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Mass Spectrometry Method Development and Application to Investigate Plant-Microbe and Microbiome-Host Symbiosis

By

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Chapter 10

Investigation of Symbiotic Relationships through Mass Spectrometry: Application to Plant/Bacteria Symbiosis

Written for the Wisconsin Initiative for Scientific Literacy in order to describe this thesis for a broader audience.

Keywords: Symbiosis, *Medicago truncatula*, microbiome, metabolites, peptides

Symbiotic relationships

The diversity of life forms on Earth is truly astounding. Organisms can range from tiny single cells not visible to the naked eye to massive multi-organ animals. Across all levels of life, from the tiniest microorganisms (microbes) at 1/1000th of a millimeter or less in size to complex organisms weighing tons, relationships exist. These relationships can involve food, such as predator-prey relationships, but do not have to be harmful to one member of the relationship. Multitudes of organisms exhibit mutually beneficial, or symbiotic relationships.

My research explores two very different types of relationships. In one case, Rhizobia bacteria invade the plant *Medicago truncatula* (Medicago) to facilitate the exchange of nutrients between the two organisms. In the other, trillions of microbial cells inhabit the human intestines; these cells are commonly referred to as the gut microbiota and greatly impact human health. While the microbiota residing in our bodies are typically beneficial, disturbances in the microbiota community are associated with a number of harmful diseases. In this chapter, I will focus solely on my work on the Medicago, Rhizobia symbiotic relationships.

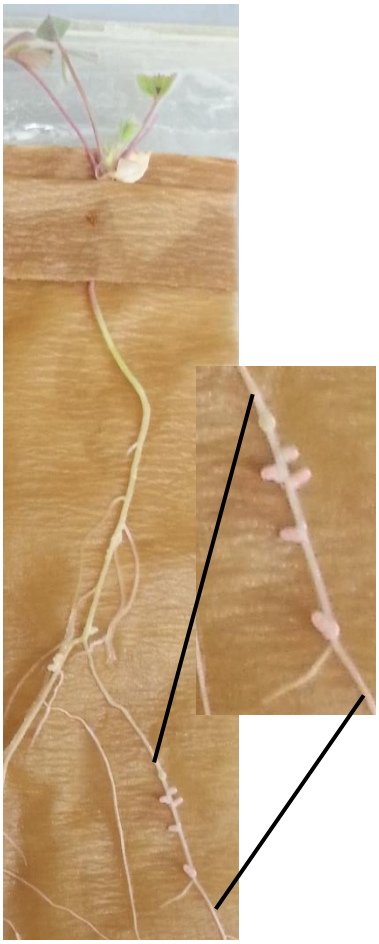

Figure 1. Picture of a Medicago Plant used for this study. An enlarged section of roots containing root nodules is also shown.

Medicago truncatula-rhizobia Symbiosis

While atmospheric nitrogen (N_2) is the most abundant gas in the atmosphere, only a handful of organisms, namely certain bacteria and archaea, which are single-celled organisms without a defined nucleus, can use the N_2 in the atmosphere for their nitrogen

needs by converting N_2 into ammonia (NH_3), which is the form of nitrogen most organism require. This process is called biological nitrogen fixation. To meet crops' nitrogen demand, farmers use nitrogen fertilizers, which increase available nitrogen.[1] Fertilizers require considerable resources to manufacture,[2] and fertilizer leaching contributes to environmental pollution through release of greenhouse gases.[3] Also, fertilizer usage can lead to eutrophication of waterways, where excess nutrients results in an overgrowth of algae, depleting oxygen and resulting in the death of aquatic life. Thus, it is beneficial to use biological nitrogen fixation in agriculture to reduce fertilizer usage.[4] This is accomplished through plants that form a symbiotic relationship with nitrogen fixing bacteria. For example, legume plants (i.e. peas, beans, soybeans) can form a symbiotic relationship with rhizobia bacteria, which can perform biological nitrogen fixation. The rhizobia bacteria provide fixed nitrogen for the plant, and in exchange, the plant provides a protected environment and a carbon source for the rhizobia bacteria.[5] The use of legume plants in agriculture reduces the requirements for nitrogen fertilizers.[6, 7]

Medicago truncatula (plant)-*Sinorhizobium meliloti* (bacteria) is a model legume-rhizobia symbiotic relationship capable of performing biological nitrogen fixation.[8] When rhizobia bacteria are near Medicago plants, they detect chemical signals produced by the plant and then secrete their own chemical signals, which starts a series of signaling events in the plant to from specialized organs on the plant roots, called root nodules (**Figure 1**), that provide an environment for rhizobia to live in.[9, 10] My research develops and applies analytical methods involving an instrument called a mass spectrometer to further understand the molecules present in the root nodules during biological nitrogen fixation.

Mass Spectrometry

When studying complex systems, mass spectrometry (MS) is a powerful tool, as it can detect a wide variety of biomolecules present in a system. These biomolecules include proteins, peptides, and metabolites, among others. Proteins are encoded for in our genes and play a wide variety of roles in the cells, such as structural support and transport and storage. Peptides are often signaling molecules throughout our body (insulin, for example), but can have other roles, such as defense. Metabolites are produced by proteins and also have a wide variety of biological roles, including energy, defense, structure, and signaling. My research primarily focuses on studying the metabolite and peptide content in plant tissue via mass spectrometry, including developing methods to more effectively analyze these molecules.

MS measures the mass to charge ratio (*m/z*) of a charged molecule (either a positively or negatively charged ion). Ions are created when uncharged molecules either lose electrons, which are negatively charged particles, creating a net positive electrical charge, or gain electrons, creating a net negative electrical charge. The general set-up of a mass spectrometer is (1) ion source (2) mass analyzer and (3) detector (**Figure 2**). The ion source creates charged molecules in the gaseous phase, allowing ions to enter the mass spectrometer. The ion source is important because uncharged ions are not able to travel through various parts of the mass spectrometer. In the mass analyzer, the ions are separated based upon their mass to charge ratio (*m/z*). Thus, a molecule with +1 charge will have an *m/z* twice as large as a molecule of the same mass but with a +2 charge. The detector monitors the ions as they are separated by the mass analyzer and sends signals produced by the ions hitting the detector to a computer to create a mass spectrum. The mass spectrum plots intensity (y axis) for all the *m/z* (x axis). An example mass spectrum is shown in **Figure 2**.

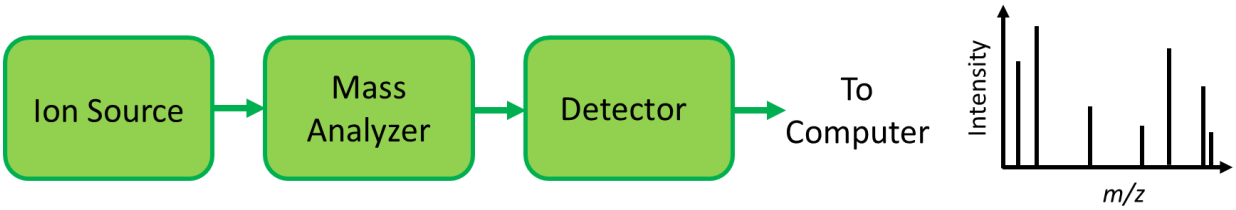

Figure 2. Basic diagram depicting the set-up of a mass spectrometer.

The basic workflow for analyzing the molecular content of a tissue involves homogenizing the tissue, *i.e.* making the sample uniform in nature, and extracting molecules from the tissue into an appropriate solvent. However, during the homogenization procedure, the spatial information of where the molecule was located in the tissue is lost. Mass spectrometry imaging (MSI) analyzes tissue sections directly in order to preserve the spatial locations of the molecules within the tissue. Although there are multiple different ionization techniques (i.e. different ion sources for creating ions from the tissue sections), my research utilizes a specific ionization technique utilizing a laser to selectively ionize specific locations of the sample at a time. The general workflow for MSI is shown in **Figure 3**. The sample is sliced into thin sections (16 µm, about ¼ the width of a human hair) and a small molecule, called the matrix, is applied uniformly across the tissue section to aid in ionization. After inserting the sample into the instrument, a box is drawn around the area to be imaged and the box divided into pixels. To acquire the image, a laser is fired at each pixel across the matrix covered tissue section. This generates a mass spectrum at each pixel. After acquisition, software is used to extract the ion intensity of specific *m/z* from each mass spectrum to create a heatmap for the *m/z* across the tissue section. Different distributions can be observed for different *m/z* (**Figure 3**). In this way, hundreds of different molecules can be imaged in a single run. First, I will discuss my efforts to improve MSI methods to study symbiosis in the plant Medicago, then I will discuss the application of developed methods to study the plant's response to stress.

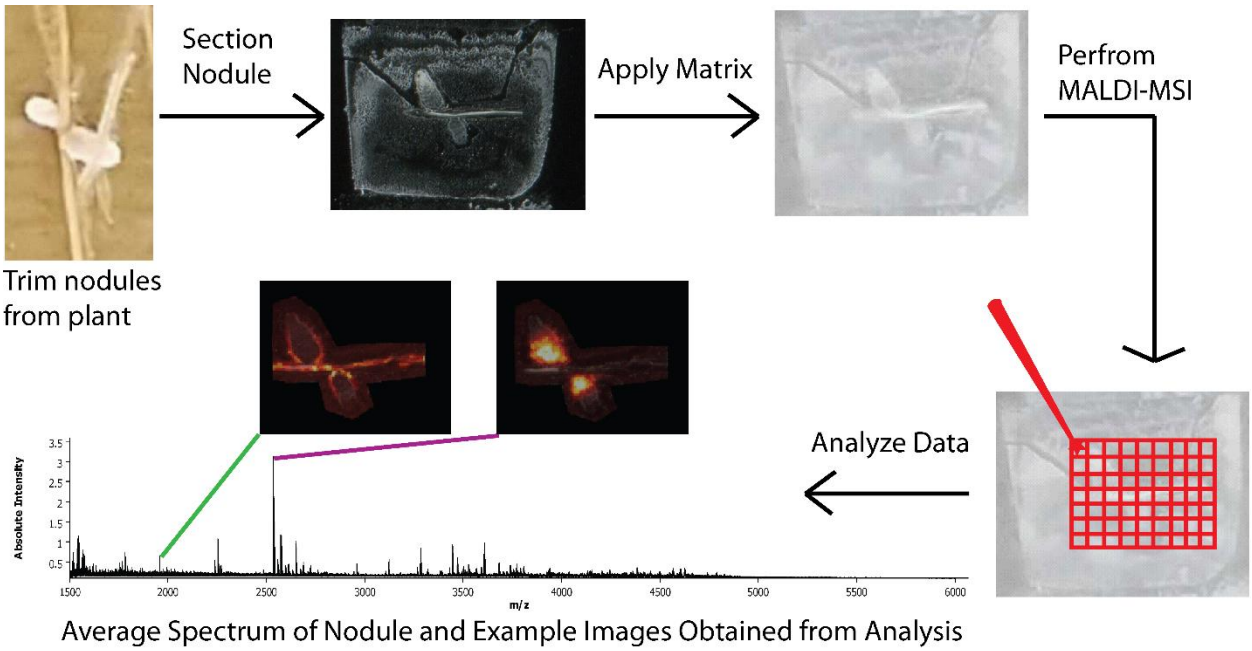

Figure 3. Typical workflow for MSI on Medicago root nodules.

Experimental Results- Developing a MSI Method to Study Peptides in *Medicago truncatula* Rhizobia Symbiosis

My research uses mass spectrometry to not only study symbiotic relationships, but also to develop and improve methods that increase our ability to study symbiotic relationships. Previously, our lab developed a MSI method to image metabolites in the root nodules of Medicago.[11] Since then, MSI of plant metabolites has become increasingly more prevalent. Peptides, however, are much less frequently studied by MSI despite their importance in plants. Signaling peptides, in particular have important roles in communication within the plant, but they are difficult to investigate with MSI due to the fact that they are present at very low concentrations. The various roles of signalling peptides in plants include growth, development, and symbiosis.[12] Thus, I set out to develop a MSI method to image signalling peptides in Medicago root nodules, specifically focusing on a class of peptides that are critical for proper formation of root nodules capable of performing biological nitrogen fixation. To accomplish this, I altered the sample

preparation workflow in **Figure 3**. Traditionally, metabolite MSI sample preparation involves sectioning the tissue section and then matrix application. I added a washing step in between the sectioning step and the matrix application step in the MSI workflow (**Figure 4**). By washing tissue sections with organic solvents (ethanol, chloroform, for example), interfering molecules are removed. Removing these species increases our ability to detect peptides. **Figure 4** shows the increased signal of larger peptides after washing.

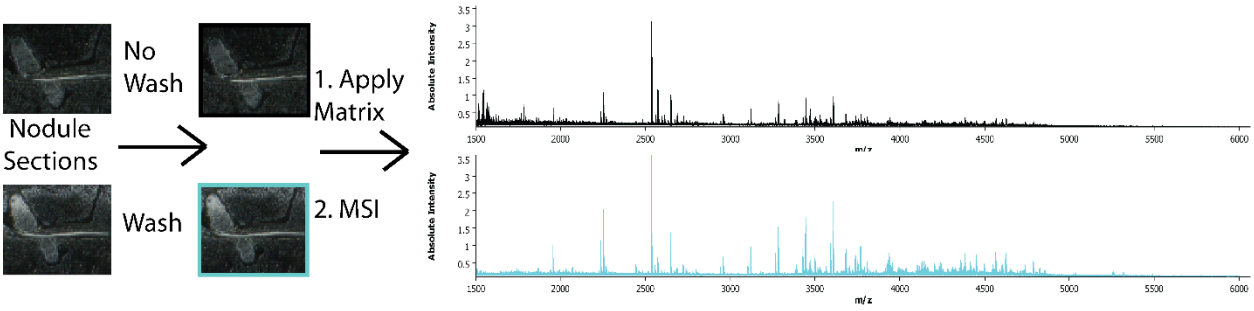

Figure 4. Optimization of MALDI-MSI for detection of larger endogenous peptides in plants.

Experimental Results: Studying *Medicago truncatula* Rhizobia Symbiosis in Salt Stress

Although basic understanding of symbiotic relationships is important, additional knowledge of what occurs when these relationships are disturbed is highly relevant as well. Stressors can cause harm to either or both organisms present in symbiotic relationships. One example of an environmental stressor is soil salinity. For example, in Medicago, high levels of salt in the soil has major effects on the root nodules, despite the relatively minor effects of salt stress on other aspects of plant growth. Due to the importance of biological nitrogen fixation in the root nodules, it is important to understand why salt has such a relatively large impact on root nodules.

To study salt stress, I utilized a MSI method based upon a new ionization source to study the metabolites in root nodules. The source used here to study salt stress allows for easier and faster insertion of the sample into the instrument. The employed source also allows for higher

spatial resolution (decreased pixel size) due to the smaller laser diameter. Higher spatial resolution allows for better resolution of smaller features in the tissue, in a similar way that higher resolution cameras better resolve images.

The developed method was applied to study the metabolites present at high salt and low salt conditions. **Figure 5** shows example MSI results for small molecule changes due to salt stress in Medicago. Each image in **Figure 5** shows control (low salt) nodules on the left side and high salt (salt) nodules on the right side. **Figure 5 (A,E)** provide the picture of the root nodule sections (optical image) prior to MSI analysis. The MSI images are shown on a hot intensity scale, which goes from black to red to yellow. Thus, the area on the tissue with the highest amount of the molecule will be shown in yellow. Example *m/z* whose distribution shows a higher amount in control root nodules **Figure 5 (B-D)** and salt root nodules **Figure 5 (F-H)** are shown. This analysis reveals which molecules are being altered in salt stress, providing insights into how the plant adapts to the stress.

Conclusions and Future Directions

I have discussed my research focusing on determining molecular distribution changes in Medicago by using MSI to image *m/z* in the plant. On the one hand, I have developed a MALDI-MSI method to investigate higher molecular weight peptides in Medicago root nodules and developed an MSI method to study metabolites in the Medicago root nodules. However, I have also applied developed methods to study small molecule changes due salt stress in the root nodules. In the future, my method to study peptides in the root nodule, could be applied to better understand the role of peptides in root nodules by comparing wild-type root nodules to root nodules from a non-functional mutant version of Medicago. Comparing peptides observed in functional and non-

functional nodules could determine peptides that are critical for forming nodules capable of biological nitrogen fixation. This would improve understanding of the needed molecule components for formation of legume-rhizobia symbiosis.

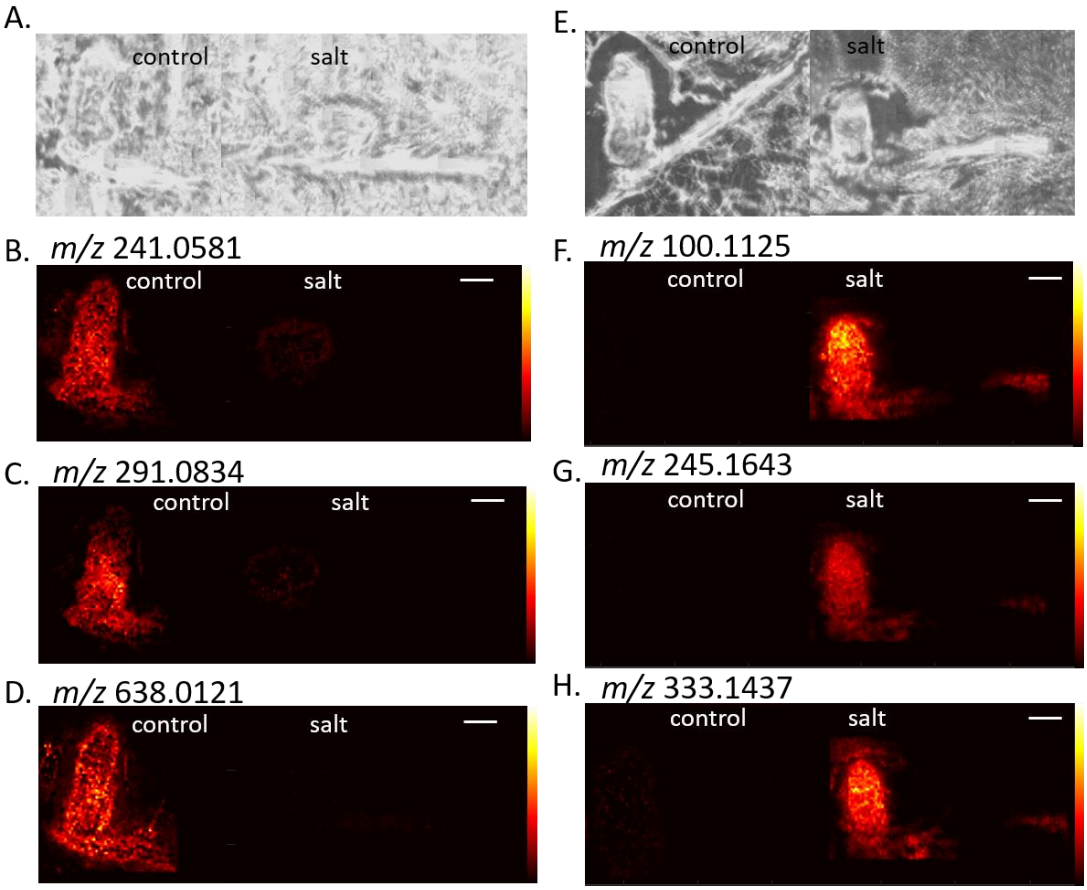

Figure 5. Example MALDI-MSI images for *m/z* changing during salt stress. **(A,E)** are the optical images and **(B-D)** are *m/z* higher in control nodules, while **(F-H)** are *m/z* higher in salt nodules. The white scale bar indicated 1 mm.

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