Communicating Research to the General Public

At the March 5, 2010 UW-Madison Chemistry Department Colloquium, Prof. Bassam Z. Shakhashiri, the director of the Wisconsin Initiative for Science Literacy (WISL), encouraged all UW-Madison chemistry Ph.D. candidates to include a chapter in their Ph.D. thesis communicating their research to non-specialists. The goal is to explain the candidate's scholarly research and its significance to a wider audience that includes family members, friends, civic groups, newspaper reporters, program officers at appropriate funding agencies, state legislators, and members of the U.S. Congress.

Over 20 Ph.D. degree recipients have successfully completed their theses and included such a chapter.

WISL encourages the inclusion of such chapters in all Ph.D. theses everywhere through the cooperation of Ph.D. candidates and their mentors. WISL is now offering additional awards of $250 for UW-Madison chemistry Ph.D. candidates.

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Method Development and Application of Mass Spectrometry Imaging to Study Symbiotic Relationships Between Bacteria and Host Organisms

By

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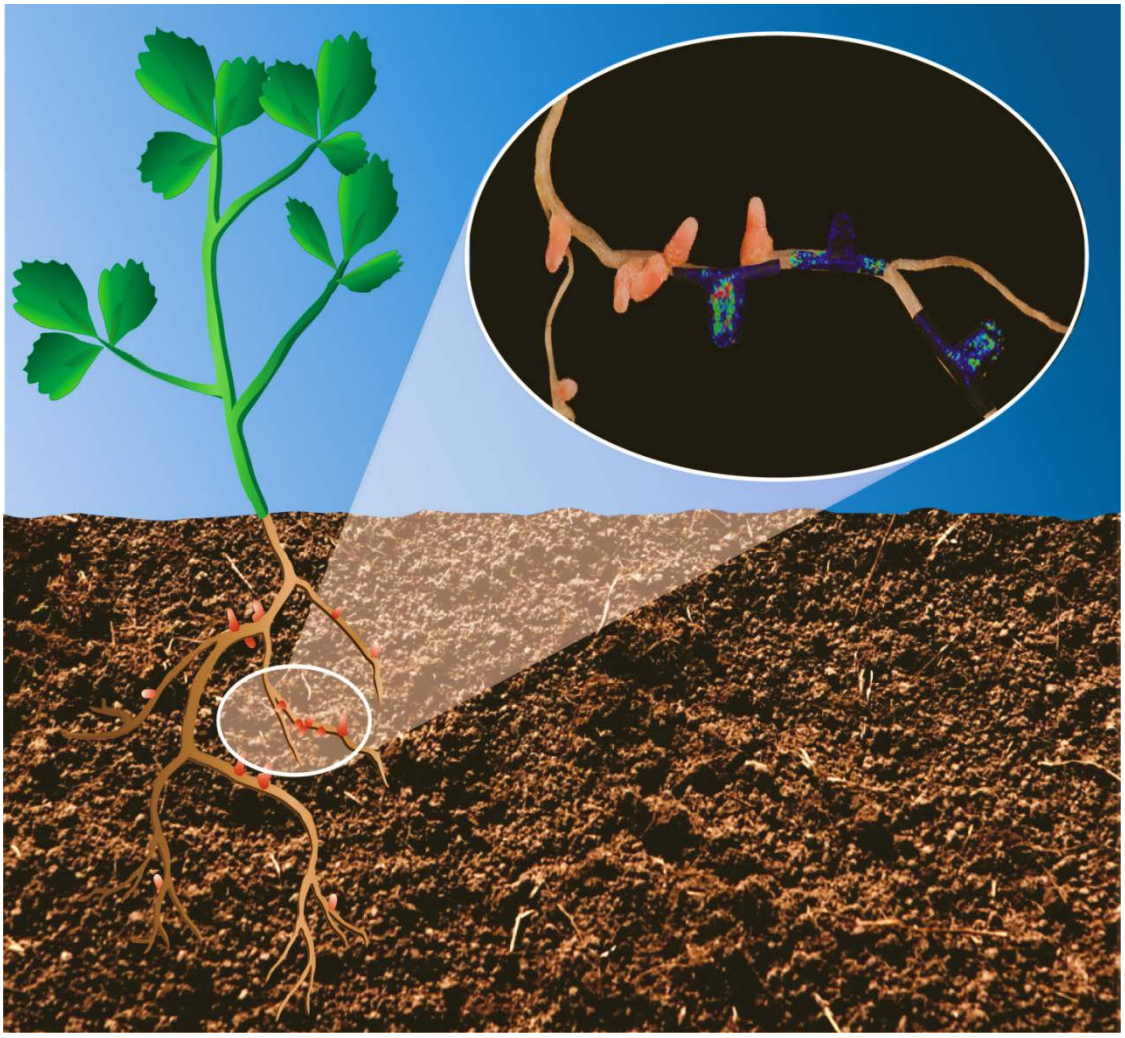
Chapter 8

Summary of

“Method Development and Application of Mass Spectrometry Imaging for the Study of Symbiotic Relationships between Bacteria and Host Organisms”

for the Wisconsin Initiative for Science Literacy



Introduction

In order to understand how biological systems work, it is important to study their underlying chemistry. Diseased organisms have different biochemistries than healthy organisms. We can find new ways to detect and treat diseases by comparing the differences in chemical activity between healthy and diseased states. There are many different classes of chemicals inside an organism that we can study, from proteins to peptides to small molecules/ metabolites. Your genes encode information to create proteins and proteins produce small molecules/ metabolites that the cells in our bodies directly use for fuel, structure, cell signaling, defense, and many other key roles. Organic acids (citric acid, amino acids, etc.), sugars (glucose, fructose, sucrose, etc.), alcohols, antioxidants, and lipids are all classified as metabolites. For my research I chose to study these different metabolites because by looking at the small molecules that are directly produced and used by the cell, you get a picture of what is going on in the cell at a given time.

How does my research relate to the study of metabolites?

My research over the last 4 ½ years has largely focused on the analysis of metabolites in plants by developing analytical techniques and methods to detect changes in metabolites between normal and mutant plants. I want to know what kinds of metabolites are in my plants and where they are located within the plants. In my research, I use a tool called a mass spectrometer to detect metabolites. The mass spectrometer allows me to measure the mass of hundreds of metabolites the plant samples and determine which ones are found in my normal plant samples and not found in the mutant plants. The type of mass spectrometry I use is a special technique

called mass spectrometry imaging. Using mass spectrometry imaging, I not only get the mass of each metabolite found in my plants, but I can also see a picture of the location of those metabolites within the plant (roots, leaves, stem, etc.).

Why plants?

In the introduction above, I used the example of wanting to know the differences between healthy and diseased states. For my research, I am actually looking at the difference in metabolites between normal and mutated legume plants. Legume plants, like soybeans, alfalfa, chickpeas, and other types of beans, are very important crops to the agriculture industry. Think back to elementary school when you learned about crop rotations. Farmers rotate what types of crops they plant in their fields because different crops suck up different nutrients from the soil. Legumes are one class of crops that can actually put nutrients back into the soil. The nutrient we are talking about is nitrogen. Legume plants have developed a symbiotic relationship (meaning the relationship is beneficial for both partners) with a bacteria that lives in soil, called rhizobia. The rhizobia take nitrogen from the air and "fix" it, or turn it into a form of nitrogen that the plant can actually use, in a process known as biological nitrogen fixation. In return, the plant provides a source of carbon for the rhizobia, which the bacteria need to survive. The bacteria are attracted to the roots of the legume plants and make their way inside the plants' roots. When bacteria enter a plant's roots, the roots form special structures, called root nodules, shown in **Figure 1**, and this is where the bacteria fix nitrogen for the plants.

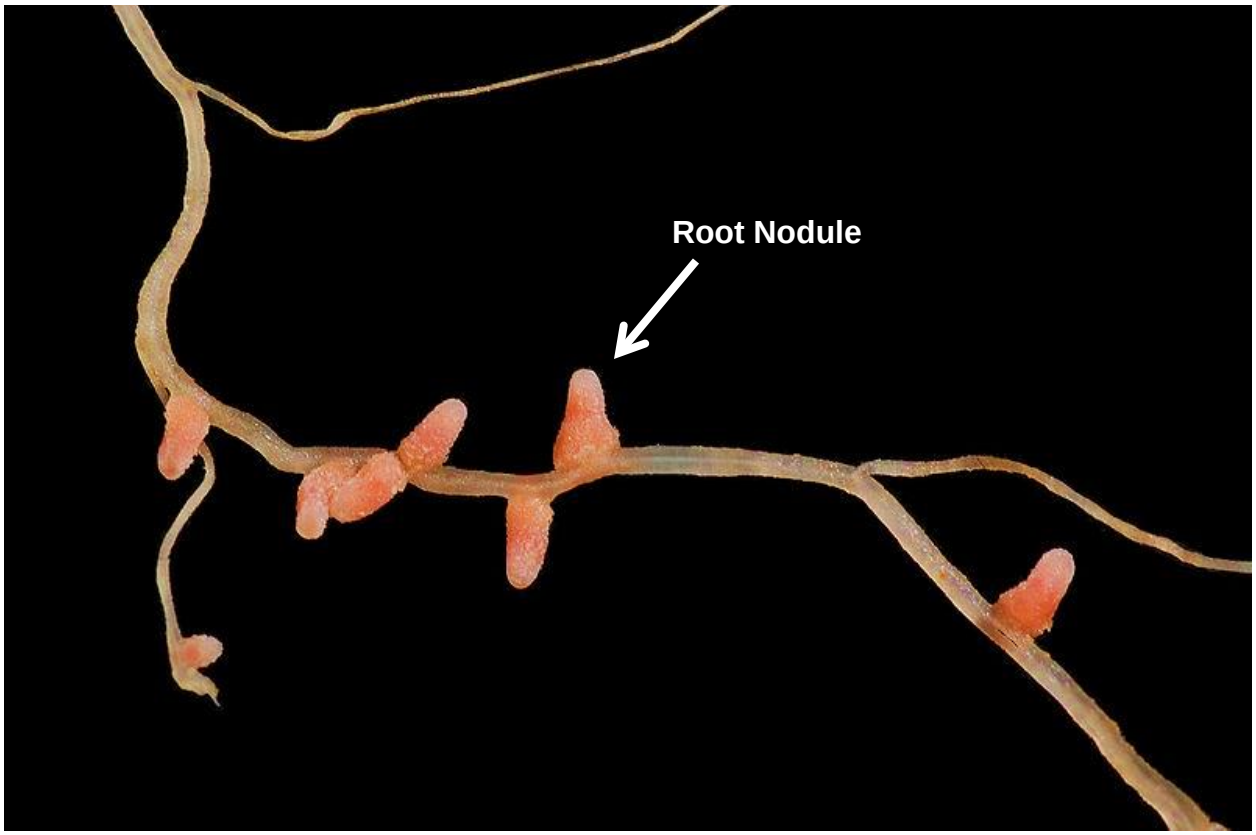


Figure 1. Photograph of legume root nodules. Photo courtesy of the Jean-Michel Ané research group in the department of agronomy at the University of Wisconsin- Madison

For my research, the normal plants are capable of fixing nitrogen while the mutant plants have a genetic mutation that makes them unable to fix nitrogen. My goal was to detect and identify the metabolites produced and exchanged between the plant and the bacteria when biological nitrogen fixation happens. To do this I compared the metabolites in the root nodules of normal plants to the metabolites in the mutant plants to figure out what the mutant plants were missing that made them unable to fix nitrogen. The goal of my research is to help biologists understand the mechanisms behind biological nitrogen fixation so scientists can make this process more efficient for the agriculture industry. The end goal is to put more nitrogen back into the soil so other plants can use this natural nitrogen instead of nitrogen fertilizers. By

increasing the supply of natural nitrogen in the soil, scientists hope crop yields will increase and the need for costly and potentially harmful nitrogen fertilizers will decrease.

What is mass spectrometry and how does it work?

Mass spectrometry is one of the most popular techniques used for analyzing metabolites because it offers speed, sensitivity to detect tiny amounts of molecules, and the ability to detect hundreds of metabolites at once. There are three basic parts of a mass spectrometer. The first part is an ionization source, which is very important because a mass spectrometer can only detect ions, which are molecules that have a positive or a negative charge. To be more precise, a mass spectrometer measures a molecules mass-to-charge ratio (represented as *m/z*). The molecules must have a positive/ negative charge in order for the mass spectrometer to measure them because mass spectrometers use electric and/or magnetic fields to move ions through the various parts of the mass spectrometer. MALDI, which stands for Matrix-Assisted Laser Desorption/ Ionization, is the main type of ionization source on the mass spectrometers I use in my research. Although the name seems long and daunting, the acronym tells you exactly what is going on in this phenomenon. **Figure 2** illustrates this process. Matrix-Assisted: First, I apply a matrix over my sample. A matrix is just a type of small, organic acid that forms crystals and can absorb ultraviolet (UV) light. Laser: Next, we shoot our matrix-coated sample with a laser. Desorption/ Ionization: The matrix absorbs the UV light from the laser and heats up, causing the matrix to vaporize, releasing a cloud of matrix ions and metabolite ions from the sample.

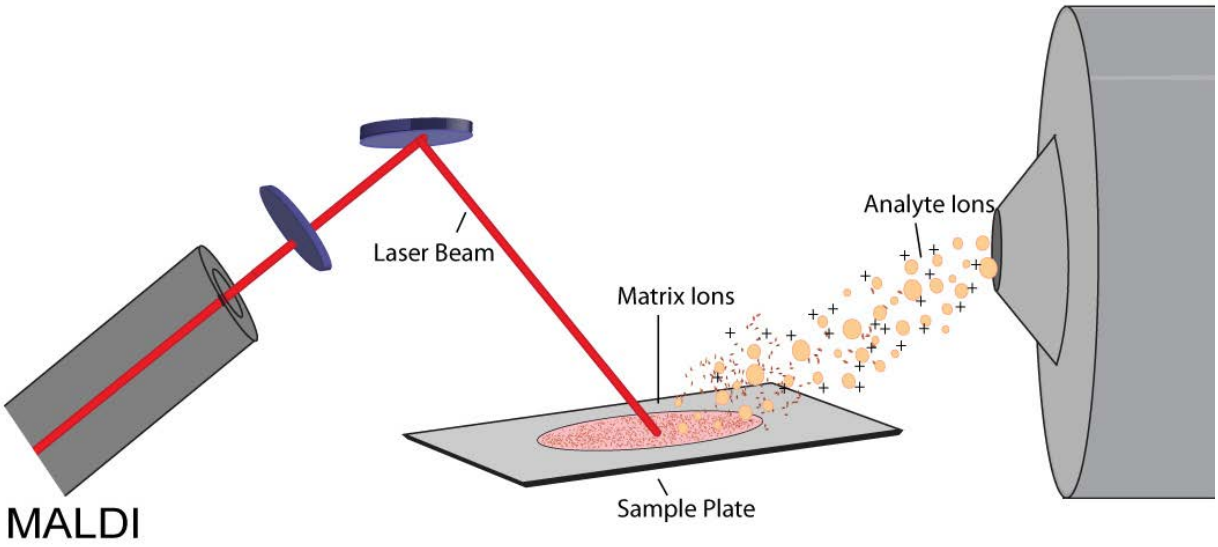


Figure 2. Cartoon showing the MALDI ionization method. A laser shoots the matrix-coated sample creating a cloud of charged matrix and analyte molecules. An applied electric/ magnetic field guides ions into the mass spectrometer. Analyte ions are the metabolite ions from the sample underneath the matrix.

Once the electric field guides ions into the mass spectrometer, the second part of the mass spectrometer, called the mass analyzer, separates the charged ions based on their mass-to-charge ratio (*m/z*). The mass of the molecule is roughly equal to the *m/z* number measured by the mass spectrometer divided by the charge of the molecule. My research focuses on small molecules and since the molecules are small in size, they can typically only fit one charge on them before other charges start to repel each other. For example, if we brought the positive ends of two magnets close to each other, they would repel each other in the same way two positive charges would repel each other if they were trying to fit too close to each other on the surface of a small molecule. Since the charge on metabolites is almost always +1, the mass-to-charge ratio essentially tells us the mass of the molecule that was detected by the mass spectrometer; for example, if the molecule had a +2 charge, the *m/z* number shown by the mass spectrometer would be equal to the mass of the compound divided by 2. Back to the mass analyzer: to separate the ions, the instrument applies an electric field that makes the charged ions accelerate

toward the end of the instrument. As they accelerate, they separate due to the differences in their masses. If you pushed a skateboard and a truck with the same amount of force, you can imagine that there would be a difference in how long it takes each to reach the finish line.

The last component of the mass spectrometer is the detector, which sits at the "finish line" and detects the masses of the ions as they reach it. A computer converts the signal from the mass spectrometer, typically an electric current, into a mass spectrum that scientists can interpret. **Figure 3** shows a mass spectrum of a plant sample. The y-axis shows the abundance of each ion and the x-axis is the mass-to-charge ratio of each ion. Each peak on the mass spectrum represents a unique molecule detected by the mass spectrometer.

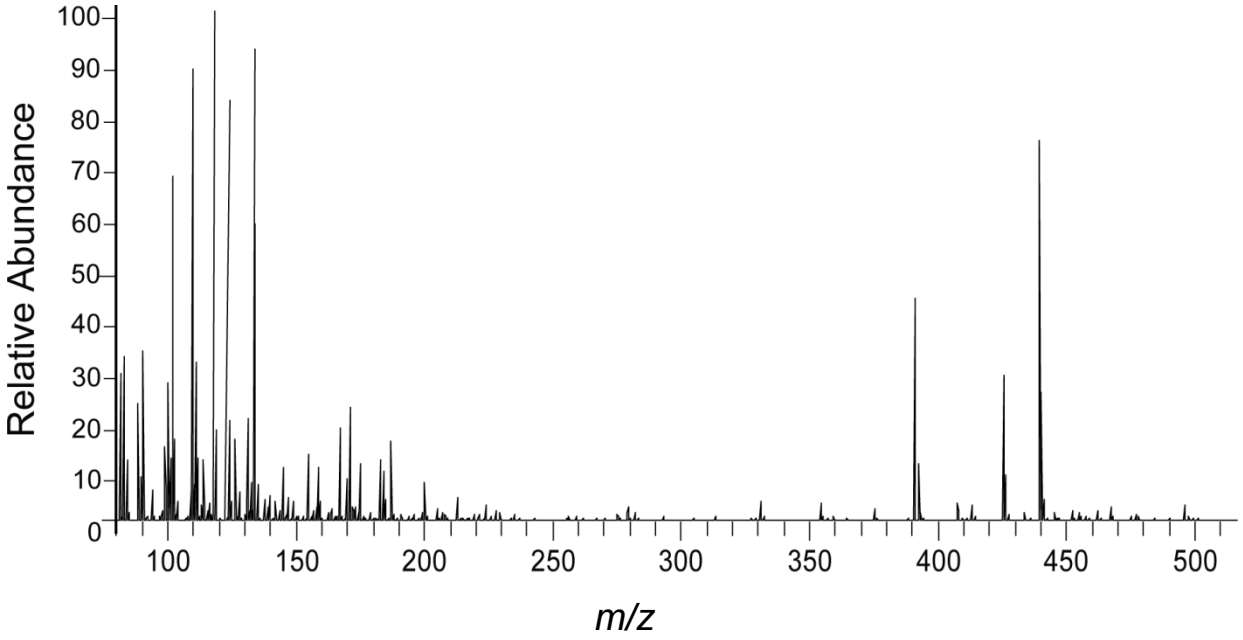


Figure 3. A mass spectrum of a matrix-coated plant sample. Each peak represents a molecule (either matrix or plant analytes). *m/z* stands for mass-to-charge ratio. The relative abundance means the height (or intensity) of the peaks relative to each other.

What is mass spectrometric imaging (MSI)?

So we have learned about the different parts of the mass spectrometer and the type of information we can learn by using a mass spectrometer. I mentioned before that my research involves a special type of mass spectrometry, called mass spectrometry imaging (MSI). This technique not only gives me an array of mass spectra, but also gives me a picture that shows where each molecule is located within the plant. Let's go into a little more detail about MSI.

For MSI experiments, I slice my plants into very thin sections (about 16 micrometers thick) and lay them on a glass microscope slide. I coat the plant sections with matrix, and shoot it with the laser just as I did in the regular MALDI experiment discussed above. Instead of shooting my plant with the laser one time, I use software to draw a grid over my plant section and shoot the laser at every point on the grid. Instead of collecting a single mass spectrum, as we see in Figure 3, we collect a mass spectrum at every grid point. The computer software can then put the mass spectra together and show us an image similar to a heat map for each *m/z* measured by the mass spectrometer. In these heat map images, the part of the plant with the highest concentration of the particular *m/z* is shown in red and the area of the plant with the lowest concentration is shown in blue, with a range of colors in between. **Figure 4** outlines the MSI process and shows an example of an MS image.

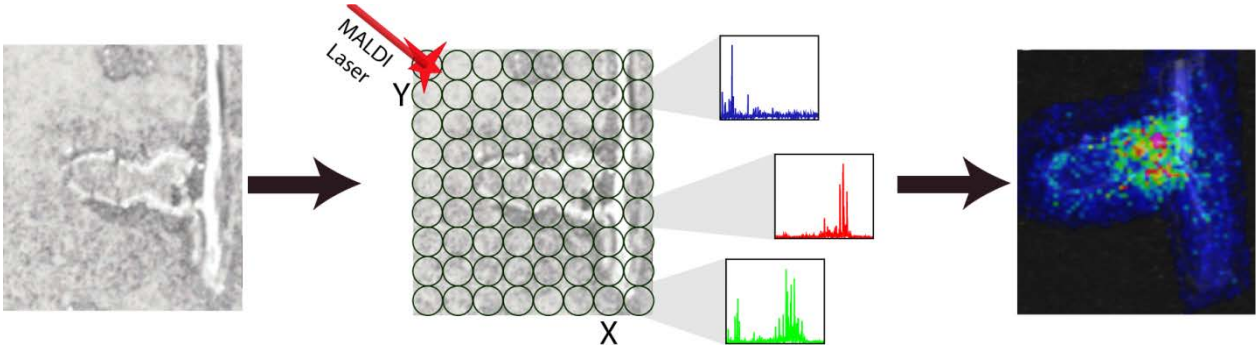
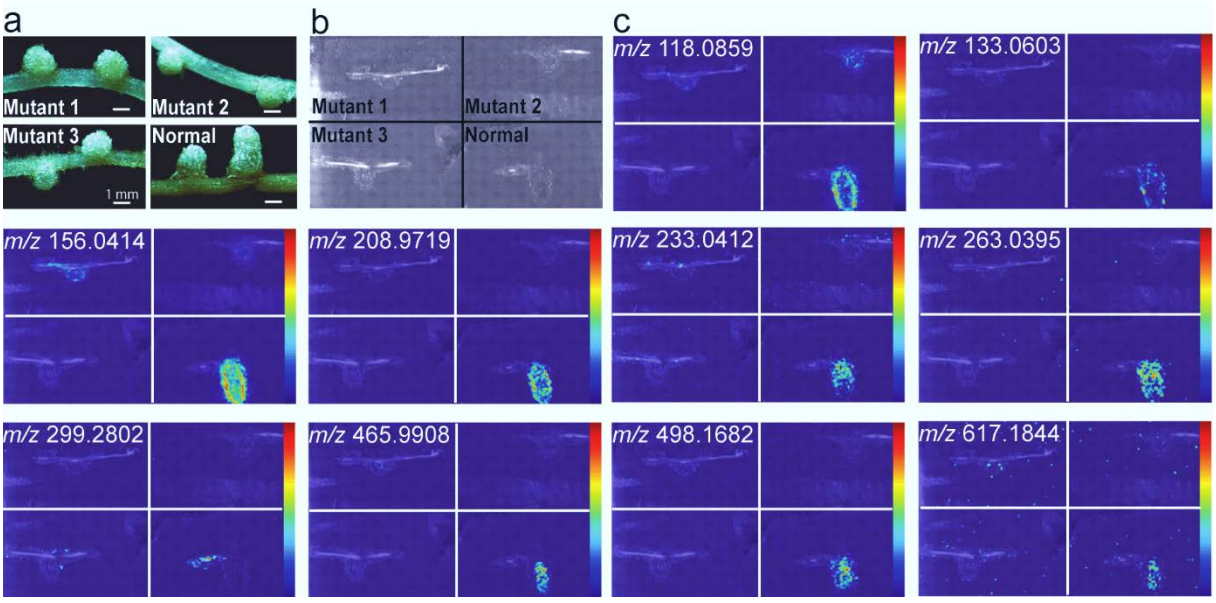


Figure 4. Outline of the MSI process. I cut the plant into a thin slice, lay it on a glass slide, and coat the slide with matrix. We use software to lay a grid pattern over the plant tissue so the laser knows every point it needs to shoot. We collect a mass spectrum at every grid point. The software compiles all the mass spectra into a heat map image for each specific *m/z* detected.

Results of my research

For my research I used MSI to look at the differences in metabolites found in normal plants (capable of fixing nitrogen) and those in mutant plants (unable to fix nitrogen). I detected and identified over 30 metabolites produced by normal plants that were absent from the non-nitrogen-fixing mutants. **Figure 5a** shows the root nodules of the normal plants (bottom right corner) compared to the three types of mutant plant I used. **Figure 5b** shows a picture of what the plants look like after I take a thin slice of the plant root, lay it on a glass microscope slide, and coat the slide with matrix. **Figure 5c** shows sample images of several of the metabolites that are clearly present in the normal plants but not in any of the three mutant plants.



Finally, I compiled a list of over 30 identified metabolites that I detected only in the normal plants, shown in **Table 1**.

Table 1. List of metabolites found only when nitrogen fixation occurs in legume root nodules

Metabolite	Measured *m/z*
Aminobutryic acid	104.0708
methyl-piperidin-iumone [M+]	114.0915
Proline	116.0706
Aminopentene-diol*	118.0863
Isoleucinol	118.1228
Asparagine*	133.0607
Glutamic Acid	148.0603
Asparagine [M+Na]*	155.0425
3-thiophen-1-yl propanoic acid	158.0397
ethyl-aminocyclopentane carboxylic acid	158.1173
Phenylalanine	166.0859
Asparagine [M+K]*	171.0165
Arginine	175.1187
Tyrosine	182.0810
methyl-alpha-galactopyranoside	195.0862
ethyl-aminocyclopentane carboxylic acid [M+K]	196.0731
Proclavaminic acid*	203.1024
methyl-alpha-galactopyranoside [M+Na]*	217.0678
Proclavaminic acid [M+Na]	225.0840
Proclavaminic acid [M+K]	241.0577
dihydroxybenzoic acid succinimido ester	252.0500
5-amino-2-(aminomethyl)-6-butoxyoxane-3,4-diol [M+Na]	257.1462
9H-fluoren-9-yl-di(propan-2-yl)phosphane	283.1618
N-(4-guanidinobutyl)-3-methyldecanamide	299.2802
SAM (S-adenosyl-L-methionine)	399.1441
2-hydroxy-5-[[2-phenyl-2-[4-(phenylcarbamoylamino)phenyl]sulfanylacetyl] amino]benzoic acid	514.1423
2-[hydroxy-[(2R)-3-hydroxy-2-[(9E,12E)-octadeca-9,12-dienoyl]oxypropoxy]phosphoryl]oxyethyl-trimethylazanium [M+]	520.3395
Oleoyl lysophosphatidylcholine	522.3552
18-[(4Z)-4-[(2-hydroxy-5-nitrophenyl)hydrazinylidene]-3-oxocyclohexa-1,5-dien-1-yl]octadecanoic acid [M+]	541.3125
3-[[[[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy-hydroxy phosphoryl]oxy-hydroxy phosphoryl]oxy-hydroxyphosphoryl]oxypropanoic acid	580.0223
[[(2R,3S,5R)-5-[4-amino-5-(4-aminobutyldisulfanyl)-2-oxopyrimidin-1-yl]-3-hydroxy oxolan-2-yl]methoxy-hydroxyphosphoryl] phosphono hydrogen phosphate [M+]	602.0052
(Z)-4-oxo-2-[(Z)-1-oxooctadec-9-enyl]-12-henicosenoic acid	603.5340
1,3-dilinolenin	613.4814
Heme	617.1844

The MS images give us a lot more information about the 30 molecules listed above that I identified only in the normal plants. The MS images show that some of these molecules are found on the inside of the nodule while others are primarily on the outside. There are even some that are only in the roots of the plant but not in the actual nodule. Biologists can use this additional information to determine how the plant might use these molecules. Biologists already figured out that some of the metabolites listed in the table above are used by the plant to transport nitrogen. What's more exciting is that biologists have no idea what some of the metabolites in the table are used for or that they had any part in the nitrogen fixation process. Now biologists can use this new information to figure out exactly how the plants use these chemicals for biological nitrogen fixation. Once they know this information, biologists can make this process more efficient, either by ensuring the plants themselves put more nitrogen back into the soil, or by genetically engineering other crops to fix their own nitrogen. This will hopefully increase crop yields and decrease the need for environmentally damaging artificial nitrogen fertilizers.