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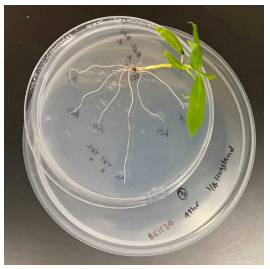
Protocol for Roots and Rhizodeposition Metabolite extraction from Plant-Agar Plates for LC-MS/MS analysis



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We use this protocol and it's working

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Abstract

Our research has demonstrated that *Populus* root-associated microbiomes assembled in a plant-agar system formed a nearly identical composition to that observed in a clay soil matrix. This finding suggests that a plant agar matrix can replicate a soil-like environment while providing a more controlled and reproducible workflow for studying rhizodeposition. Building on this, we developed a novel biphasic extraction method specifically tailored for rhizodeposition studies using the plant agar-plate system. Rhizodeposition encompasses a diverse mixture of metabolites with varying polarities, from polar organic acids and amino acids to nonpolar phenolics and lipophilic compounds. To capture this full range, we employed a 50/50 water and ethyl acetate biphasic extraction mixture to enable a comprehensive profiling of root-derived metabolites involved in rhizodeposition

Preparing Plant-agar Plate for Spatial Metabolite Extraction

1 **Prepare Agar Media:** Mix agar with the appropriate growth media according to the specifications of the experiment. Adjust pH as needed, then autoclave to sterilize. Pour the agar media into sterile Petri dishes (typically 90 mm or 100 mm diameter) and allow it to cool and solidify under sterile conditions.

2

Note

Note that while this metabolite extraction protocol is compatible with both agar and agarose, using agar may introduce a higher background of matrix compounds in comparison to agarose. Although one can manipulate the amount, we have tested and can only recommend 2% agar and 0.3% agarose.

3 **Transplant Plant onto Agar Plate:** In a sterile laminar flow hood, using sterile forceps, carefully place roots gently on the agar surface, avoiding damage to the root tissue. To place the cuttings on the plates, lay the root system down on the surface of the media and use sterile forceps to spread the roots out so that one root traverses the middle of the plate and the other roots are pushed away from each other. Use the sterile forceps to gently push the roots into the media so they are fully embedded.

4

Note

Example of container used when growing plants with agar inoculated with microbes.

5 **Close container:** Place each plate with a cutting on an inverted lid of a sterile plastic container and position an inverted container over the cutting and use the sidewall to hold the cutting upright, then seal the container by pressing it down and snapping it in place with the inverted lid. You now have an open plate with a cutting embedded in the media and sitting inside a sterile inverted plastic container. Label the container and place the cuttings in a light room or plant incubation chamber.

Metabolite Extraction from Root Tissue

6 **Excise Root Tissue:** Excise roots from each plant with a sterile scalpel.

7 **Freeze and Store:** Transfer the root tissue from each plant into a separate vial. Freeze the sample vials immediately in liquid nitrogen and store at -80°C until further processing.

8 **Freeze-dry and Record Weights:** Freeze-dry the root tissue (e.g., Labconco, USA) and record the dry weights for each sample.

9 **Grind Root Tissue:** Using a cryogrinder (e.g., SPEX Sample Prep 2010 GENO/GRINDER) or mortar and pestle, grind the freeze-dried root tissue in liquid nitrogen into a fine powder and store samples on ice.

10 **Preparation of Solvents for Metabolite Extraction:** Mix HPLC-grade ethyl acetate with HPLC grade water in a 1:1 ratio to create the hydrated ethyl acetate solvent. Transfer the hydrated ethyl acetate and HPLC grade water to separate containers, and chill both on ice prior to extraction.

11 **Perform Metabolite Extraction:** Add equal volumes of ice-cold, hydrated LC-MS grade ethyl acetate and LC-MS grade water (total volume = 2 mL) to each powdered root sample. Vortex the samples at maximum speed for 1 minute to mix thoroughly. Ensure samples remain cold throughout this process by keeping them on ice, then transfer them to a fridge set at 4°C and incubate on ice overnight.

> Note
>
> Total solvent volume can be adjusted depending on the sample amount.

12 **Centrifuge Samples:** Centrifuge the samples at maximum speed for 10 minutes at 4°C.

13 **Separate Fractions:** Using aspiration, carefully separate the organic (ethyl acetate) and aqueous supernatant fractions. Ensure samples remain cold throughout this process by keeping them on ice.

14 **Prepare Organic Fraction:** Dry the ethyl acetate extracts completely in a chemical fume hood. Resuspend each extract in 50 µL of organic solvent (70% acetonitrile with 0.1% formic acid) prior to LC-MS/MS measurement.

15 **Prepare the Aqueous Fraction:** Pre-wet 10 kDa centrifugal filters (e.g., Sartorius VivaSpin). Filter the aqueous extracts by centrifuging at 4,500 x g at 4°C to remove

residual media and particles. Lyophilize the aqueous extracts (e.g., Labconco, USA), then resuspend in 50 µL of aqueous solvent (98% LC-MS water, 2% acetonitrile with 0.1% formic acid) prior to LC-MS/MS measurement.

16 **Store Extracts:** Store both organic and aqueous extractions at 4°C for short-term storage until LC-MS/MS analysis.

Metabolite Extraction from Media (Rhizodeposits)

17 **Collect Spatially Resolved Rhizodeposits:** Use an inverted 1 mL pipette tip to cut and collect agar plugs of equal volume from distinct spatial regions surrounding the plant roots (e.g., root base, root tips, and regions distant from the root base and tip).

18 **Freeze and Store Agar Plugs:** Immediately freeze the plugs in liquid nitrogen and store them at -80°C until further processing.

19 **Freeze-dry:** Freeze-dry the agar plugs (e.g., Labconco, USA).

20 **Perform Metabolite Extraction:** Add equal volumes of ice-cold, hydrated LC-MS grade ethyl acetate and LC-MS grade water (total volume = 2 mL) to each agar plug sample. Vortex the samples at maximum speed for 1 minute to mix thoroughly. Ensure samples remain cold throughout this process by keeping them on ice, then transfer them to a fridge set at 4°C and incubate on ice overnight.

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Acknowledgements

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