High-Throughput *in situ* Molecular, Spatial, and Temporal Characterization of Bioactive Secondary Metabolites by the SepQuant® *droplet* Probe™

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Overview

Objective

- To generate structural, spatial, temporal, and ecological functionality data on the secondary metabolites of three distinct organisms through *in situ* analysis using the SeqQuant® *droplet*Probe™ coupled to an HPLC-HRMS/MS system

Methods

- Direct surface sampling of all specimens was performed using the SeqQuant® *droplet*Probe™ with 5 µL droplet microextractions using MeOH-H_2O (1:1).
- Droplets were injected into an Acquity UPLC system coupled to an electrospray QExactive Plus MS.

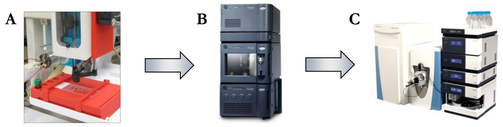

Figure 1. Generalized workflow for all experiments. (A) All specimens were sampled via the SeqQuant® *droplet*Probe™, (B) the droplets were separated using an Acquity UPLC system with PDA detection, and (C) data on eluates were collected using an electrospray QExactive Plus MS.

Results

- The secondary metabolite profiles of each plant tissue of *A. triloba* were similar, but the ovaries, a previously unstudied source of acetogenins, were discovered to contain the most extensive number of acetogenins.
- Griseofulvin, the fungistatic secondary metabolite of *X. cubensis,* remains in the younger tissue of the fungus, while polyhydroxyanthraquinones, the secondary metabolites of *P. restrictum,* are excreted.
- When grown in co-culture, the spatial and temporal distributions of griseofulvin remained the same as the monoculture, but the distribution of polyhydroxyanthraquinones from *P. restrictum* was altered.

Introduction

The field of pharmacognosy has been rapidly advancing to identify several novel therapeutics from a variety of natural products. However, research continues to be limited by a lack of understanding of the ecological and biological contexts of potential compounds. In this study, we set out to use an automated surface sampling system coupled to high performance liquid chromatography-high resolution mass spectrometry/mass spectrometry (HPLC-HRMS/MS) system to investigate the molecular, spatial and temporal profiles of bioactive secondary metabolites of both a plant, *Asimini triloba* (paw paw), and of two species of fungi, *Xylaria cubensis* and *Pencillium restrictum*. We hypothesized that our *in situ* analysis method would provide novel insight into the structure and function of secondary bioactive metabolites from these species, thus presenting a high-throughput methodology to aid in the prioritization of compounds in pharmacognostic or natural products research.

Figure 2. (A) Photograph of *Asimina triloba* and magnification of the fruit. (B) Images of *X. cubensis* (G536) grown in a glass Petri dish and placed in a sterile plant tissue-cultivating container (Plant Con®).

Methods: *A. triloba*

- *A. triloba* was collected from Pfafftown, NC, USA
- Seeds, pulp, and twigs were all directly sampled with the SeqQuant® *droplet*Probe™
- As the leaves, petals and ovaries of *A. triloba* are covered with a waxy, hydrophobic surface, small (0.5x0.5 cm) pieces of each were immersed in Tissue-Tek optimum cutting temperature embedding medium and placed in a -80°C freezer. Once frozen, each 15 µm cross-sections of each tissue were obtained using the Leica CM1100 cryostat (Leica Biosystems Inc., Buffalo Grove, IL) and allowed to thaw on a microscope slide.
- Microextractions of 5 µL were performed using the SeqQuant® *droplet*Probe™ with MeOH-H_2O (1:1).
- Droplets were injected into an Acquity UPLC system (Waters Corp. Milford, MA).
- Data on the eluates were collected on a Qexactive Plus MS (Thermofisher, San Jose, CA)
- Compound Discoverer (ThermoFisher, San Jose, CA) was used to perform mass defect filtering. (±100 Da and ±25 mDa around m/z 603.4807).

SepQuant *droplet*Probe™

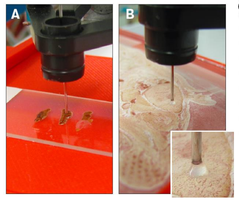

Figure 3. Extraction liquid dispensed by the needle in locked position on (A) a tissue biopsy sample and on (B) a brain thin tissue section. Zoomed in view in inset in (B) shows of liquid microjunction between the tip of the needle and the sample.

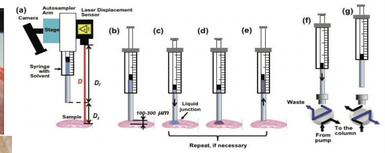

Figure 4. Schematic of the surface sampling process including (a) determining syringe-to-surface distance, (b) the positioning of the syringe needle 100-300 µm above a defined surface spot, (c) dispensing a discrete volume of extraction solvent onto a surface creating a liquid junction between the needle and the surface, (d) dissolution of the analyte in the extraction solvent, e) the liquid is drawn back into the syringe needle after a predefined extraction time, and (f) loaded into the sample loop followed by (g) injection for a consecutive HPLC-MS/MS analysis. Steps (c)-(e) may be repeated to improve extraction efficiency.

Results: Spatial Mapping of Annonaceous Acetogenin Analogues in *A. triloba*

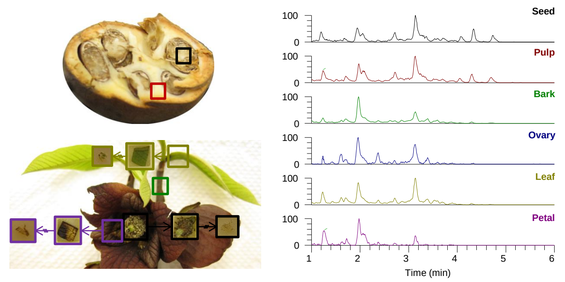

Figure 5. (A) Locations of paw paw where the droplet-LMJ-SSP directly sampled seed (black), pulp (red), and twig (green) and the portions that were cross-sectioned: ovary (blue), leaf (yellow), and petal (purple). (B) The mass defect filtered chromatograms around annonacin; 603.4807 ± 100 Da with a mass defect of ±25 mDa.

Methods: *X. cubensis* & *P. restrictum*

- Fungi were isolated as endophytes from surface sterilized plant tissue segments, and cultures were maintained are UNC-Greensboro.
- Both strains were identified via morphological and molecular methods.
- After fermentation on rice media, the *X. cubensis* was extracted using biphasic MeOH-$CHCL_3$ (1:1) separation method and filtered.
- The extracted material was separated via normal phase flash chromatography on a CombiFlashRf system using a 4 g RediSep Rf Si-gel Gold column (Teledyne0Isco, Lincoln, NE, USA).
- HPLC was performed on fraction 2.
- Griseofluvin eluted at 6.9 min and yielded 1.06 mg.. The structure was confirmed via NMR on a JEOL ECS-400 NMR spectrometer (400 MHz; JEOL Ltd. Tokyo, Japan) and HRMS-UPLC system.
- *X. cubensis* was transferred onto six plates of MEA and allowed to grow for 10 days. *P. restrictrum* was introduced, and the plates were covered and allowed to grow for 30 days when the cultures started to grow towards each other. Controls of each fungus were kept separately on PDA plates.
- Microextractions of 5 µL were performed using the SeqQuant® *droplet*Probe™ with MeOH-H_2O (1:1).
- Droplets were injected into an Acquity UPLC system (Waters Corp. Milford, MA).
- Data on the eluates were collected on a Qexactive Plus MS (Thermofisher, San Jose, CA)

HPLC Parameter	*A. triloba*	*X. cubensis and P. restrictum*
Flow Rate	300 µL/min	
Column	BEH C_{18} column, equilibrated at 40°C A 2 mM solution of LiF in MeOH was infused post-column at a rate of 5 µL/min in the A. triloba experiments only.	
Mobile Phase	Fisher optima LC-MS grade CH_3CN-H_2O (acidified with 0.1% formic acid), 70%-100% CH_3CN, increasing linearly over 8 min	Fisher optima LC-MS grade CH_3CN-H_2O (acidified with 0.1% formic acid), 15%-100% CH_3CN, increasing linearly over 8 min
PDA Detection Range	200-500 nm (4nm resolution)	

MS Parameter	*A. triloba*	*X. cubensis and P. restrictum*
Spray Voltage	3.7 kV	3.7 kV
Capillary Temperature	350°C	350°C
N2 Sheath Gas	25 arb	25 arb
N2 Auxiliary Gas	5 arb	5 arb
S-lens RF Level	50	50
HCD Normalized Collison Energy	60.0	N/A

Results: Spatial Mapping of Griseofulvin Production of *X. cubensis*

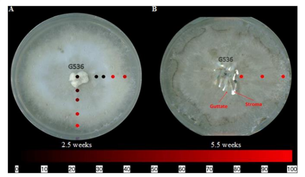

Figure 6. The spatial distribution of griseofulvin on *X. cubensis* (G536) grown on MEA at (A) 2.5 weeks and (B) 5.5 weeks of growth displaying the locations of guttate and stroma formations.

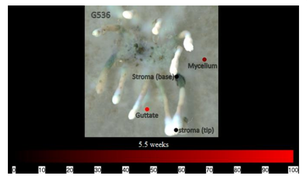

Figure 7. The spatial distribution indicating the relative intensities of griseofulvin on fungal culture *X. cubensis* (G536) for the stroma, mycelium, and guttates at 5.5 weeks.

Note: On Figures 6, 7, 9, 10, and 11, each circle represents a sampled location. The compound signal was most intense where the circles are the brightest, and the bottom bar indicates the relative amount of signal as measured via mass spectrometry.

Results: Co-Culture of *X. cubensis* & *P. restrictum*

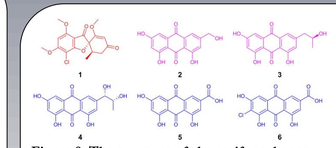

Figure 8. The structure of the antifungal agent, griseofulvin (1; red), from *Xylaria cubensis* (G536). The polyhydroxyanthraquinones **(2–6)** from *Penicillium restrictum* (G85) were grouped into two categories (purple and blue) based on their distributions in co-culture.

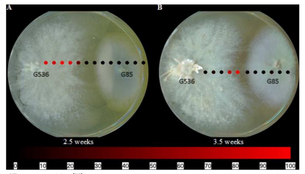

Figure 10. The spatial distribution of griseofulvin from *X. cubensis* (G536) grown in co-culture with *P. restrictum* (G85) at (A) 2.5 and (B) 3.5 weeks.

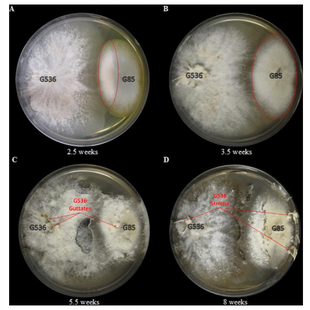

Figure 12. The visible discoloration of *P. restrictum* (G85) while in co-culture with the griseofulvin-producer, *X. cubensis* (G536), at (A) 2.5 weeks and (B) 3.5 weeks. The discolored regions of *P. restrictum* (G85) are circled in red. The visible expansion of *X. cubensis* (G536) in co-culture with *P. restrictum* (G85) at **(C)** 5.5 weeks and **(D)** 8 weeks. The guttates were attributed to *X. cubensis* (G536) due to the detection of griseofulvin. The stroma were attributed to *X. cubensis* (G536) since *P. restrictum* (G85) does not produce stroma.

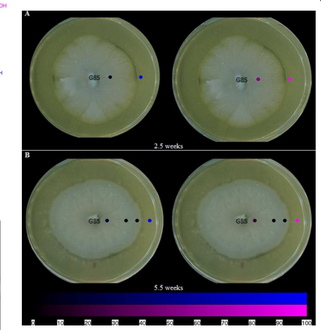

Figure 9. The spatial distribution of both groups of polyhydroxyanthraquinones on fungal isolates of *P. restrictum* at (A) 2.5 weeks and (B) 5.5 weeks. The color coding corresponds to the structures in **Figure 8**.

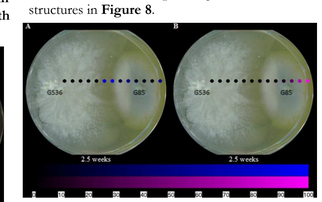

Figure 11. The spatial distribution of the (A) blue and (B) purple groups of polyhydroxyanthraquinones on fungal isolates of *P. restrictum* (G85) while grown in co-culture with *X. cubensis* (G536) at 2.5 weeks. The color coding corresponds to the structures in **Figure 8**.

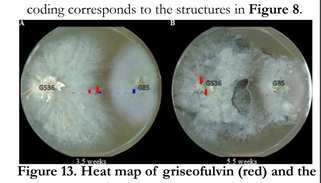

Figure 13. Heat map of griseofulvin (red) and the *P. restrictum* (G85) metabolites (blue group only; purple metabolites were undetectable) at (A) 3.5 weeks and (B) 5.5 weeks. The heights of the bars are relative to their intensity from the HRMS data.

Conclusions

- Through mass spectrometry mapping techniques, we were able to provide structural, spatial, temporal, and ecological functionality data on the secondary metabolites of three distinct organisms.
  - Specifically, the flowers of *A. triloba*, a previously unstudied organ, were found to be a rich source of analogues acetogenins.
  - The fungistatic ability of griseofulvin likely correlated to the concentration of the younger tissue of the fungus by which we hypothesize that *X. cubensis* gains a competitive advantage through spreading its mycelium throughout the host plant by inhibiting the growth of other fungi, thus ensuring a symbiotic relationship with the host.
  - As *X. cubensis* dramatically reduced the biosynthesis of two of the five polyhydroxyanthraquinones excreted by *P. restrictum* in the interaction zone between the two fungi, it is likely that *X. cubensis* stunts the growth of other fungi through inhibiting the production of secondary metabolites.
- We conclude that analysis of natural products by the SepQuant® *droplet*Probe™ coupled to an HPLC-HRMS/MS system represents a methodology to efficiently generate various types of data that are critical to the discovery, prioritization, and understanding of new bioactive chemical entities.

References

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Sica VP, El-Elimat T, Oberlies NH. In situ analysis of Asimina triloba (paw paw) plant tissues for acetogenins via the droplet-liquid microjunction-surface sampling probe coupled to UHPLC-PDA-HRMS/MS. *Anal Methods*. 2016;8(32):6143-6149.

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