RESEARCH ARTICLE

Symbiosis of Legumes and Soil Bacteria Explored by Single Cell Mass Spectrometry

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Received: 29/08/2022 **Accepted (online first):** 23/10/2022 Vol./Issue/Year: 3(1), 2022

Competing interests: Author(s) stated no compete of interest. **Edited by**: Lowy, A.D., Mátyás, B.

How to cite: *Vertes, A. "Symbiosis of Legumes and Soil Bacteria Explored by Single Cell Mass Spectrometry" DRC Sustainable Future 2022, 3(1): 33-40, DOI: 10.37281/DRCSF/3.1.4*

ABSTRACT

Biological nitrogen fixation, a highly beneficial process responsible for improved plant health and crop production, is performed by bacteroids transformed from rhizobia in the root nodules of legumes. Infected and uninfected cells in the root nodules of soybean (*Glycine max*) and symbiont soil bacteria, e.g., *Bradyrhizobium japonicum,* are interspersed at a cellular level. To explore the metabolic differences between these cellular phenotypes, and plant cells in general, new single-cell mass spectrometry methods have been developed. Here we describe three emerging methods, optical fiber-based laser ablation electrospray ionization, laser desorption ionization on silicon nanopost arrays, and capillary microsampling electrospray ionization for the mass spectrometry of plant cells. These new techniques enable the capture of cellular heterogeneity, and the discovery of metabolic states and hidden cellular phenotypes in symbiotic plant-microbe systems performing biological nitrogen fixation.

Keywords: nitrogen fixation, soil, bacteria, spectrometer, Glycine max, soybean, Bradyrhizobium japonicum, electrospray.

1. Introduction

At the beginning of the twentieth century, access to water-soluble nitrogen compounds became crucial for both agricultural (fertilizers) and military (explosives) applications. The main source of these compounds at the time was sodium nitrate ($NaNO_3$), available mostly in the form of Chile saltpeter mined in the desert salt flats of Chile (Cisternas and Galvez 2014). As nitrates were a main component of explosives, they had a major impact around the outbreak of World War I, when the British prevented German shipments of Chile saltpeter (Benbow 2008). Around the same time, in Germany, Fritz Haber developed a process for converting nitrogen gas from air into water-soluble ammonia. This great advance in synthetic chemistry relied on a quite simple process. At 200 atm and 450 °C in the presence of an iron catalyst, nitrogen and hydrogen gas combine to form ammonia (Erisman, Sutton *et al.* 2008). Ammonia synthesis has triggered many positive changes, but also very dark and negative events in the world. On one hand, it led to the development and production of synthetic fertilizers on an industrial scale. On the other, by converting ammonia to nitrates via catalytic oxidation, it enabled the production of various explosives used on a large scale in World War I. With synthetic fertilizers farmers achieved high crop yields to supply food for a lot more people. In ~1900, before the industrial scale production of

ammonia, there had been ~1.6 billion people living on earth, a number that jumped to ~7.7 billion by 2019 through a century of exponential population growth (see Figure 1). Another measure for the impact of this discovery is that today 80% of the nitrogen atoms found in the proteins of the human body comes from the Haber process (Howarth 2008). These momentous changes, however, come at a price. Fertilizer production involves significant energy consumption, and the overuse of synthetic fertilizers has a negative environmental impact.

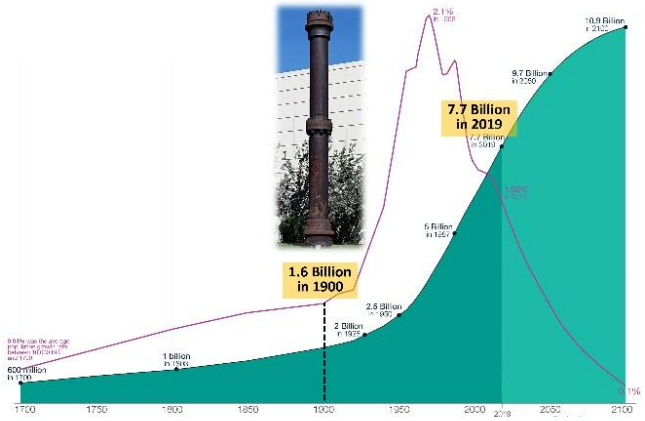

Figure 1. Introduction of synthetic fertilizers, based on Haber process, contributed to 20-th century population explosion. Inset shows high pressure reactor for ammonia synthesis from 1921. (Figure derived from plot by Max Roser and image by Drahkrub, licensed under CC-BY-SA.)

In this paper, we focus on biological nitrogen fixation (BNF), an elegant alternative to the Haber process created by nature that eliminates many of the negative consequences associated with the industrial process (Vicente and Dean 2017). In nature, nitrogen fixation from air is a process that takes place under much milder conditions, i.e., at ambient temperature and pressure. Rhizobium soil bacteria, such as *Bradyrhizobium japonicum*, live on the roots of legumes and by infecting the plant they induce the formation of root nodules, where in a symbiotic process nitrogen from air is converted into ammonia using the nitrogenase enzyme (Itakura, Saeki *et al.* 2009). Based on this process, legumes self-fertilize by receiving as much soluble nitrogen compounds as needed at the place of their utilization. Thus, there is no negative environmental impact, and there is no excess energy needed to drive the production of ammonia. The desire to devise broader applications of BNF prompted us to take a closer look at the symbiotic relationship with cellular resolution using the new techniques explained in the next section.

2. Methods

2.1 Analysis of individual plant cells by laser ablation electrospray ionization

Over a decade ago, optical fiber-based laser ablation electrospray ionization (f-LAESI) has been developed in our laboratory for the analysis of single cells (Shrestha and Vertes 2009). A mid-IR (λ = 2.94 μm) laser pulse is delivered to the selected cell via a sharpened optical fiber for ablation sampling of the cell content. The generated plume is intercepted by an electrospray for efficient ionization. The produced ions are analyzed by high-performance mass spectrometry (MS) for the identification and relative quantitation of cell metabolites. The f-LAESI-MS method analyzes single cells in situ, i.e., in their original tissue environment, with minimal to no sample preparation.

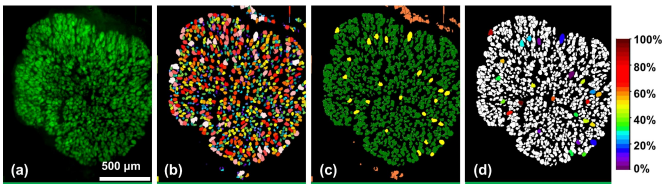

Figure 2. (a) Fluorescence image of infected cells in soybean root nodule section. (b) Image segmentation identifies cells as objects for morphometric analysis. (c) Larger than average cells are selected for analysis by f-LAESI-MS. (d) Relative abundances of jasmonic acid in these cells are represented on a false color scale. Adapted with permission from (Stopka, Wood et al. 2021). Copyright 2021 American Chemical Society.

Recently, combining automated cell targeting, enabled by the analysis of brightfield and fluorescence microscopy images, and f-LAESI-MS resulted in a high-throughput technique to study the metabolic heterogeneity of cell populations (n > 1000 cells) (Stopka, Wood *et al.* 2021). Morphometric analysis or fluorescence labeling of the cells in the segmented images can help to pinpoint rare cells for analysis. For example, the fluorescence image of the infected cells in a soybean root nodule section (see Figure 2a) can be segmented to identify the centroids of individual cells (see Figure 2b), followed by morphometric analysis to pinpoint the larger than average cells (see Figure 2c). These cells can be analyzed by f-LAESI-MS for the abundances of multiple metabolites. As an example, Figure 2d shows the relative intensity of jasmonic acid ions on a false color scale detected from the larger than average cells. Combining f-LAESI with 21 T Fourier transform ion cyclotron resonance (21T-FTICR) MS yielded the first demonstration of capturing isotopic fine structures (IFSs) for compounds in single cells (Samarah, Khattar *et al.* 2020). This unique system helped to identify 47 known compounds and to discover the elemental formula of 11 unknown chemical species in these cells.

2.2 Laser desorption ionization from silicon nanopost arrays

The efficiency of ion production from certain biooligomers can be greatly enhanced by a nanophotonic ionization technique we have introduced over a decade ago (Walker, Stolee *et al.* 2010). Nanofabricated silicon nanopost arrays (NAPAs) can serve as a substrate for

laser desorption ionization (LDI) both in local MS analysis and MS imaging (MSI) mode (Stopka, Rong *et al.* 2016). To determine the distribution and degree of polymerization of polyhydroxybutyric acid (PHB), a carbon and energy storage polymer for the bacteroids, in soybean root nodules, we used NAPA-LDI-MS imaging. The photonic interaction of the laser pulse and the nanostructure caused the ionization of PHB and other metabolites present in the sample and the generated ions were recorded by MS (Samarah, Tran *et al.* 2021).

2.3 Capillary microsampling electrospray ionization for single cell analysis by mass spectrometry

Capillary microsampling electrospray ionization (ESI) MS had been used to probe the composition of single human (Zhang and Vertes 2015) and plant cells (Zhang, Foreman *et al.* 2014) for relatively low cell numbers. Here, we have adapted this technique to sample the xylem sap and the content of single parenchymal cells from live soybean plants for ESI-MS (Samarah, Tran *et al.* 2020). For this project, the capillary is drawn to a sharp tip with its shape optimized for sap sampling or penetration into a plant cell through its sturdy wall. The collected sample is ionized by ESI and the produced ions are analyzed by MS. Analysis of the xylem sap is performed for both uninfected plants and plants infected by rhizobia, and the spectra are processed by multivariate statistical analysis.

3. Results

3.1 Analysis of individual plant cells by laser ablation electrospray ionization mass spectrometry

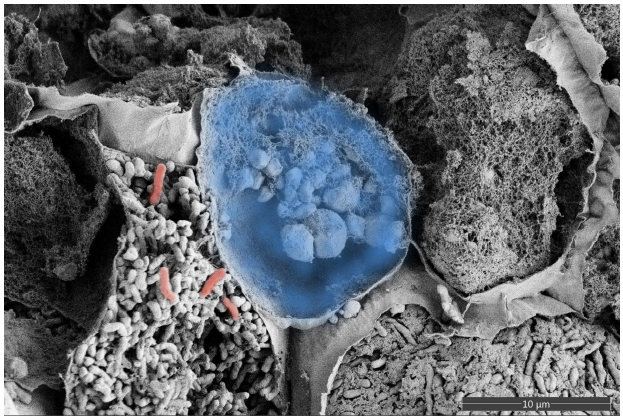

Figure 3. Colorized scanning electron micrograph of infected and uninfected (blue) plant cells and bacteroids (red) in soybean root nodule. Reprinted with permission from (Dolatmoradi, Samarah *et al.* 2022). Copyright 2022 Wiley-VCH GmbH.

In the infection zone of soybean root nodules, infected and uninfected plant cells are interspersed. The electron micrograph in Figure 3 reveals that the bacteria in the infected cells multiply until they completely fill the inside of the plant cell. Uninfected plant cells retain their internal structure. As a result of the interaction with the plant cell they inhabit, the bacteria are transformed into bacteroids that lose their ability to move and divide. These bacteroids perform BNF in a symbiotic relationship with the plant, where the former supply nitrogen containing compounds in exchange for carbon and energy sources produced by the latter.

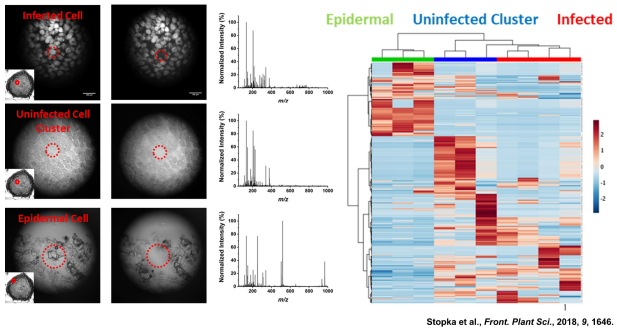

Figure 4. (Left panel) Targeting and laser ablation sampling of infected, uninfected, and epidermal cells in soybean root nodule. (Middle panel) Mass spectra of the three cell types. (Right panel) Hierarchical cluster analysis separates peak combinations specific to cell types.

To understand and separate biochemical processes in the two cell states, individual infected cells or small homogeneous uninfected cell clusters must be analyzed. The image pairs in the left panel of Figure 4 show the selective sampling of infected cells (top), uninfected cell clusters (middle), and epidermal cells (bottom) by optical fiber-based mid-IR laser ablation. The infected cells are visualized by fluoresce microscopy because the rhizobia used for infection are labeled with green fluorescent protein, whereas the uninfected and epidermal cells are imaged in the brightfield mode. Infected cells and the epidermal cells on the outside of the nodule were analyzed individually, and as they were much smaller, small groups of uninfected cells were examined. The f-LAESI mass spectra corresponding to the three cell types (see the middle panel in Figure 4) exhibit significant differences in the presence and abundance of metabolite-related peaks. After performing hierarchical cluster analysis of these spectra, the separation of cell-type specific peak subsets is observed (see the right panel in Figure 4). The heat map helps to identify metabolite combinations characteristic of infected, uninfected, and epidermal cells (Stopka, Khattar *et al.* 2018). Further refinement and validation of the cell-type related peaks can result in the identification of biomarkers. This early version of f-LAESI-MS required the manual selection of individual cells for targeting and analysis thereby limiting its capabilities to low cell numbers.

To get a wider understanding of cellular heterogeneity in seemingly homogeneous cell populations, automated cell targeting, and analysis had to be implemented.

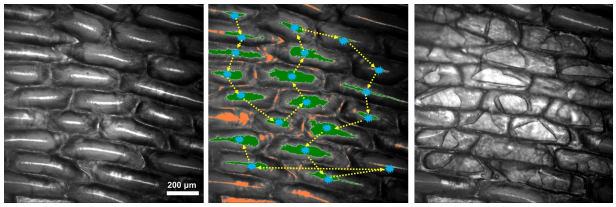

Figure 5. Brightfield image of onion bulb epidermal cells before sampling (left), determination of cell centroids and sampling trajectory by image analysis (middle), and confirmation of cell-selective laser ablation sampling (right). Adapted with permission from (Stopka, Wood *et al*. 2021). Copyright 2021 American Chemical Society.

Automation and testing of this capability was applied to onion bulb epidermis made of a single layer of cells. Figure 5 shows the individual cells in the onion epidermis used for method development (Stopka, Wood *et al.* 2021). The brightfield image of the epidermis reveals individual cells in the tissue (left panel in Figure 5). Image analysis and morphometry results are used to identify the cell centroids and the planned sampling trajectory (middle panel in Figure 5). Brightfield image taken after the analysis confirms successful sampling of the selected cells and the lack of co-sampling adjacent cells (right panel in Figure 5). In the sampling process, cell content is ejected, the formed plume is ionized by the electrospray, and the mass spectrum of the produced ions is recorded.

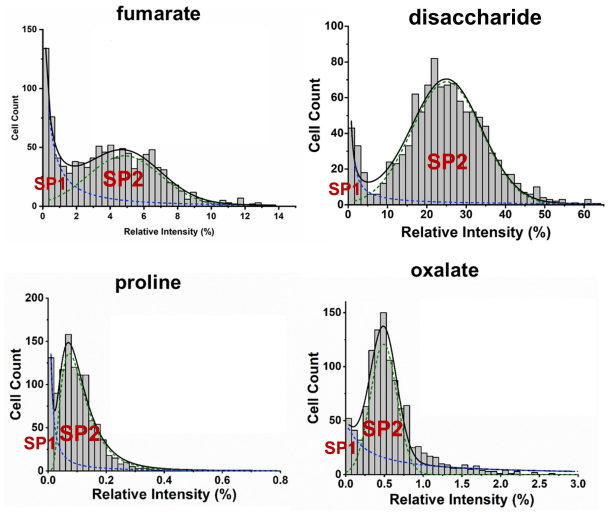

Figure 6. f-LAESI-MS analysis of a large number (n > 1000) of individual onion epidermal cells allows accurate determination of numerous metabolite abundance distributions. Fumarate, disaccharide, proline, and oxalate shows bimodal distributions, whereas other components exhibit normal or lognormal distributions (not shown). Adapted with permission from (Stopka, Wood *et al.* 2021). Copyright 2021 American Chemical Society.

The automated process enabled the fLAESI-MS analysis of over 1000 onion bulb epidermal cells and the determination of cellular metabolite level distributions. These distributions characterize the differences in cellular metabolism, i.e., the metabolic heterogeneity in the cell population. Because cell function is influenced by many stochastic external and internal factors, one would expect metabolite levels in the cells of a given population to follow a normal or Gaussian distribution. (This is a consequence of the central limit theorem.)

However, this is not the case for all metabolites. While many metabolites exhibit normal or lognormal distributions with single maxima, there are some with bimodal abundance distributions. For example, when disaccharide levels in onion epidermal cells are examined, a bimodal distribution is observed (see Figure 6). There is a subpopulation with low disaccharide levels (SP1), and there is another subpopulation in which this metabolite appears at higher levels (SP2). Similar bimodal distributions are found for some other metabolites, such as oxalate, proline, and fumarate.

According to these findings, the cells present in this plant tissue fall into two hidden phenotypes. One in which certain metabolites show low abundance and another in which they are present in higher concentrations.

Such differences are well known in human populations, where metabolically distinct subpopulations, e.g., diabetics with higher average blood glucose levels than the non-diabetic population, can exist. Similarly, different metabolic states might occur in plant cell populations, resulting in differences in the average levels of certain metabolites.

3.2 Discovery of unknown compounds in single cells by capturing isotopic fine structure

Mass spectra of biological samples in the low mass range often contain a large number (up to 70%) of unidentified peaks, sometimes called the “dark metabolome.” This reflects the existence of numerous metabolites not yet discovered. Determination of isotopic fine structures (IFSs) is an important tool for identifying the elemental compositions and molecular formulas of such unknown compounds. Because of its ultrahigh performance (mass resolution and mass accuracy), Fourier transform ion cyclotron resonance (FTICR) MS is the method of choice to capture IFSs of unknown compounds. Combining a top-of-the-line 21 tesla (21T) FTICR-MS with a LAESI ion source enabled the determination of IFSs for >70 ions directly from biological tissues (Stopka, Samarah *et al.* 2019). Implementing an f-LAESI ion source on the 21TFTICR-MS yielded an instrument configuration capable of capturing IFSs in single cell analysis mode. Figure 7 displays the targeting and ablation of an infected

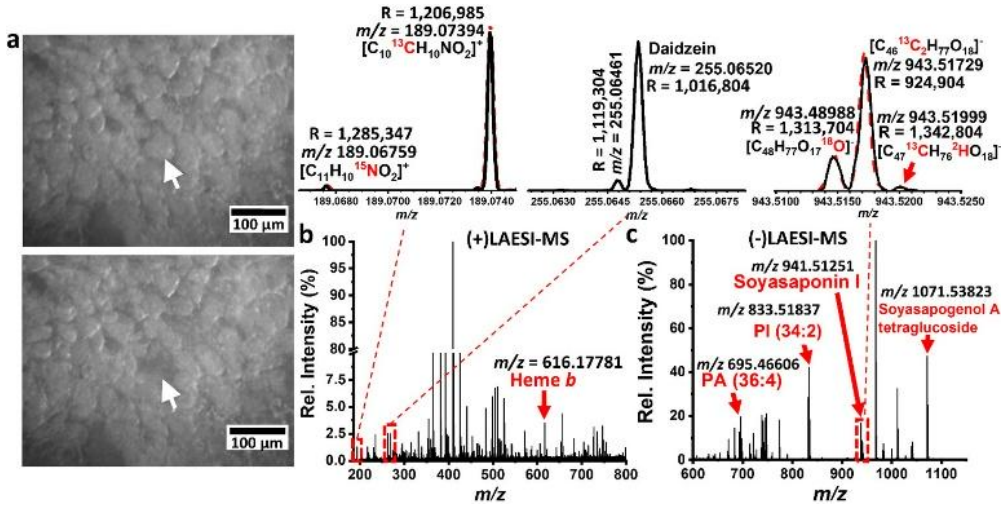

Figure 7. Determination of metabolite elemental formulas from a single infected root nodule cell by f-LAESI-21T-FTICR. (a) Selection (top image) and laser ablation sampling (bottom image) of a single infected root nodule cell. Determination of isotopic fine structures from (b) positive and (c) negative ion spectra afforded by the ultrahigh mass resolution and mass accuracy of the 21T-FTICR-MS. Reprinted with permission from (Samarah, Khattar *et al.* 2020). Copyright 2020 American Chemical Society.

soybean root nodule cell, its mass spectra in positive and negative ion modes, and some associated IFSs (Samarah, Khattar *et al.* 2020). Ultrahigh mass accuracy and resolution help with the identification of known metabolites with close-to-isobaric alternatives. For example, they can distinguish between [histidine + K]+ at m/z 194.03318 and [phosphocreatine + H H_2O]+ at m/z 194.03307 that differ by only 0.11 mDa. Determination of IFSs can help the determination of elemental formulas for previously unknown metabolites. fLAESI21TFTICR-MS examination of single soybean root nodule cells infected with B. japonicum allowed the high-certainty identification of 47 previously known compounds. More interestingly, the elemental composition of 11 previously unknown compounds could also be determined.

Our measurements on 124 single infected cells revealed bimodal abundance distributions for NAD+, hexose phosphate and acetylcarnitine. To rationalize the presence of the two subpopulations with low and high NAD+ concentrations we hypothesized that they represented plant cells harboring proliferating bacteria and dormant bacteroids, respectively. As the bacteria multiply in the plant cells, they eventually fill the cell volume, transform into bacteroids, exit the cell cycle, and enter into a resting (quiescent) state (G0). At rest, metabolism is slower, as in this state cell division and migration cease, and much less synthesis is required. This might explain why NAD+ shows lower average concentrations in some cells and higher abundances in others.

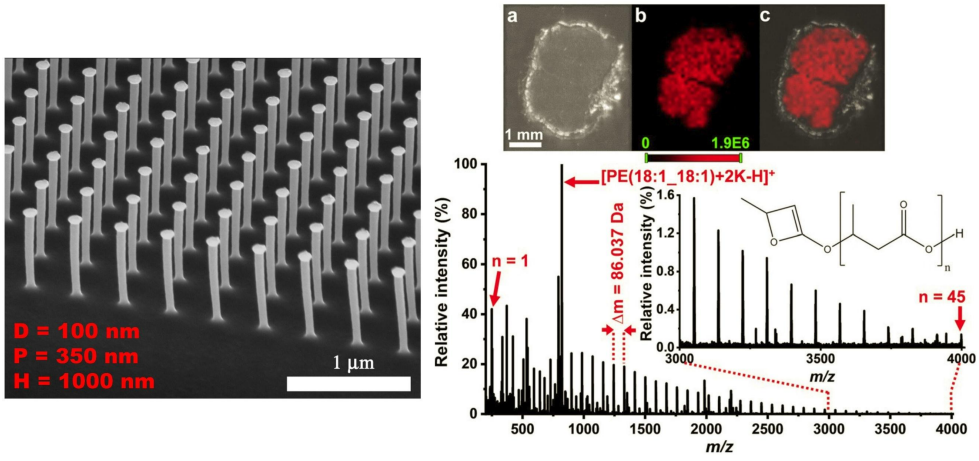

Figure 8. (Left panel) Scanning electron micrograph of a silicon nanopost array with optimized geometry. Adapted with permission from (Walker, Stolee *et al.* 2010). Copyright 2010 American Chemical Society. It can serve as a substrate for NAPA-LDI-MSI (Stopka, Rong *et al.* 2016). (Right panel) Mass spectrum collected from a soybean root nodule tissue section by NAPA-LDI reveals the presence of PHB oligomers with $1 \leq n \leq 45$ repeat units. The inset shows the PHB structural formula. At the top, (a) optical image of the tissue section, (b) distribution of the n = 9 oligomer in the tissue, and (c) the combined image. Reprinted with permission from (Samarah, Tran *et al.* 2021). Copyright 2021 Wiley-VCH GmbH.

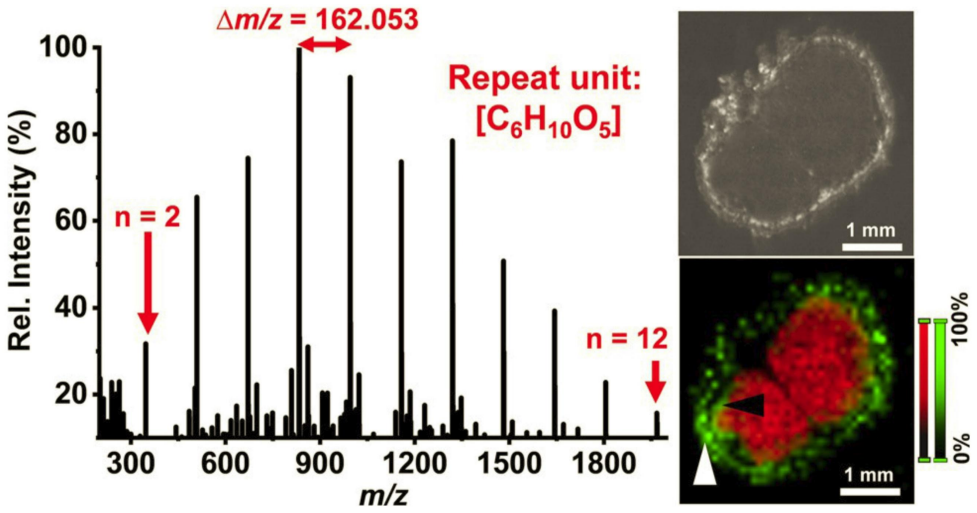

Figure 9. (Left panel) NAPA-LDI mass spectrum from soybean root nodule cortex shows the presence of oligohexoses with $2 \leq n \leq 12$ repeat units. (Right panel) At the top, optical image of the tissue section, and at the bottom combined chemical image of the n = 7 oligohexose and the n = 11 PHB oligomer. Reprinted with permission from (Samarah, Tran *et al*. 2021). Copyright 2021

3.3 Mass spectrometry imaging of bio-oligomer distributions

While there are several analytical methods for mapping the distributions of heteropolymers, such as proteins or peptides, in biological tissues, there are relatively few methods for the molecular imaging of homopolymers, e.g., amylose or amylopectin. In soybean root nodules, a specific homopolymer, polyhydroxybutyric acid (PHB, $H(C_4H_6O_2)nOH$), is produced by the bacteroids to store carbon and energy. Whereas matrix-assisted LDI (MALDI) is routinely used for MSI of proteins and lipids, its performance for the imaging of PHB distributions is limited by low ion yields. The matrix-free NAPA-LDI-MSI technique, based on optimized silicon nanostructure substrates (Walker, Stolee *et al.* 2010) (see left panel in Figure 8), offers molecular coverage complementary to MALDI-MSI (Fincher, Korte *et al*. 2020). Taking advantage of this complementarity, we have developed new methods for the MSI of PHB and other homopolymers, including oligosaccharides and polyglutamic acid oligomers (Samarah, Tran *et al.* 2021). The right panel in Figure 8 shows the NAPA-LDI mass spectrum from a soybean root nodule section with a prominent series of peaks corresponding to PHB oligomers where the number of detected repeat units, n, is in the $1 \leq n \leq 45$ range. At the top of the right panel (a) an optical image of the tissue section, (b) a chemical image for the n = 9 oligomer of PHB at m/z 899.3353, and (c) the combination of the optical and chemical images are depicted. Spatial distributions for all the detected PHB oligomers can be determined by NAPALDIMSI. This method can also be used to explore the variations in PHB polydispersity within the nodule.

3.4 Xylem sap analysis by capillary microsampling electrospray ionization mass spectrometric

Long-distance transport of nutrients in legumes relies on the xylem and phloem organized into vascular bundles. To explore the transfer of nitrogen containing compounds produced in the root nodules to the rest of

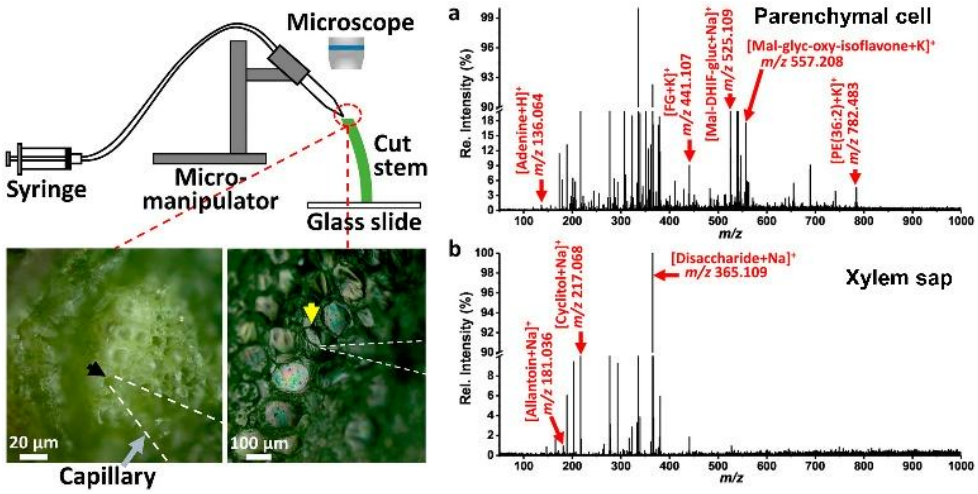

Figure 10. Capillary microsampling of xylem sap and single parenchymal cells in soybean stem (left panel) and associated ESI mass spectra (right panel). Adapted with permission from (Samarah, Tran *et al.* 2020). Copyright 2020 American Chemical Society.

the plant, we have implemented capillary microsampling of the xylem sap and single parenchymal cells for ESIMS analysis (Samarah, Tran *et al.* 2020).

The microcapillary, drawn to a sharp tip with ~5 μm opening, is either touched to the end of the xylem to extract ~1 pL of sap, or it is inserted into a selected parenchymal cell to aspirate a portion of the cell contents. By selectively sampling and analyzing the xylem sap and comparing their composition for soybean plants infected or uninfected by rhizobia, compounds participating in the transport of nitrogen containing compounds from the roots to the rest of the plant are identified. Xylem sap obtained from infected plants contained higher concentrations of metabolites derived from BNF, such as allantoin, allantoic acid, hydroxymethylglutamate, and methylene glutamate.

4. Conclusions

In summary, we have developed and applied three new methods based on dedicated forms of MS for the analysis of single cells and xylem sap in the mutualism of legumes and rhizobia. These new methods enabled us to study various aspects of this symbiosis and the related metabolism with unprecedented single cell granularity. We hope that the knowledge gathered in this way can help to extend BNF to plants that are currently not capable of producing their own nitrogen fertilizers (Vicente and Dean 2017).

Acknowledgement

The author thanks several generations of students and postdocs, who have participated in his research team and conducted the experiments described in this article. We are also grateful to our collaborators, Gary Stacey and Beverly Agtuca at the University of Missouri, and Christopher Anderton, Ljiljana Paša-Tolić, and David Koppenaal at the Pacific Northwest National Laboratory. Parts of this work was funded by the U.S. Department of Energy, Office of Biological and Environmental Research under award number DESC0013978, and by the U.S. National Science Foundation Plant Genome Program under award number IoS1734145.

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