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CEDRIC

BEST PRACTICE 4

Root exudate sampling and analysis

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Root exudates are a complex mixture of organic and inorganic compounds actively released by plant roots into the surrounding environment. These compounds include sugars, amino acids, organic acids, phenolics, secondary metabolites, and signaling molecules, and they play a key role in shaping plant–microorganism interactions, nutrient mobilization, and stress responses.

The qualitative and quantitative composition of root exudates is highly dynamic and depends on multiple factors, including plant species, developmental stage, nutritional status, environmental conditions, and the presence of biotic interactions. For this reason, the collection and analysis of root exudates require carefully standardized methodologies to ensure reproducibility and meaningful biological interpretation.

Hydroponic systems are widely used for root exudate studies because they allow a high level of control over growth conditions and facilitate the collection of exudates with minimal interference from soil particles. However, hydroponic cultivation also introduces specific limitations that must be considered when interpreting results.

In this Best Practice, a refined protocol for root exudate sampling and analysis is described, based on experiments conducted within the CEDRIC project. The protocol aims to provide general guidelines that can be adapted to different plant species, experimental objectives, and analytical platforms, while highlighting critical steps, potential sources of variability, and methodological trade-offs. As part of the CEDRIC project, barley plants were grown hydroponically in controlled conditions for few weeks. To half the plants, optimal amounts of nutrients were supplied; to the other half the micronutrient iron was not. Each week, the root exudates of Fe-deficient and Fe-sufficient barley plants were collected. Nutrient deficiency was used as it induced a much larger release of exudate. Root exudates were analysed via Mass spectrometry after liquid chromatography separation. Experiments were conducted to refine the methodology of collection, chemical and data analysis, which is described in detail below for the benefit of anyone interested.

Although the protocol described above refers to barley plants grown under iron-sufficient and iron-deficient conditions, the general approach can be applied to a wide range of plant species and experimental designs. Growth duration, nutrient composition, and container size should be adjusted according to plant morphology, growth rate, and experimental objectives.

Nutrient deficiency or other controlled stresses (e.g. nitrogen or phosphorus limitation, salinity, or pH variation) can be used to stimulate root exudation; however, care must be taken to distinguish between physiologically relevant responses and stress-induced artifacts. When nutrient deficiencies are applied, the use of deionised water is strongly recommended to avoid uncontrolled ion inputs from tap water.

Environmental parameters such as temperature, light intensity, photoperiod, and relative humidity should be kept as constant as possible, as fluctuations can significantly influence plant metabolism and exudation patterns. If optimal conditions cannot be maintained, plant growth duration and sampling timing should be adjusted accordingly.

1) Plant growth

- **Plant materials:** Seeds of suitable cultivar/specie

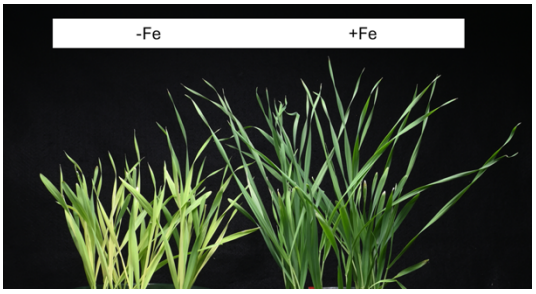
- **Tools:** Absorbent paper, boxes (at least 300x300x250mm), floating rafts for hydroponics (slightly smaller than box size), 1-L bottles, glass/plastic wares to prepare solutions, measuring glass/plastic wares; small scale (if salts are used); pH meter, air pump for aquarium (at least 600 L/h); greenhouse (with illumination if needed)

- **Consumables:** salts (or fertilizers in liquid form)

Example of a detailed optimal protocol if +Fe/-Fe barley plants are used. Barley seeds (Hordeum vulgare L., cv. Bianca), are rinsed with water, insert into the floating raft. Fill the box up to few cm from the top of the box with aerated (with the aquarium

pump) Fe-free nutrient solution and grown for 21 days in the box. The Fe-free nutrient solution contains the following nutrients: $Ca(NO_3)_2$ 2 mM; $MgSO_4$ 0,5 mM; K_2SO_4 0,7 mM, KCl 0,1 mM, KH_2PO_4 0,1 mM, H_3BO_3 1 µM; $MnSO_4$ 0,5 µM; $CuSO_4$ 0,5 µM; $ZnSO_4$ 0,5 µM; Na_2MoO_4·0,01 µM. pH should be around 6.5 if not, correct with 0.1 M NaOH or HCl. Fe-sufficient plants are grown in the nutrient solution described above with the addition of 100 µM Fe-EDTA. The nutrient solution must be renewed twice a week. The controlled climatic conditions should be as follows: day/night photoperiod, 16/8; light intensity, 220 µE $m^{-2}s^{-1}$; temperature (day/night) 25/20°C; RH 70-80%.

- Size of the box, growth time and nutrients' content must be adapted for each plant species
- Prepare a stock solution for each nutrient at a 100-1000x concentration and add each to a half-full box with water mixing constantly. Or use liquid complete fertilizer solutions and follow the manufacturer's instructions (in this case it is difficult to impossible to induce nutrient deficiency).
- If deficient inducing growth solutions are used water should be deionised not tap water
- Best solution would be to keep the plant in a growth chamber with all the environmental parameter controlled. In alternative, a small greenhouse could be use (the temperature should not be too high, above 28°C, or too low, below 18°C). Natural light can be use if sufficient or use led illumination with a timer to control the photoperiod). If the environmental conditions are not optimal the duration of plant growth must be adjusted accordingly.



2) Root exudate collection

- **Tools:** At least two boxes of the same size of the one used in point 1; sterile deionised water; filtration system at 0.2 µm; sterile bags or containers (e.g., tubes, freezer bags), denatured alcohol, -20°C freezer for storage
- **Labelling:** Prepare durable labels, permanent markers, and a notebook for notes.
- **Sterility:** All tools should be sterilized before sampling (sterilized with 70% ethanol)

Each week, sterilised boxes are partially filled with sterile water to rinse the roots, transfer the floating rack with the plants for two minutes moving the roots out and in the water few times to help the rinsing, repeat this whole process three times. Fill with a minimum amount (just enough to immerse all the roots) of sterile deionised water in a sterilised box. Transfer the floating rack with the rinsed roots on to the solution for 1-4 hours. Put back the floating racks with the plants in the box to continue plant growth. The solution containing the exudates must be filtered at 0.2 µm, transfer into sterile labelled containers and store at -20°C until further processing.

The rinsing steps performed prior to exudate collection are essential to reduce the carryover of residual nutrient solution and loosely associated microorganisms. Nevertheless, it should be noted that complete sterility cannot be achieved, and microbial activity may still occur during the collection period.

Sterile deionised water is commonly used as a collection medium because it minimizes background signals during chemical analysis; however, the absence of nutrients may also induce short-term stress responses in plants. The duration of exudate collection therefore represents a compromise between maximizing compound recovery and minimizing microbial degradation or stress-induced artefacts.

Collection time should be carefully selected based on the experimental goal, and consistency across samples is critical for reliable comparisons.

- Late morning/midday is generally the best time of the day to collect root exudates as the amount of their release is higher
- The duration of root exudate collection is difficult to choose. Longer will result in a higher amount of root exudate released, however it increases the probability of exudate degradation. Bear in mind that even if all the tools are sterilised and the roots are carefully rinsed, some microorganisms will always be present and thus they will possibly consume root exudates. One hour will be use if the goal is to minimise the effect of microorganisms. Two to four hours are used if the amount of root exudate recovered is critical.

Box 1. Plant growth conditions and their impact on root exudation

Plant growth conditions have a strong influence on both the amount and composition of root exudates. Light intensity and photoperiod affect carbon assimilation and allocation to the roots, while temperature and humidity modulate metabolic rates and transpiration. Nutrient availability not only alters plant physiology but also directly shapes the qualitative profile of exuded compounds.

To ensure comparability between experiments, it is recommended to carefully document growth conditions, including photoperiod, light intensity, temperature, humidity, nutrient composition, and growth duration. Even small variations in these parameters can result in significant differences in exudate profiles, potentially confounding biological interpretation.

When experiments are conducted outside controlled growth chambers, additional variability should be expected and explicitly considered during data analysis.

3) Root exudate analysis

Root exudate analysis is commonly performed using liquid chromatography coupled to mass spectrometry (LC–MS), which allows the detection of a wide range of organic compounds with high sensitivity. Depending on the analytical setup, targeted or untargeted approaches can be applied.

Due to the typically low concentration of exuded compounds, particular attention should be paid to sample concentration, background contamination, and signal normalization. Whenever possible, internal standards should be included to assess analytical variability and improve quantitative reliability. Data normalization to root biomass, root length, or collection time is strongly recommended to allow meaningful comparisons between samples.

- **Tools:** Liquid chromatography system with column and detector for organic molecules

Box 2. Root exudation collection problematics: hydroponics vs soil-grown plants

Root exudate collection can be performed using either hydroponic systems or soil-grown plants, each approach presenting specific advantages and limitations. Hydroponic cultivation allows a high degree of experimental control and facilitates direct recovery of exuded compounds, minimizing interference from soil particles and organic matter. This makes hydroponics particularly suitable for qualitative and quantitative chemical analyses.

However, hydroponic systems do not fully reproduce the physical, chemical, and biological complexity of soil environments. In soil-grown plants, a substantial fraction of root exudates can be

rapidly adsorbed onto soil particles, metabolized by microorganisms, or transformed through abiotic reactions, making direct collection more challenging. At the same time, soil-based systems provide a more realistic representation of plant–soil–microbe interactions.

The choice between hydroponic and soil-based approaches should therefore be guided by the specific objectives of the study. Hydroponics is recommended when precise chemical characterization and reproducibility are priorities, whereas soil-based systems are more appropriate when ecological relevance and interaction dynamics are the main focus.

4) Data analysis

Data analysis of root exudate profiles requires careful consideration of biological variability, analytical noise, and normalization strategies. Raw chromatographic and mass spectrometry data should be processed using appropriate software for peak detection, alignment, and compound annotation.

To enable meaningful comparisons between samples, exudate signals should be normalized using biologically relevant parameters, such as root fresh or dry weight, root length, number of plants per sample, or collection time. The inclusion of blank controls (e.g. sterile water subjected to the same collection and filtration procedures) is essential to identify background contaminants and artefacts.

Whenever possible, biological replicates should be prioritized over technical replicates, as they capture natural variability between plants. Statistical analyses should be selected according to the experimental design and data distribution, and multivariate approaches may be particularly useful for identifying treatment-specific exudation patterns.

Box 3. Difficulties in analysing the quality and quantity of organic molecules in a solution

The analysis of root exudates presents several technical challenges due to the low concentration and high chemical diversity of the compounds released. Many metabolites are present at trace levels and may fall below the detection limit of analytical instruments. In addition, some compounds are chemically unstable and may degrade during collection, storage, or sample preparation.

Matrix effects, such as the presence of salts or residual contaminants, can interfere with ionization efficiency during mass spectrometry, affecting quantitative accuracy. Adsorption of metabolites to plastic or glass surfaces may further reduce recovery, particularly for hydrophobic compounds.

To mitigate these issues, careful selection of materials, rapid sample processing, appropriate storage conditions, and the use of internal standards are strongly recommended.

Box 4. Collection solution and duration: impact on root exudate profiles

The choice of collection solution and the duration of root exudate sampling can significantly influence both the quantity and composition of recovered compounds. Sterile deionised water is commonly used to minimize background interference during chemical analyses; however, prolonged exposure to nutrient-free conditions may induce stress-related exudation responses.

Short collection times (e.g. 1 hour) reduce the impact of microbial degradation and stress responses but may result in low metabolite recovery. Longer collection periods (2–4 hours) increase the amount of exudate collected but also raise the risk of compound degradation and microbial consumption.

Researchers should therefore carefully balance collection duration and analytical requirements, maintaining consistency across samples and clearly reporting these parameters in experimental metadata.

More information:

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