A multimodal metabolomics approach using imaging mass spectrometry and liquid chromatography-tandem mass spectrometry for spatially characterizing monoterpene indole alkaloids secreted from roots

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Abstract Plants release specialized (secondary) metabolites from their roots to communicate with other organisms, including soil microorganisms. The spatial behavior of such metabolites around these roots can help us understand roles for the communication; however, currently, they are unclear because soil-based studies are complex. Here, we established a multimodal metabolomics approach using imaging mass spectrometry (IMS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to spatially assign metabolites under laboratory conditions using agar. In a case study using Catharanthus roseus, we showed that 58 nitrogen (N)-containing metabolites are released from the roots into the agar. For the metabolite assignment, we used 15N-labeled and non-labeled LC-MS/MS data, previously reported. Four metabolite ions were identified using authentic standard compounds as derived from monoterpene indole alkaloids (MIAs) such as ajmalicine, catharanthine, serpentine, and yohimbine. An alkaloid network analysis using dot products and spinglass methods characterized five clusters to which the 58 ions belong. The analysis clustered ions from the indolic skeleton-type MIAs to a cluster, suggesting that other communities may represent distinct metabolite groups. For future chemical assignments of the serpentine community, key fragmentation patterns were characterized using the 15N-labeled and non-labeled MS/MS spectra.

Key words: imaging mass spectrometry, liquid chromatography-tandem mass spectrometry, metabolomics, monoterpene indole alkaloid, secretion.

Introduction

The development of “omics” technologies has shed light on soil studies (Huang et al. 2019). Sequencing technologies have generated huge quantities of microorganisms’ genome/transcriptome data, thereby helping us understand different populations or behaviors in the soil (Brunel et al. 2020). Metabolome data are key to the understanding how plants communicate with organisms, e.g., how metabolites keep enemies from roots or attract beneficial organisms. Recent studies have shown that specialized metabolites alter rhizosphere microbiota (Huang et al. 2019; Lucke et al. 2020; Sugiyama 2019).

To date, metabolomics approaches have been poorly developed for soil studies. The soil chelates or degrades metabolites, which are released from roots. In addition, metabolite chemical composition and specificity can also exacerbate the development of such approaches. Sequencing technologies have been used to successfully annotate genomes or gene information from sequencing data using huge comprehensive databases; however, it is difficult for metabolomics to adequately assign metabolite information because of poor database resources. Thus, new approaches are required to understand how metabolites function in and around roots and what type of metabolites are released from the roots, even under experimental conditions.

Matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance-imaging mass spectrometry (MALDI-FTICR-IMS) is an important technology for the understanding of the spatial...
metabolome of plant tissue longitudinal or cross sections with ultra-high resolution and accuracy of ion peaks (Dong et al. 2020; Nakabayashi et al. 2017; Yamamoto et al. 2019). Matrix reagents are sprayed onto the sections to extract metabolites from surfaces. After this, mixed crystals of the matrix reagent and extracted metabolites are generated on these surfaces. Then, MALDI-FTICR-IMS analysis is conducted on the sections. Spatial signal intensities of metabolites are used for visualization, where higher and lower signal intensities reflect metabolite localization.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) chemically assigns specialized (secondary) metabolites (Aron et al. 2020; Nothis et al. 2018; Tohge et al. 2020; Xue et al. 2020). From analyses, structural information such as retention times, mass to charge (m/z) values of precursor and product ions, and fragmentation patterns are acquired. Metabolite identification using authentic standard compounds is recommended, but annotation using reported spectra or characterization using deconvoluted spectra is also important in identifying metabolites (Tsugawa et al. 2019).

Here, we developed a multimodal metabolomics approach using MALDI-FTICR-IMS and LC-MS/MS under experimental conditions, using agar. Even though metabolite behavior in agar is theoretically different from the soil, the generation of fundamental data will support field experiments. We used the medicinal plant Catharanthus roseus because this plant biosynthesizes monoterpene indole alkaloids (MIAs) (Nakabayashi et al. 2020; O’Connor and Maresh 2006; Yamamoto et al. 2019), the functions of which are currently unknown. The workflow comprised: 1) sectioning agar and roots; 2) performing MALDI-FTICR-IMS on sections; 3) performing LC-MS/MS on agar, roots, and leaf samples; and 4) reusing N-labeled and non-labeled metabolome data (Nakabayashi et al. 2020) for the chemical assignment of nitrogen (N)-containing metabolites, including MIAs. From this approach, we characterized 58 N-metabolites including MIAs from roots.

Materials and methods

Chemicals

Ajmalicine [Sigma-Aldrich Japan (Tokyo, Japan)], catharanthine [Sigma-Aldrich Japan (Tokyo, Japan)], serpentine [FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation, (Osaka, Japan)], and yohimbine [FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation, (Osaka, Japan)] were used in this study.

Plant materials and growth conditions

Catharanthus roseus (Equator White Eye, Sakata Seed Corporation) was used in this study. Fifty ml of Murashige and Skoog agar medium [FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation, (Osaka, Japan)] was prepared in plant boxes (125 ml, VWR, US). The agar samples were harvested from the boxes in which two plants were grown for three months under a log day (16h day/8h night) condition at 24°C. As a negative control, a box without the plants was incubated at the same time and was harvested. Leaves and roots of the plants were also harvested from the plants. All the samples were immediately lyophilized at −55°C. The lyophilized materials were stored at room temperature with silica gel. Four independent samples were analyzed in this study.

Preparing sections

For the IMS analysis, agar was cut with a razor, embedded with a compound (Surgipath FSC22: Leica Microsystems, Germany) and frozen in a −75°C acetone bath (Histo-Tek Pino; Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The frozen sample block was placed on a cryostat specimen disk and was cut with the knife blade until the desired tissue surface appeared. Transfer tape (Adhesive Tape Windows, Leica Microsystems, Germany) was placed on the face of the block to obtain sections (each with a thickness of 20µm) in the CM3050S cryostat (Leica Microsystems, Germany). Two sections were transferred to conductive Cu tape (double-sided, No. 796) (TERAOKA SEISAKUSHO, Co., Ltd.) on a glass slide (ITO coating, Bruker Daltonik GmbH). The section on the glass slide was freeze-dried overnight at −30°C in the cryostat.

MALDI-FTICR-IMS analysis

A 2,5-dihydroxybenzoic acid (DHB) matrix solution (15 mg ml−1 in 90% ACN, 0.1% TFA) was sprayed on the prepared section that was on the glass slide using TM-sprayer (HTX TECHNOLOGIES, LLC) running modified parameters (nozzle temperature, 60°C; pump device, LC pump; plate rate 0.125 ml min−1; z-arm velocity, 700 mm min−1; number of passes, 14; moving pattern, CC; track spacing, 3 mm) for a wet condition. The freeze-dried section with the matrix was analyzed in the FTICR-MS SolariX 7.0 T instrument (Bruker Daltonics Inc.). The MALDI parameters are as follows: geometry, MTP 384 ground steel; plate offset, 150 µm; deflector plate, 200 V; laser power, 40.0%; laser shots, 100; frequency, 2,000 Hz; laser focus, small; raster width, 30 µm. The MS conditions were as follows: mass range, m/z 100.33–500.00; average scan, 1; accumulation, 1,000 s; polarity, positive; source quench on; API high voltage, on; resolving power, 66,000 at 400 m/z; transient length, 0.4893 s; Mode (data storage: save reduced profile spectrum, on; reduced profile spectrum peak list, on; data reduction, 97%; auto calibration: online calibration, on; mode, single; reference mass, m/z 362.926338); API Source (API source: source, ESI; capillary, 4,500 V; end plate offset, −500; source gas tune: nebulizer, 1.0 bar; dry gas, 4.01 min−1; dry temperature, 180°C); Ion Transfer (source optics: capillary exit, 180 V; detector plate, 200 V; funnel 1, 150 V; skimmer 1, 10 V; funnel RF amplitude, 120 Vpp; octopole: frequency, 5 MHz; RF amplitude, 250 Vpp;
quadrupole: Q1 mass, 100.0 m/z; collision cell: collision voltage, –3.0 V; DC extract bias, 0.3 V; RF frequency, 2 MHz; collision RF amplitude, 600.0 Vpp; transfer optics: time of flight, 0.400 ms; frequency, 6 MHz; RF amplitude, 200.0 Vpp; Analyzer (infinity cell: transfer exit lens, –20.0 V; analyzer entrance, –10.0 V; side kick, 0.0 V; side kick offset, –1.0 V; front trap plate, 0.700 V; back trap plate, 0.550 V; sweep excitation power, 12.0%; multiple cell accumulations: ICR cell fills, 1).

Visualizing IMS data
Visualization was performed using ScilS Lab software version 2019c (Bruker Daltonik GmbH, Bremen, Germany).

Metabolite extraction for LC-MS/MS analysis
The freeze-dried agar samples (each, ~700 mg DW) were extracted with 4.8 ml of 80% MeOH using a mixer (EYELA CUTE MIXER CM-1000) at 1,500 rpm for 7 min. After centrifugation with the following conditions (force, 13,000 g; running time, 10 min; and temperature, 4°C), 3.6 ml of supernatant was dried up. Extracts were resolved with 3 ml of 2.5% MeOH. Extraction solvents were applied to HLB cartridges (3 cc, Waters) after equilibration according to the protocol. The cartridges were washed with 3 ml of 0.1% acetic acid twice, and then were extracted with 3 ml of 90% MeOH. The elution solvents were concentrated and dried up completely. The extracts were resolved with 100 µl of 80% MeOH including 2.5 µM lidocaine and then were filtered through Ultrafree MC centrifugal filter, Millipore.

Extraction of leaf and root samples was performed according to the previous research (Nakabayashi et al. 2020).

LC-MS/MS analysis
The analysis was performed according to the previous research (Nakabayashi et al. 2020). Extracts (1 µl) were analyzed using LC-QTOF-MS instrument (LC, Waters Acquity UPLC system; MS, Waters Xevo G2 Q-Tof). Analytical conditions were as follows: LC: column, Acquity bridged ethyl hybrid C18 (1.7 µm, 2.1 mm × 100 mm, Waters); solvent system, solvent A (water including 0.1% formic acid) and solvent B (acetonitrile including 0.1% formic acid); gradient program, 99.5%A/0.5%B at 0 min, 99.5%A/0.5%B at 0.1 min, 20%A/80%B at 10 min, 0.5%A/99.5%B at 10.1 min, 0.5%A/99.5%B at 12.0 min, 99.5%A/0.5%B at 12.1 min, and 99.5%A/0.5%B at 15.0 min; flow rate, 0.3 ml/min at 0 min, 0.3 ml/min at 10 min, 0.4 ml/min at 10.1 min, 0.4 ml/min at 14.4 min, and 0.3 ml/min at 14.5 min; column temperature, 40°C; MS detection: polarity, positive; capillary voltage, +3.00 kV (positive); cone voltage, 25.0 V; source temperature, 120°C; desolvation temperature, 450°C; cone gas flow, 501/h; desolvation gas flow, 8001/h; collision energy, 6 V; mass range, m/z 50–1500; scan duration, 0.1 s; interscan delay, 0.014 s; data acquisition, centroid mode; lockspray, leucine enkephalin; scan duration, 1.0 s; interscan delay, 0.1 s. MS/MS data was acquired as the following analytical conditions: (1) MS: polarity, positive; mass range, m/z 50–1500; scan duration, 0.1 s; interscan delay, 0.014 s; data acquisition, centroid mode and (2) MS/MS: polarity, positive; mass range, m/z 50–1500; scan duration, 0.02 s; interscan delay, 0.014 s; data acquisition, centroid mode. In this mode, MS/MS spectra of the top 10 ions (>1,000 counts) in an MS scan were automatically obtained. If the ion intensity was less than 1,000, MS/MS data acquisition was not performed and moved to next top 10 ions.

Data analysis for N-metabolites
The 15N-labeled and non-labeled metabolome data acquired in the previous study (Nakabayashi et al. 2020) were used for assignment of N-metabolites. Using the data, ions derived from N-metabolites were assigned to the current data with the following conditions (retention time, ≥0.2 min; m/z value, ±0.01) (Supplementary Table S1).

Alkaloid network analysis
MS/MS similarities were calculated using dot product algorithm (Tsigawa et al. 2019). The similarities more than 0.8 was used for the network. The ions were clustered using spinglass algorithm of R (https://igraph.org/r/doc/cluster_spinglass.html). Parameters on gamma and spins were changed to 1.5 and 200, respectively. Parameters on the network are available in Supplementary Data S1. Visualization of the network was performed using the PlaSMA database (http://plasma.riken.jp/).

Results and discussion
To detect root-secreted metabolites, MALDI-IMS or a combination approach of MALDI-IMS and LC-MS/MS was developed previously (Sasse et al. 2020; Veličković et al. 2020). In this study, we developed a multimodal metabolomics approach using MALDI-FTICR-IMS and LC-ESI-QTOF-MS/MS. To have higher spatial and peak resolution, the IMS analysis requires a certain time for ion accumulation to detect MS spectra. For acquiring MS/MS spectra, it requires extra time. It is therefore not easy to acquire both MS and MS/MS spectra with higher spatial and peak resolution in a high-throughput way. To supplement this point, we combined the IMS analysis with the LC-MS/MS analysis to consider MS and MS/MS spectra with retention times. In IMS and LC-MS/MS analysis, common adduct ions were detected even with MALDI and ESI ionization modes (Nakabayashi et al. 2017). Previous studies revealed that plants secrete a wide range of specialized metabolites including alkaloids (Korenblum et al. 2020; Strehmel et al. 2014). A characterized role of harmine in soil communications (Walker et al. 2003) suggests that MIAs secreted from roots of Catharanthus roseus play roles in the communications. However, because of the complexity of MS/MS fragmentation pattern and the lack of authentic standard compounds, it had been difficult to characterize
MIAs in MS-based metabolomics. By using the previously reported data as reference (Nakabayashi et al. 2020), this approach realized both spatial and qualitative analysis in a high-throughput way.

Plants were grown in plant boxes for three months. Agar around the roots was cut away from the box (Figure 1A). In this study, we used a part of the roots (0–3 mm depth from the surface of agar). Cross sections, including the agar and the roots, were prepared using a cryostat. Sections were then placed on conductive tape using transfer tape (Nakabayashi et al. 2019). As shown in Figure 1B, the roots were placed in the center. To consider spreading out metabolites from the root during spraying a matrix reagent, the TM sprayer was selected for a much shorter spraying time. The sprayer generated fine-grained mixed crystals of the reagent and metabolites from the root. A larger crystal generated incorrect localization in highly spatial resolution analysis. The spatial resolution was set at 30 µm. IMS analysis was performed in approximately 3 mm² areas to detect metabolites. Thus, ions from root metabolites were detected at approximately 500 µm around the root (Figure 1C).

To chemically assign MIAs, agar samples were analyzed using LC-MS/MS together with leaf and root samples. In addition, agar, where plants were never grown, was analyzed as a negative control. A comparative analysis using both agar samples suggested that large quantities of MIA metabolites are secreted from this. Ions from the LC-MS/MS data were matched using conditions (retention time, ±0.2 min; m/z value, ±0.01) to the previous data of 15N-labeled and non-labeled plants (Nakabayashi et al. 2020). After this, ions detected by IMS and LC-MS/MS were paired with a filter condition (m/z value, ±0.005). Finally, LC-MS/MS-based 58 ions were characterized as N-metabolites [Figure 2A and Supplementary Table S1]. In consideration of overlapping m/z values, of which, 28 ions could be visualized using the IMS data (Supplementary Figures S1). The IMS analysis showed different localization patterns even under the experimental conditions using agar. Most of the ions were extensively detected around the root, but the ions at m/z 337.191, 397.212, 409.211, 413.207, 423.191, and 427.222 were detected close to the root. These localizations suggest that N-metabolites have specific spatial behaviors outside of the root. An ion at m/z 349.154 was detected in the agar and root samples. This had no other ions detected at the same m/z value. To discuss the localization of a metabolite, this was the best candidate for identification. Observed m/z value suggested serpentine providing precursor ion as [M+H]+ at m/z 349.1546 in positive ion mode. Using the authentic standard compound, we identified as the ion is derived from serpentine. In addition, three ions were identified as derived from ajmalicine, catharanthine, and yohimbine. Including those, 16 ions were derived from MIAs, which were characterized in the previous study, as having an indolic skeleton. To characterize structural features, an alkaloid network analysis was performed using both MS/MS similarity network and spinglass cluster detection algorithms. Communities 1 to 4 were connected on the basis of similarity scores. Nodes that have similarity scores less than 0.8 were classified to the community named Isolated. Detailed information of nodes, edges, and communities was provided in Supplementary Data S1. The analysis generated five communities as upper layer and MS/MS similarity network as lower (Figure 2B, Supplementary Table S1). The communities were generated based on MS/MS similarities. When nodes in a cluster had a similarity to others in another community, both communities were connected as shown (Figure 2B, upper). Interestingly, ions derived from identified MIAs were included in community 1, suggesting the cluster represented indolic skeleton-type MIAs. The ion derived from serpentine was included in community 3. All ions except for the serpentine ion were uncharacterized (Figure 2B, lower). Because their MS/MS patterns were similar to each other, a deconvoluted serpentine pattern will be useful for further analysis. Here, a fragmentation pattern was...
elucidated using $^{15}$N-labeled and non-labeled MS/MS spectra (Figure 2C). By considering mass shifts from labeling, we successfully characterized fragmented bonds leading to substructures. The information of the substructures will be exploited to determine the structure of serpentine-like alkaloids.

Plants secrete various metabolites from their roots. Primary metabolites such as carbohydrates and organic acids and specialized metabolites are also secreted, including glucosinolates from *Arabidopsis thaliana*, isoflavones from *Glycine max*, and momilactones from *Oryza sativa* (Baltes and Voytas 2015; Sugiyama et al. 2016). Secreted metabolites play various roles, e.g., nutrient uptake (Rengel and Marschner 2005), defense against pathogens and/or other plants (Berendsen et al. 2012), and the regulation of rhizosphere microorganisms (Berendsen et al. 2012). Some studies reported on root exudates containing MIAs; the hairy roots of *Catharanthus* secrete several MIAs including ajmalicine, serpentine, and catharanthine into the medium (Monribot-Villanueva et al. 2015; Ruiz-May et al. 2009). Interestingly, endophytic infection induces ajmalicine and serpentine biosynthesis in roots of *Catharanthus*, suggesting that these MIAs have roles in rhizosphere interactions with microorganisms (Etalo et al. 2018).

In this study, we established the multimodal metabolomics approach using MALDI-FTICR-IMS and LC-MS/MS to profile MIAs secreted from *Catharanthus* roots under experimental conditions using agar. This approach identified 58 $^N$-containing metabolites from the roots. Although the functions of these metabolites are still unknown, these data will facilitate more comprehensive soil/root studies. Four MIAs—ajmalicine, catharanthine, serpentine, and yohimbine—were identified using authentic standard compounds. Applying the MIAs to the soil characterizes the alternation of the microbiota. A comparative analysis using mutants lacking MIAs (using genome editing technologies) will uncover functional profiles and relationships with microbiota. Combining this approach with other omics...
approaches will help us understand how these plants alter soil microbiota via MIAs.

**Author Contributions**
RN designed the research. RN, NT-K, TM, TN, and KT prepared the samples. RN and TM acquired and analyzed the metabolome data. YY performed the alkaloid network analysis. RN, MU, and KS discussed the research. RN wrote the manuscript.

**References**