root exudate analysis

The method for root exudate analysis has been optimized for both targeted and untargeted analysis of root exudates and has a run time of 28 minutes for each sample in negative ionization mode. The details of this method will be discussed in a forthcoming publication from the group but is described briefly below.

Preparation of samples for UHPLC analysis. Before sample preparation, sample solutions in vials were allowed to thaw at room temperature. An internal standard of ketopinic acid (10µg/mL in MilliQ water) was prepared for spiking the samples. This allows checking for injection efficiency and gauging sample signal strength in LC-MS analysis. 180 µL exudate sample was added to 20 µL of internal standard and filtered using 0.22 µm Polytetrafluoroethylene (PTFE) hydrophobic syringe filters and finally transferred to 2 mL vials with inserts.

HPLC-MS analysis. The samples were analyzed on a Thermo UltiMate 3000 UHPLC coupled to a quadrupole time-of-flight mass spectrometer, qTOF (Bruker, Compact). The column was a Waters Acquity UHPLC HSS T3 column, which had a length of 100 mm and a diameter of 2.1 mm and a particle diameter of 1.8 µm. The column temperature was 45°C. The mobile

phase consisted of 0.1% acetic acid (AA), in MilliQ-water (eluent A) and 0.1% AA in acetonitrile (eluent B). The flow rate was 0.200 mL min^{-1} for the entire analysis, and the injection volume was 2µL. The UHPLC was coupled to the qTOF, through an electrospray ionization (ESI) inlet in the negative ionization mode. Prior to each run, the MS was calibrated using 5 mM sodium acetate solution (HCOONa). Operating settings for the MS were as follows: the capillary voltage was 4.5 kV, the drying gas (N_2) was 5.5L min^{-1}, the nebulizer pressure was 2.5 bar, the capillary temperature was 200°C, and the scanning interval was 50–1000 m/z. Data were acquired and processed using Bruker software (DataAnalysis) followed by MZmine3 and SIRIUS 5.

The data obtained was first analyzed using the proprietary software Bruker Compass DataAnalysis 4.3, where background noise levels, signal intensities, signal-to-noise ratios, etc. can be investigated in depth. The open access software OpenChrom and MZmine are some freely available alternatives for such manual examination of the data. MZmine 3 is an open-source software for mass-spectrometry data processing, especially from LC-MS data. Peak alignment and noise filtering were carried out with Mzmine3. The noise threshold was estimated as $1.0E^2$, and the minimum feature height threshold to be considered a true signal was kept at least three times the noise level. Applying appropriate filters from control peaks, the chromatogram peaks were then aligned across samples. All the relevant features were further analyzed with SIRIUS 5 software another open access Java software for analyzing metabolites from tandem mass spectrometry data[40,41]. SIRIUS provides a fragmentation tree to each relevant peak (feature), describing the fragmentation reaction from each molecule from the ESI. The CSI:FingerID feature can then be used to search in molecular structure databases from the internet for tentative identification of compounds[42].

Notably, SIRIUS 5's natural product classification (NPC) allows clustering of annotated compounds into class, superclass, and pathway categories, which significantly enhances our understanding of the biological interpretations of the metabolites[43]. In the analysis conducted using both positive and negative ionization modes, a higher number of features were identified in the negative mode (379) compared to the positive mode (76). As a result, we will focus on the negative mode results in this context to highlight its capability in detecting and identifying a significant number of compounds[44].

Results

Out of the 35 Col-0 phytometers that were used in the experiment, 28 were successfully sampled for root exudates. The remaining plants either did not survive or were too small to be sampled. Figure 2 shows the chromatograms obtained from the untargeted analysis in negative ionization mode of Col-0 accessions using the above-described method overlaid over controls (in green). MZmine3 annotated and aligned 378 features/chemical compounds in the 28 Col-0 samples, whereas the 10 random controls consisted of 68 features (see *Underlying data*[45]). After subtracting the features present in

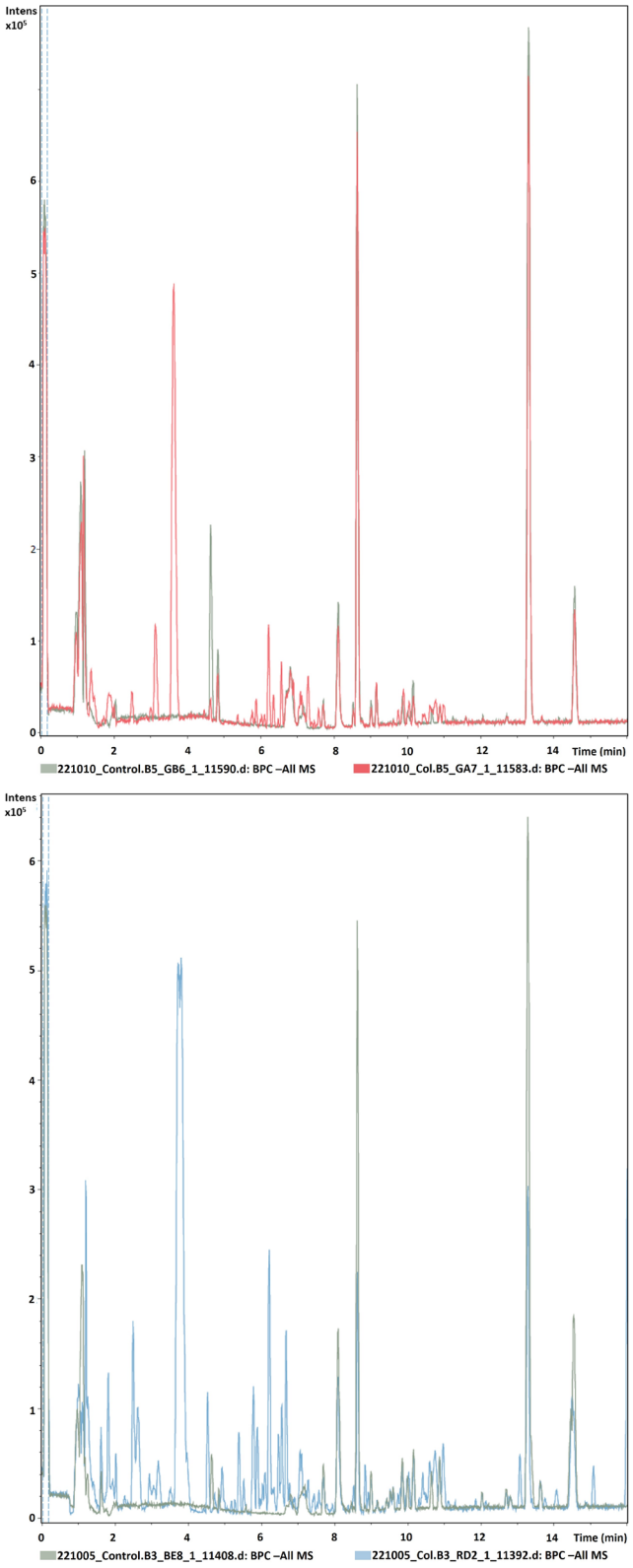


Figure 2. Representative chromatograms of two different Col-0 accessions (m/z 121–832, rt 0.99–15 minutes) obtained from root exudates overlaid over blank samples by UHPLC/ESI-QTOFMS in negative mode.

controls, we identified 354 metabolic features present only in Col-0 accessions (see Supplementary Table 1 in *Extended data*[45]). The CSI:FingerID web service feature of SIRIUS 5 classified 254 compounds (around 71% of the annotated features) in the samples. Their chemical annotations are listed in Supplementary Table 2 (see *Extended data*[45]). It should be noted that the features here are categorized as 'Confidence Level 3'[41] indicating that while the metabolites have been annotated, they have not been individually confirmed via reference standards.

Using the phenotypic data collected during the experiment (number of leaves and rosette size of plants at the time of sampling), we observed a clear correlation between plant size and total peak area (*see Extended data analysis*) validating the ability of the methodology to accurately capture and quantify differences in compound abundance[46,47]. We also estimated the coefficient of variation (CV) and mean abundance for the detected compounds (*see Supplementary Table 1*). We then calculated the relationship between CV and mean abundance based on the Poisson distribution, as suggested by Ipsen and Ebbels[48], and found it to be in line with the predicted relationship for LCMS data (*see Extended data analysis*).

Discussion

Root exudates are substantially hard to study, yet the importance of root-secreted signaling chemicals in mediating plant interactions is no longer debatable. So far, the methodologies available to trap and identify root-secreted signaling chemicals have remained a serious problem[49]. This has limited our ability to identify any patterns of their natural variation on a large sample of accessions, and thus, their role in driving belowground communications remains to be elucidated.

The method developed here was tuned for large-scale root exudate sampling of *A. thaliana*, natural accessions. With this method, by reducing the time of collection and omitting orbital shaking, we aimed to diminish the shock effects experienced by plants during transfer from solid to liquid media. Furthermore, faster chemical analysis time is another significant improvement when handling hundreds of samples. Even with minute sampling solutions, the method yielded sufficient signals for the LCMS analysis, which was sensitive enough to capture microquantities of several metabolites, considering the sample sizes (*i.e.,*smaller plants showed smaller total peak areas, while larger plants exhibited larger total peak areas). This is further supported by the number of secondary metabolites potentially identified in the analysis. Assessment of reproducibility of the detected compounds through the coefficient of variation (CV) and mean abundance of detected compounds, further validates the robustness and reliability of our approach. Moreover, SIRIUS 5 was able to annotate a substantial number of features (listed in Supplementary Table 2 in *Extended data*[45]), and this information can be used to draw broad patterns and make inferential hypotheses to be validated by complementary approaches. Our method is valuable for quick pattern assessment experiments of large samples of accessions for the purpose of studying plant-environment interactions (including but not limited to plant-microbes, plant-insects, and plant-plants).

Even though hydroponic root exudate collection is not a perfect solution, as we cannot fully mimic the conditions roots may experience in nature, and there can be potential variations in the exudate profiles derived from hydroponic *vs*. soil environments[12], these methods are still highly useful setups that allow capturing exudates in sterile environments[13]. In this method, using a cost-effective plastic floater, plants were easily managed during the entire growth and hydroponic sampling steps. Furthermore, this floating setup could be translated into longer temporal collection experiments, as in similar experiments conducted on *A. thaliana*[19,24]. Using meshes of different pore sizes, one can customise this setup for root exudate collection and analysis of several individual genotypes from other plant species.

Verifying the biochemical functions of these metabolites is without a doubt, a mammoth feat, requiring an interdisciplinary approach. Analyzing root exudates in axenic conditions gives a broad overview of their chemical composition, and they can be further validated for their roles using complementary methodologies spanning multiple disciplines. For example, compounds tentatively identified by SIRIUS 5, can be quantified using a targeted approach if authentic standards are available. Many root exudate studies have previously confirmed an altered pattern of root behavior once their ability to sense the exudates of neighboring plants is removed[3]. Targeting specific components of exudates for validating this behavior could also present an interesting next step. Deep investigation into the biochemical pathways of these metabolites by creating gene knockouts could also reveal their exact roles in plant behavior modulation. As a preliminary validation step, we successfully confirmed the accuracy of SIRIUS5 within our team by targeting the identification and quantification of standards, such as ketopinic acid, tryptophan and cyclic GMP, for a subset of samples analyzed in this study[50].

Studying root exudate chemical signals mediating belowground interactions are important not only from an ecological context but also from an applied perspective in agriculture and forestry[1]. Root-secreted compounds are part of a complex interaction network incorporating other inter/intraspecific neighbors, bacteria, fungi and invertebrates etc., where each interacting plant might receive individual signals from multiple sources, making this exceedingly difficult to dissect. Hence, methodologies are needed to first simplify studying these interactions and identify ecologically relevant biochemical communication channels[51], which can be ultimately applied in agricultural context[1]. Scrutinizing the variation in chemical profiles of interacting partners remains an essential first step, which has been largely overlooked in root exudate studies[13], further hindered by following a targeted approach to identify already known compounds.

The method developed here takes a step forward in overcoming limitations of previously developed hydroponic systems. By developing an easy-to-manage setup for largescale sampling, we detected many interesting secondary metabolites in Col-0, which will be used for further investigations using population genomics, evolutionary ecology and analytical chemistry

approaches to answer questions relating to their roles in plant-environment interactions.

Ethics and consent

Ethical approval and consent were not required.

Data availability

Underlying data

Zenodo: Unraveling the secrets of plant roots: Simplified method for large scale root exudate sampling and analysis in Arabidopsis thaliana. https://doi.org/10.5281/zenodo.8004460[45].

This project contains the following underlying data:

- Raw datafiles.xlsx: The raw datasets obtained from MZmine 3 analysis, which contains aligned features of Columbia genotypes (Sheet1) as well as control samples (Sheet2). The dataset consists of *feature (row)ID, average (mass-to-charge ratios) m/z* and *retention times (RT)* across samples for individual features. It also includes sample-specific information including *feature status, name, m/z, RT, feature peak height,* and *area*. We also include a filtered datasheet excluding the features obtained in control as well as samples (Sheet3). Sheet 4 contains the phenotypic data on the number of leaves and rosette size of the 28 replicates at the time of sampling, along with the total peak area for each sample from MZmine data.

Extended data

Zenodo: Unraveling the secrets of plant roots: Simplified method for large scale root exudate sampling and analysis in Arabidopsis thaliana. https://doi.org/10.5281/zenodo.8004460[45].

This project contains the following extended data:

- Supplementary tables-.xlsx: The 354 metabolites obtained after filtering out control features are listed with their *mass-to-charge ratios*, *retention times, and the mean, variance and coefficient of variation of the peak areas*(Supplementary Table 1). Supplementary Table 2 details the features annotated by SIRIUS 5with their *mass-to-charge ratios, retention times, chemical formula, chemical annotations,* and corresponding *probabilities scored by SIRIUS* Extended data analysis: Contains supporting data analysis for reproducibility and validity of our method for root exudate collection and analysis in *Arabidopsis thaliana.* These extended data analyses enhance the understanding of the relationship between plant phenotypic traits, peak area, and variation in compound abundance

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgments

We would firstly like to thank Dr. Xavier Picó (CSIC, Spain) for providing the accessions used in developing this methodology. We would also like to thank Plant Molecular Biology group members, especially Dr. Alen Trgovcevic and Raffaella De Masi for their valuable insights on *in vitro* cultivation of *Arabidopsis thaliana* plants. We would additionally like to thank research group members at Dep. of Chemistry, especially Master's students Antigoni Christofili, Jeppe Clemmensen and Jonas Dahl Dueholm and for all their help with sample preparation and chemical analysis. We also would like to thank Dr. Thomas Bataillon for all his help with the data reproducibility analysis.

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Version 3

Reviewer Report 26 October 2023

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✔ **Sophie Alvarez**
Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

The authors have appropriately addressed the comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant stress, root exudate, metabolomics, proteomics, mass spectrometry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 21 July 2023

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? **Sophie Alvarez**
Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

Thanks to the authors for addressing the comments. However I still have some questions.

1. I see the authors have included time information for the steps, but can they also include

what the overall time is from beginning of sample collection to transferring samples to vials?

2. Can the authors please use the word 'annotation' or 'annotated' instead of 'identification' or 'identified' for the metabolite. Based on the supplementary information from the "identification", the annotation has clearly not been reviewed or confirmed with authentic standards, so these are only putative identifications. There are redundant annotations, and annotations not endogenous to plants.

3. Is it also unclear what the confidence scoring reported in the table is, and if there was any threshold used for any of the probabilities reported by NPC, Classyfire or SIRIUS?

4. Finally, how do authors explain the presence of a large number of compounds belonging to fatty acids in their samples, if the collection type used here is meant to be for polar compounds.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant stress, root exudate, metabolomics, proteomics, mass spectrometry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Oct 2023

Harihar Jaishree Subrahmaniam

We are grateful for the additional questions from Reviewer 1 which enable us to clarify important aspects of our methodology further. Below we have pasted our “point-by-point” response to the comments.

Reviewer 1 follow-up questions Thanks to the authors for addressing the comments. However I still have some questions.

1. I see the authors have included time information for the steps, but can they also include what the overall time is from beginning of sample collection to transferring samples to vials?
2. Can the authors please use the word 'annotation' or 'annotated' instead of 'identification' or 'identified' for the metabolite. Based on the supplementary information from the "identification", the annotation has clearly not been reviewed or confirmed with authentic standards, so these are only putative identifications. There are redundant annotations, and annotations not endogenous to plants.
3. Is it also unclear what the confidence scoring reported in the table is, and if there was any threshold used for any of the probabilities reported by NPC, Classyfire or SIRIUS?
4. Finally, how do authors explain the presence of a large number of compounds belonging to fatty acids in their samples, if the collection type used here is meant to be for polar compounds.

Response to Reviewer 1 comments

Overall time for sample collection to vial transfer: Thank you for emphasizing the importance of a comprehensive timeframe. We have updated the Methods section to provide a timeframe from beginning of sample collection to transferring samples to vials, taking into account the 120 minutes allocated for exudate collection. This brings the total estimated time to a range of 133-136 minutes per sample.

Use of 'annotation' instead of 'identification': We appreciate your insightful suggestion to use 'annotation' in lieu of 'identification' for greater accuracy. Given that the metabolites have not been confirmed by comparison with authentic standards, the term 'annotation' more appropriately reflects the level of confidence in these putative identifications. Accordingly, the manuscript has been revised to make these adjustments throughout.

Confidence scoring in tables: We understand that the confidence scoring presented in our tables might have been ambiguous. These scores are raw values sourced directly from the NPC, ClassyFire, and SIRIUS databases, and no additional thresholding has been applied. We believe presenting the unfiltered data underscores our commitment to methodological transparency. In the Results section, we have also incorporated a statement about the 'Confidence Level 3' classification for our annotated metabolites, as per the framework by Blazenovic et al. (2018).

Presence of fatty acids in samples: The observation regarding the prevalence of fatty acids in our samples is indeed pertinent. We employed a Waters Acquity UHPLC HSS T3 column, which is a C18 reverse-phase column designed for high selectivity and peak shape across a wide polarity range. While the column is capable of separating compounds with a broad range of polarities, it is particularly effective for non-polar to moderately polar compounds, including fatty acids. Unlike a hydrophilic interaction liquid chromatography (HILIC) column, which is more focused on the separation of polar compounds, our setup does not selectively exclude less polar molecules like fatty acids, particularly when considering our chosen mobile phase conditions.

We trust that these modifications adequately address the concerns and questions raised by Reviewer 1 and serve to augment the rigor and clarity of our study.

Sincerely, Harihar Jaishree Subrahmaniam on behalf of all authors

Competing Interests: No competing interests were disclosed.

Reviewer Report 11 July 2023

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Mònica Escolà Casas
Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain

I have no further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am an environmental scientist working with nature based solutions (constructed wetlands, biofilms) for the removal of contaminants of emerging concern in water. Lately I have been investigating how root exudates are affected by contaminants of emerging concern and how root exudates may help to their degradation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 06 June 2023

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?

Paul A Weston
Charles Sturt University, Wagga Wagga, New South Wales, Australia

This manuscript presents an interesting approach to quantifying and identifying metabolites from *Arabidopsis thaliana* and potentially many other plant species. The proposed method offers many advantages over other techniques that have been used previously, but there are some shortcomings in their presentation of the methods and interpretation of results. In addition, there are numerous grammatical errors that require correction.

The major shortcomings are as follows:

1. The authors provide no indication of the plant-to-plant variation in metabolite profiles. This is not a trivial concern because most metabolomic studies pool samples collected from numerous individuals to smooth out plant-to-plant variation in order to focus on larger trends in metabolite variation. Is the greater investment in time and resources involved with growing and sampling individual plants justified by the results? We can't tell because the authors provide no information on this variation.

2. Collecting exudates by suspending seedlings in pure water might significantly affect the exudates produced by the seedlings. How relevant are these exudates to those that might be produced by the seedlings in soil? How does the duration of the collection impact the

quality and quantity of exudate produced? Incidentally, one must assume the collection liquid is water, although the authors refer to the medium in the collection vessels somewhat mysteriously as “MilliQ sampling solution” on page 6.

3. The anaerobic conditions arising from exclusion of air from the collection vessels and preventing aeration of the sampling medium might be expected to significantly alter the quantity or quality (or both) of exudates produced by the seedlings. The magnitude of this effect would be influenced by the collection interval; was the collection interval examined to determine if this effect was significant?

4. It is unclear how a glove can be used to determine the level of vacuum in the desiccator.

Regarding the more mundane issue of correctness of expression, the authors use the terms aboveground and belowground inconsistently (sometimes each is presented as two words, sometimes one, and sometimes with a hyphen). Regardless of which style the authors decide on, they should use the wording consistently throughout the manuscript. As noted on the manuscript, a space should be used between numerals and units of measurement. Capitalization of numerous words (e.g. Sodium, Liquid chromatographs, etc.) is done incorrectly. I also thought the wording "to absorb any erroneous exudation pattern" on page 4 was very poor. I think you mean to say something such as "extraneous exudates", that is exudates produced by the plants prior to the sampling interval. The word "erroneous" is inappropriate to use as well; these exudates might be extraneous, but can't be said to be erroneous.

More substantively, the chromatograms in Figure 2 would be easier to see if they were compressed vertically and if the legend text was larger. Related to this figure, it is difficult to distinguish between the control and treatment chromatogram in the left panel; readers with red/green color blindness will have no hope of distinguishing the two traces, which is challenging even for readers with normal color vision.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Metabolomics, natural products chemistry, entomology, plant physiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Jun 2023

Harihar Jaishree Subrahmaniam

We would like to thank the reviewer for their valuable feedback on our manuscript. We have thoroughly reviewed all the comments and suggestions and incorporated substantial revisions to address them. The revised manuscript includes track changes to highlight all the modifications made. Reviewer 3 raised several important points that have helped us address the potential limitations of our methodology and inconsistencies in the manuscript. We have addressed these points individually.

Comment 1: The authors provide no indication of the plant-to-plant variation in metabolite profiles. This is not a trivial concern because most metabolomic studies pool samples collected from numerous individuals to smooth out plant-to-plant variation in order to focus on larger trends in metabolite variation. Is the greater investment in time and resources involved with growing and sampling individual plants justified by the results? We can't tell because the authors provide no information on this variation.

Response: We acknowledge the importance of considering plant-to-plant variation in metabolite profiles. To address this concern, we performed data analysis using established methods, including calculating the coefficient of variation (CV) and mean abundance for the detected compounds in the 28 replicates. The results of the data analysis, including CV and mean abundance, are now included in the *Extended data analysis* section of the revised manuscript. These additions provide insights into the reproducibility and plant-to-plant variability of the detected compounds across samples.

Comment 2: Collecting exudates by suspending seedlings in pure water might significantly affect the exudates produced by the seedlings. How relevant are these exudates to those that might be produced by the seedlings in soil? How does the duration of the collection impact the quality and quantity of exudate produced? Incidentally, one must assume the collection liquid is water, although the authors refer to the medium in the collection vessels somewhat mysteriously as "MilliQ sampling solution" on page 6.

Response: We agree that collection of exudates by suspending seedlings in pure water may influence the exudates produced by the seedlings compared to those produced in soil. We address the potential limitations and relevance of exudates collected using this method in the *Introduction* and the *Discussion* section. Additionally, on page 6, we removed the term "MilliQ sampling solution" and clarify that the term refers to sampling vials containing MilliQ water.

Comment 3: The anaerobic conditions arising from exclusion of air from the collection vessels and preventing aeration of the sampling medium might be expected to significantly alter the quantity or quality (or both) of exudates produced by the seedlings. The magnitude of this effect would be influenced by the collection interval; was the collection interval examined to determine if this effect was significant?

Response: We acknowledge the possible impact of anaerobic conditions and collection interval on the quantity and quality of exudates. Although our study did not examine the influence of collection interval specifically, our protocol was consistent with our previous time exposure experiments on another plant species and similar studies that suggest brief sampling periods to maintain sterility and reduce stress times. We now provide an account of these studies in the *Introduction* our revised manuscript.

Comment 4: It is unclear how a glove can be used to determine the level of vacuum in the desiccator.

Response: In the revised manuscript, we address how a glove can be used to determine the level of vacuum in the desiccator. We briefly explain in the *Methods* section how a glove indicator is used as a visual cue to assess the level of vacuum inside a sealed container like a desiccator. As the vacuum pump removes air from the desiccator, the pressure inside decreases, causing the glove attached to the desiccator to expand. The expansion of the glove indicates that the desired level of vacuum has been achieved.

Comment 5: Regarding the more mundane issue of correctness of expression, the authors use the terms aboveground and belowground inconsistently (sometimes each is presented as two words, sometimes one, and sometimes with a hyphen). Regardless of which style the authors decide on, they should use the wording consistently throughout the manuscript. As noted on the manuscript, a space should be used between numerals and units of measurement. Capitalization of numerous words (e.g. Sodium, Liquid chromatographs, etc.) is done incorrectly. I also thought the wording "to absorb any erroneous exudation pattern" on page 4 was very poor. I think you mean to say something such as "extraneous exudates", that is exudates produced by the plants prior to the sampling interval. The word "erroneous" is inappropriate to use as well; these exudates might be extraneous, but can't be said to be erroneous.

Response: We have addressed inconsistencies in terminology, capitalization, and grammatical errors to ensure adherence to scientific language conventions. The term "erroneous" has been replaced with "extraneous" for more accurate representation.

Comment 6: More substantively, the chromatograms in Figure 2 would be easier to see if they were compressed vertically and if the legend text was larger. Related to this figure, it is difficult to distinguish between the control and treatment chromatogram in the left panel; readers with red/green color blindness will have no hope of distinguishing the two traces, which is challenging even for readers with normal color vision.

Response: We have refined Figure 2 by compressing the chromatograms vertically and increasing the size of the legend text for enhanced visibility. Additionally, we have adjusted

the color schemes for better differentiation between the control (green) and sample chromatograms (in blue and red).

Competing Interests: No competing interests were disclosed.

Reviewer Report 30 May 2023

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Mònica Escolà Casas
Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain

This method article describes how to sample and analyze root exudates from Arabidopsis Thaliana by using a plastic float and a small volume of water. The analysis is made by UPLC-qTOF using a common methodology. Finally, the data curation and treatment are thoroughly done using different softwares. With this approach the authors can capture a large number of polar and semi-polar root-metabolites with minimal disturbance of the plant when transferring it to the sampling trap.

The article is well written, but it lacks some improvement in the following points:

1) The introduction should be more clear in the sense that it should follow a logical order of ideas. Also, some concepts are repeated over the paragraphs (e.g. the belowground signaling process and its importance in plants is mentioned several times). Finally, a few examples of significant root-sampling methods should be presented and compared with the method that they are about to present in their study.

2) As it is a method paper, a description of the method should put emphasis on why their approach is better than the approaches of other methods. E.g. "previous studies have found difficulties sampling in xxx way so we decided to investigate xxx possibility and found that xxx was the best solution".

3) The description of the method is well done but some specific details are missing:

3.1 What was the composition of the plastic mesh (type of plastic)? Define the type of plastic mesh and maybe the supplier should be included as it may be replicated.

3.2 What was the shape of the 15mL glass vials (400 uL in a 15 mL vial sounds like a very small height of the liquid and very limited space for the roots). Maybe Figure 1 could include measures of the vials.

3.3 The article mentions biological replicates. How were they made? Only plastic mesh with no plant?

3.4 The sampling method is crucial in this article. In this sense, the authors have chosen a 2h-collection in Milli-Q water. Have the authors tried other exposure times? Also, sampling in ultrapure water might have affected exudation by stress. The authors should dedicate some sentences explaining that (although it is a common practice).

4) Why did the authors chose the ESI negative ionization mode?

5) In the results, the authors mention that there were a few hundred chemical annotations. Have the articles verified any of the annotations by injecting some true standards? I know the goal here was not to identify compounds but it could add some value to the article if you could mention that some of the annotations were verified with a true standard.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am an environmental scientist working with nature based solutions (constructed wetlands, biofilms) for the removal of contaminants of emerging concern in water. Lately I have been investigating how root exudates are affected by contaminants of emerging concern and how root exudates may help to their degradation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Jun 2023

Harihar Jaishree Subrahmaniam

We would like to thank the reviewer for their valuable feedback on our manuscript. We have thoroughly reviewed all the comments and suggestions and incorporated substantial revisions to address them. The revised manuscript includes track changes to highlight all the modifications made. Reviewer 2 raised several important points that have helped us improve the logical order and several details of our methodology. We have addressed these points individually.

Comment 1: The introduction should be more clear in the sense that it should follow a logical order of ideas. Also, some concepts are repeated over the paragraphs (e.g. the belowground signaling process and its importance in plants is mentioned several times). Finally, a few examples of significant root-sampling methods should be presented and compared with the method that they are about to present in their study.

Response: We have restructured the *Introduction* section to follow a logical order of ideas and eliminate the repetition of concepts. We have also highlighted examples of significant root-sampling methods and compared them with the method presented in our study. These revisions aim to provide a clearer and more organized introduction that effectively introduces the novelty and importance of our approach.

Comment 2: As it is a method paper, a description of the method should put emphasis on why their approach is better than the approaches of other methods. E.g. "previous studies have found difficulties sampling in xxx way so we decided to investigate xxx possibility and found that xxx was the best solution".

Response: In response to the reviewer's comment, we have emphasized why our approach is superior to other methods. We now provide an explanation in each subsection of the *Introduction* section, highlighting the challenges faced by previous methods that led us to develop more effective solutions.

Comment 3: The description of the method is well done but some specific details are missing:

1. 1. What was the composition of the plastic mesh (type of plastic)? Define the type of plastic mesh and maybe the supplier should be included as it may be replicated

Response: The plastic mesh used in our study was made of polypropylene. We have now included this information in the *Introduction* section of the revised manuscript to ensure clarity and replicability.

1. 1. What was the shape of the 15mL glass vials (400 uL in a 15 mL vial sounds like a very small height of the liquid and very limited space for the roots). Maybe Figure 1 could include measures of the vials.

Response: In the revised version of the manuscript, we have provided a description of the shape and dimensions of the vials in the *Methods* section of the revised manuscript. The glass vials used in the study had a capacity of 20 mL and a cylindrical shape with a height of 57 mm and a diameter of 27.5 mm. Despite the limitations in terms of root space for larger plants, the short collection time span allowed us to still collect sufficient exudates.

1. 1. The article mentions biological replicates. How were they made? Only plastic mesh with no plant?

Response: The biological controls consisted of plastic meshes without plants. We have revised the manuscript to explicitly mention this and provide a clearer explanation of the controls used and their relevance for comparative analysis and validation of our methodology in the beginning of the *Methods* section.

1. 1. The sampling method is crucial in this article. In this sense, the authors have chosen a 2h-collection in Milli-Q water. Have the authors tried other exposure times? Also, sampling in ultrapure water might have affected exudation by stress. The authors should dedicate some sentences explaining that (although it is a common practice).

Response: We have addressed the importance of the sampling method and the choice of a 2-hour collection in ultrapure water in the *Introduction* of the revised manuscript. We explain that this duration is a common practice and briefly discuss the potential effects of stress caused by exposure to ultrapure water during the collection process. We also mention our previous time exposure experiments on another plant species and other references to support this collection time.

Comment 4: Why did the authors chose the ESI negative ionization mode?

Response: Although we conducted analysis in both positive and negative ionization modes, we presented the results in the negative mode as it captured a greater number of compounds. This information is included in the manuscript at the end of the *Methods* section to enhance the understanding of our analytical approach.

Comment 5: In the results, the authors mention that there were a few hundred chemical annotations. Have the articles verified any of the annotations by injecting some true standards? I know the goal here was not to identify compounds but it could add some value to the article if you could mention that some of the annotations were verified with a true standard.

Response: While our primary focus was not the annotation of compounds, we have recently undertaken a targeted approach to validate the chemical compounds we identified in the exudates. We now mention this in the *Discussion* section of the revised manuscript, highlighting the efforts made to verify the annotations using true standards.

Competing Interests: No competing interests were disclosed.

Reviewer Report 12 April 2023

https://doi.org/10.21956/openreseurope.16622.r31027

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Sophie Alvarez
Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

The authors present a new methodology for root exudate collection and analysis for *Arabidopsis* using a hybrid agar-hydroponic system to grow the plants and collect the root exudate. The aim of the new methodology described is to reduce handling of the roots to minimize damage that could bias the composition of the root exudate. The method also reduced the number of steps to collect concentrated root exudate from one individual plant for further analysis using mass spectrometry.

Although, the methodology sounds very simplified for a better representation of the composition of the root exudate. There are still some questions that authors have left unanswered to validate the relevance and reproducibility of the method.

1. The authors mentioned using paintbrush to clean the roots from agar before the washing/equilibrium step included before collection. It seems that damage and stress could still occur at that stage. Also, authors should indicate the time it takes to go through the steps from transfer of the plants from plates to the collection and storage of root exudates, to evaluate/estimate the feasibility of larger experiments. Can all the 28 replicates be done within the same time period of the day, so diurnal changes are not a variable in the experiment?

2. There is no justification for the 2 hours sample collection. Was a time course done to evaluate that 2 hours was the minimum required to get the levels high enough to be detected by untargeted LC-MS, but not detrimental to the plant (cell leakage or damage)?

3. The authors share the data from the detected compounds with peak area, but did not perform any data analysis of the reproducibility of the data collected from the 28 replicates to evaluate the experimental variation of the methodology, and to also determine how many technical replicates would be necessary to compare a large population of Arabidopsis. There is no correlation included between the data collected from the root exudate and the possible phenotype observed (in the case plants have “chlorosis”). This is essential to be able to validate their methodology.

4. There is no description of what the biological controls used to compare the data from Col0 are. What’s the relevance of it? Please explain in the manuscript.

5. It is also difficult to evaluate the methodology and its relevance with regards to identification and quantification of the root exudate content using an untargeted LC-MS metabolomics approach, when the authors voluntarily excluded the annotation of the compounds in the extended data.

Minor comments:

1. The method section is poorly written compared to the rest of the manuscript.

2. There is a typo in Figure 1. “recorded”

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant stress, root exudate, metabolomics, proteomics, mass spectrometry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 27 Jun 2023

Harihar Jaishree Subrahmaniam

We would like to thank the reviewer for their valuable feedback on our manuscript. We have thoroughly reviewed all the comments and suggestions and incorporated substantial revisions to address them. The revised manuscript includes track changes to highlight all the modifications made. Reviewer 1 raised several important points that have helped us improve the clarity and reproducibility of our methodology. We have addressed these points individually:

Comment 1: The authors mentioned using paintbrush to clean the roots from agar before the washing/equilibrium step included before collection. It seems that damage and stress could still occur at that stage. Also, authors should indicate the time it takes to go through the steps from transfer of the plants from plates to the collection and storage of root exudates, to evaluate/estimate the feasibility of larger experiments. Can all the 28 replicates be done within the same time period of the day, so diurnal changes are not a variable in the experiment?

Response: We acknowledge the concern regarding potential damage and stress during the cleaning step using a paintbrush. To minimize any bias introduced at this stage, we allowed the roots to remain in the cleaning solution for 1-2 minutes, as suggested by Oburger and Jones (2018) to capture any extraneous root exudation caused by stress. Additionally, we have provided detailed information in the *Methods* section on the time required to go

through each step of the methodology, including the transfer of plants from plates to the collection and storage of root exudates. We confirm that all 28 replicates can be performed within the same time period of the day to minimize diurnal changes as a variable in the experiment.

Comment 2: There is no justification for the 2 hours sample collection. Was a time course done to evaluate that 2 hours was the minimum required to get the levels high enough to be detected by untargeted LC-MS, but not detrimental to the plant (cell leakage or damage)?

Response: In the revised version, we have included references in the *Introduction* to support the use of the 2-hour sampling period based on previous studies and briefly mention our previous time exposure studies on a different plant species to support the collection period. These references provide evidence that 2 hours is sufficient to capture root exudates at detectable levels without causing exudate re-uptake or detrimental effects such as cell leakage or damage to the plants.

Comment 3: The authors share the data from the detected compounds with peak area, but did not perform any data analysis of the reproducibility of the data collected from the 28 replicates to evaluate the experimental variation of the methodology, and to also determine how many technical replicates would be necessary to compare a large population of Arabidopsis. There is no correlation included between the data collected from the root exudate and the possible phenotype observed (in the case plants have “chlorosis”). This is essential to be able to validate their methodology.

Response: In the revised manuscript, we have now included data analysis of the reproducibility *(in Extended data analysis)*, including the coefficient of variation (CV) and mean abundance for the detected compounds. We also calculated the relationship between CV and mean abundance based on the Poisson distribution, as suggested by Ipsen and Ebbels (2012), and found it to be in line with the predicted relationship for LCMS data. Moreover, we have discussed the correlation between plant size and total peak area as empirical evidence validating the methodology's ability to capture and quantify differences in compound abundance in the *Discussion section*.

Comment 4: There is no description of what the biological controls used to compare the data from Col0 are. What’s the relevance of it? Please explain in the manuscript.

Response: We acknowledge the lack of description regarding the controls used to compare the data from Col0. In our study, we used plastic meshes without plants as controls. These control meshes were treated in the same manner as the plants during sample preparation and analysis. They served as a baseline reference for comparison, allowing us to assess the specific contribution of root exudates from Col0. We have included an explanation of controls in the beginning of the *Methods* section. We acknowledge that the term "biological controls" may have caused confusion, and we have revised the manuscript to provide a clearer explanation of the controls used and their relevance for comparative analysis and validation of our methodology. We apologize for any confusion caused and appreciate the opportunity to clarify this in the revised manuscript.

Comment 5: It is also difficult to evaluate the methodology and its relevance with regards to identification and quantification of the root exudate content using an untargeted LC-MS metabolomics approach, when the authors voluntarily excluded the annotation of the compounds in the extended data.

Response: We have now provided the full chemical annotations of the identified compounds in the *Supplementary data file,* which includes a comprehensive list of the identified compounds along with their annotations

Minor comments:

1. The method section is poorly written compared to the rest of the manuscript.
2. There is a typo in Figure 1. “recorded”

Response: We have also addressed the minor comments, particularly the method section, and made substantial revisions to improve its clarity and coherence. Furthermore, we have corrected the typographical error in Figure 1 as pointed out by the reviewer, ensuring that the revised version of the figure accurately represents the recorded data.

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 3

Reader Comment 26 Aug 2026

Muhammad Imran Yamin, Physical Geography, Henan University College of Geography and Environmental Science, Kaifeng, China

I have read your interesting methodological paper on root exudate collection in *Arabidopsis thaliana*. I would like to point out three issues:

1. **Storage temperature:** You mention storing samples at -18°C (Methods) and -16°C (Figure 1, Step 6) after freezing in liquid nitrogen. For untargeted metabolomics, -80°C is the standard for long-term storage to prevent enzymatic degradation. I kindly suggest a correction.
2. **Inconsistency:** There is a discrepancy between the Methods section (-18°C) and Figure 1, Step 6 (-16°C). Please unify the temperature.
3. **Applicability for soil-grown horticultural plants:** This method works well for small *Arabidopsis* seedlings. However, for soil-grown plants like tomato and other horticultural plants at later stages (e.g., flowering, fruiting), How this method is feasible due to root size, soil removal difficulties, and lack of a recovery period.

I hope this feedback is helpful.

Competing Interests: No competing interests were disclosed.