VERSION 1

FEB 05, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.5qpvo36wdv4o/v1

Protocol Citation: Veronica Roman-Reyna, Nathaniel Heiden, Jules Butchacas, Hannah Toth, Jessica L. Cooperstone, Jonathan M. Jacobs 2024. Extraction and analysis of primary metabolites during Xanthomonas-Barley interaction. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvo36wdv4o/v1

Extraction and analysis of primary metabolites during Xanthomonas-Barley interaction V.1

Veronica Roman-Reyna[1,2,3], Nathaniel Heiden[2,3], Jules Butchacas[2,3], Hannah Toth[2,3], Jessica L. Cooperstone[4,5], Jonathan M. Jacobs[2,3]

[1]Department of Plant Pathology and Environmental Microbiology, Pennsylvania State University, University Park, PA, USA;
[2]Department of Plant Pathology, The Ohio State University, Columbus, OH, USA;
[3]Infectious Diseases Institute, The Ohio State University, Columbus, OH, USA;
[4]Department of Horticulture and Crop Science, The Ohio State University, Columbus, OH, USA;
[5]Department of Food Science and Technology, The Ohio State University, Columbus, OH, USA.

Veronica Roman-Reyna
Pennsylvania State University

ABSTRACT

Intercellular host-associated bacteria shape the chemistry of the living eukaryotic environment. Pathogenic microorganisms like barley-and wheat-associated *Xanthomonas translucens*, swiftly overtake the inner leaf tissue becoming the dominant community member during disease development. To define the metabolic signals associated with inner leaf colonization, we used untargeted metabolomics to characterize *Xanthomonas translucens* pathovars translucens (Xtt) and undulosa (Xtu)primary metabolism signatures associated with mesophyll growth.

This protocol indicates how to perform the infections, extract apoplastic fluids, perform GCMS, and conduct the subsequent data analysis.

GUIDELINES

To avoid changes in the sugar profile, it is good to use liquid nitrogen to snap-freeze the samples before storing them at -80C and extracting them.

Metabolite content is not uniform across samples, therefore it is good to have several samples and pool them to reduce the variation.

Protocol status: Working
We use this protocol and it's working

Created: Jan 27, 2024

Last Modified: Feb 05, 2024

PROTOCOL integer ID: 94264

Keywords: primary metabolites, GCMS, Xanthomonas translucens, barley mesophyll, untargeted metabolomics

MATERIALS

Barley cv. Morex
Xtu, Xtt
Nutrient Agar
Methanol, Chloroform
Ribitol to spike water for extraction
Liquid Nitrogen

Sample preparation

1 For sample preparation you need:
- Barley cv. Morex seeds
- *Xanthomonas translucens* pv. undulosa strain UPB513,
- *Xanthomonas translucens* pv. translucent strain UPB886

1.1 Barley Plants

The Barley cv. Morex was used for in-planta studies. All plants were sown at 20 °C, 60% humidity in Promix soil. Plants were grown for three weeks before inoculation. Plants should have at least two fully developed leaves.

1.2 Bacteria

Xanthomonas translucens pv. *undulosa (Xtu*) and *Xanthomonas translucens* pv. translucens (Xtt) were taken out of -80 °C glycerol stock and plated on nutrient agar media (3 g/L beef extract, 5 g/L peptone, 15 g/L agar) a week before the experiment and incubated at 28 °C.

Three days before the experiment, a single bacteria colony was plated again on nutrient agar and incubated at 28 °C.

On the day of inoculation, a bacterial loop was suspended in sterile water to achieve an absorbance of 0.1 at 600 mn (10^8 CFU/mL).

Bacterial and mock inoculation

2 The youngest fully expanded leaf of the three-week-old plants was used for inoculations.

Fill a 1 mL needleless syringe (insulin-type syringae) with the inoculum. Press the syringe against the abaxial surface of the leaf and place a finger on the opposite side of the syringe to keep firm contact between the syringe and the leaf. Gently plunge the syringe, inoculating roughly 100-500 µL into the leaf. Most leaves will have a temporary transparency to the infiltrated area.

The leaves were syringe-infiltrated with either 1-2 mL of bacterial resuspension or mock water.

Aim to inoculate at least 30-40 leaves with each strain to have data to collect at each time point and to pool samples.

Apoplast collection

3 Leaves were collected after **6 h**, **12 h**, and **24 h post-inoculation**.

To eliminate any surface microorganisms, the leaves were cleaned using 75% ethanol. A pool of four leaves was cut at the base for fluid extractions and then weighed.

A 10 ppm ribitol solution was used to vacuum infiltrate all the leaves. The ribitol was added for metabolite normalization. After infiltration, the leaves were weighed again to determine the amount of water infiltrated; water density was assumed to be 1 g/ml.

We followed the protocol from Roman-Reyna and Rathjen 2017 for the mesophyll fluid extraction and test cytoplasmic contamination.

CITATION

Roman-Reyna V, Rathjen JP (2017). Apoplastic Sugar Extraction and Quantification from Wheat Leaves Infected with Biotrophic Fungi..

LINK

https://doi.org/10.1007/978-1-4939-7249-4_11

We used 50 mL syringes to create the negative pressure. At each time point, 2 µL of Apoplastic fluid from each sample was plated in nutrient agar to count the colony-forming units (CFU) and ensure that all samples had the same number of bacteria. The CFUs were 5 to 200 based on the time point. After that, fluids were flash-frozen to avoid metabolite degradation.

For analysis, four pools were selected from each time point that has a similar number of bacteria.

Polar and semi-polar metabolites extraction

4 A polar and semi-polar extraction was chosen for this project.

For the extraction, 500 µL of apoplast was mixed with 1 mL of a mixture of methanol, chloroform, and water in a ratio of 5:2:2, respectively.

The sample was vortex and incubated for 5 min at 28 °C in a 20 rpm.

Samples were then centrifuged at 2200*g*, 15 °C, 15 min. The upper phase (methanol and water) was collected and 200 µL were sent to the West Coast Metabolomics Center (UC Davis) for untargeted metabolomic analysis by gas chromatography time of flight mass spectrometry (GC-TOF MS).

Untargeted Primary Metabolism with GC-TOF MS.

5 Samples were sent to the West Coast Metabolomics Center (UC Davis) for untargeted analysis. Samples were processed with the assay: "Primary Metabolism by GC-TOF MS:
carbohydrates and sugar phosphates, amino acids, hydroxyl acids, free fatty acids, purines, pyrimidines, aromatics". (Website: https://metabolomics.ucdavis.edu/core-services/assays-and-services).

5.1 Derivatization

Samples were extracted using 1 mL of 3:3:2 ACN:IPA:H2O (v/v/v). Half of the sample was dried to completeness and then derivatized using 10 µL of 40 mg/mL of methoxyamine in pyridine. They are shaken at 30 °C for 1.5 hours. Then 91 µL of MSTFA + FAMEs were added to each sample and were shaken at 37 °C for 0.5 hours to finish derivatization.

5.2 For data acquisition, processing, and raw data normalization the information can be found in the document shared by the Center:
https://www.rsc.org/suppdata/c6/en/c6en00093b/c6en00093b1.pdf

Further analyses

6 West Coast Metabolomics Center (UC Davis) provided the normalized data and annotated the peaks based on retention times and mass spectra compared to the MassBank of North America.

6.1 Metabolights

The Raw/derived files (cdf extension) are available on https://www.ebi.ac.uk/metabolights project MTBLS7676.

CITATION

Yurekten O, Payne T, Tejera N, Amaladoss FX, Martin C, Williams M, O'Donovan C (2024). MetaboLights: open data repository for metabolomics..
LINK
https://doi.org/10.1093/nar/gkad1045

6.2 MetaboAnalyst

West Coast Metabolomics Center provided a table with a list of KEGG IDs, names, and peak intensities, which was used as input for MetaboAnalyst. Statistical analyses were conducted in MetaboAnalyst 5.0.

CITATION

Xia J, Wishart DS (2011). Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst..
LINK
https://doi.org/10.1038/nprot.2011.319

We selected the module "Statistical Analysis [One Factor]" for the analysis. With the datatype "Peak Intensities" the input data was the CSV table from the West Coast Metabolomics Center.

For principal component analysis (PCA), the table was uploaded as a single file. For Fold-Change (FC) analysis data, the data was separated into six files based on time points (6, 12, 24)and Xt strains (UPB886, UPB513) before uploading.

PCA and FC analysis data underwent the same normalization steps. All data was imported into the Statistical Analysis module with the data type “Peak intensities”. The statistical filter used was Standard deviation and for sample normalization, we selected the option ' Normalization by reference feature'. The reference feature was Ribitol, which was spiked into the extraction buffer. Finally, to reduce data skewness we selected Log transformation (base 10).