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Review

Recent developments in MALDI MSI application in plant tissue analysis*

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Mass spectrometry imaging (MSI) combined with matrix-assisted laser desorption/ionization (MALDI) is an efficient technology applied in plant metabolomics research. This technique allows for visualization of spatial distribution of metabolites, such as: lipids, proteins, peptides and DNA sequences, by determining the x, y coordinates of the compounds present exactly in the plant tissue. Simplicity of such tissue preparation without the need for prior exact knowledge about the analytes is a great advantage of this method. In this review, we provide an overview of experimental workflow, including sample preparation, data acquisition and analysis, methodology, and some recent applications of MALDI MS imaging in plant metabolomics research.

Key words: MALDI MSI, mass spectrometry, metabolomics, imaging

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Abbreviations: MS, mass spectrometry; MALDI, Matrix-Assisted Laser Desorption/Ionization; MSI, Mass Spectrometry Imaging; DHB, 2,5-dihydroxybenzoic acid; CHCA, α-cyano-4-hydroxycinnamic acid; TiO_2, titanium dioxide; TOF, Time of Flight

INTRODUCTION

Mass spectrometry imaging (MSI) is a powerful technique used for visualising spatial distribution of molecules *in situ* without prior labelling. It allows for performing the analysis from tissues to cells without any *a priori* knowledge of the potential target (Yang *et al.*, 2020). MSI has been used in various studies of compounds, such as lipids, proteins and peptides. The targeted analytes can range in their molecular mass from large proteins of 100 kDa or more, to small endogenous metabolites that are less than 1 kDa (Schwamborn & Caprioli, 2010). The MS principle is based on ionisation of chemical compounds, followed by ion separation based on the mass to charge ratio (m/z) and recording it as a spectrum. When it comes to MSI, the mass spectra are collected from every scanned point on the tissue and next they are converted into maps which visualize the spatial distribution of molecules by their molecular masses. Compounds of interest can be extracted from the mass spectrum as peaks, resulting in several different maps obtained from a single experiment (Bjarnholt *et al.*, 2014). The principle of MSI is presented in Fig. 1.

The most popular ionisation method used for mass spectrometry imaging is Matrix Assisted Laser Desorption/Ionisation (MALDI). MALDI is a soft ionisation technique which allows to analyse large molecules without causing excessive fragmentation and in majority produces singly charged ions. (Michno *et al.*, 2019). Additionally, ionized proteins and peptides usually retain their post-translational modifications, e.g. phosphorylation. When using the MALDI technique, it is necessary to use a crystallising organic compound called matrix, which assists in sample ionisation under a UV laser beam (Nakashima *et al.*, 2020). MALDI is a fast and sensitive tech-

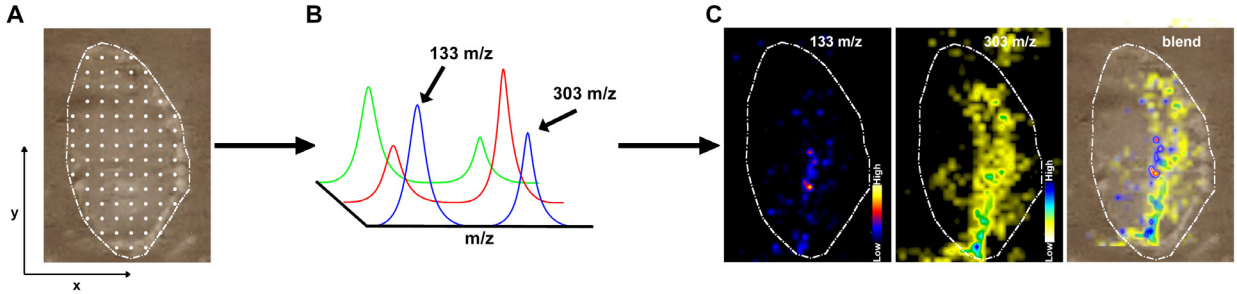

Figure 1. The principle of MALDI MSI illustrated with an example of the pea root nodule.
A – Root nodule slice with selected region of interest and the spots where the mass spectra will be acquired. B – Examples of mass spectra obtained from different sample spots. The peaks of interest at 133 and 303 m/z are marked with an arrow. C – Chemical maps of the selected peaks and blend of the maps with the visible image. Color of the spots relates to the intensity of the peak and is illustrated on the color scale.

nique, which can be combined with MS imaging, that allows for both – chemical analysis of metabolites, as well as visualization of their distribution (thanks to determining the x, y coordinates of many compounds directly in the tissue) (Seaman *et al.*, 2014). Its spatial resolution ranges from 1.4 μm to 100 μm (Kompauer *et al.*, 2016; Sun *et al.*, 2020). The advantage of this method is the simplicity of sample preparation without the need to isolate compounds selected for analysis from the tissue fragments.

MALDI MSI is a tool widely used for visualization of the plants metabolites in tissues. This tool enables non-selective identification and visualization of the metabolite distribution in the tissue slices. Moreover, for the metabolite identification no reference sample is needed (each metabolite can be identified by its m/z value). However, identification and quantification of metabolites is only half the battle when studying plant tissues. The other half is the study of their distribution. Such knowledge can be used for identification of the plant organ that contains the highest amount of the secondary metabolite of interest, so that it might be extracted later. It can be also used to discover the best method for the metabolite extraction, e.g. if the metabolite is inside the vacuole, the cells should be homogenized. The knowledge of the metabolite distribution might be also used for understanding of the primary and secondary metabolism mechanisms in the plant. The latter might be used for better plant use, for studies of novel methods of fertilization or other methods to increase the harvest.

SAMPLE PREPARATION FOR MALDI MSI

Preparation of the sample is crucial when using MALDI MSI. MSI analysis usually employs fresh-frozen tissues. It is also possible to analyse tissues embedded in formalin or paraffin after appropriate processing, but this increases the time of sample preparation. It can also interfere with the MS detection (Michno *et al.*, 2019). After collecting and freezing the sample, tissue sectioning needs to be performed. Sections are prepared in a cryostat microtome cooled from –15°C to –30°C, depending on the tissue type. The thickness of the section can affect its durability or result in its corrugation. It can also affect the efficiency of analyte extraction from the tissue. Typically, 5–20 μm thick sections are prepared for MALDI MS imaging (Morisasa *et al.*, 2019). The frozen sections are then transferred to a metal or glass plate, previously kept at room temperature. Because of the temperature difference, the tissue would be thaw-mounted on the plate (Gemperline & Li, 2014). The plate prepared with the examined tissue should be stored at –80°C until analysis in order to maintain the metabolites' stability.

Despite the fact that this procedure is a standard for sample preparation, it might cause some of measurement distortions. The sample is first flash-frozen with liquid nitrogen, which inactivates enzymes and causes formation of ice crystallites that are too small to destroy the cells. However, during thaw-mounting the sample is first thawed and then frozen again without the use of liquid nitrogen. This may cause disruption of some cells and organelles. Thus, this process may result in a slight displacement of the metabolites. Moreover, thawing of the sample also causes reactivation of the enzymes and that might change the metabolome of the sample (Schiller *et al.*, 2000). Those problems might be overcome by another flash freezing with liquid nitrogen right after thaw-mounting, or by using slides with mounting properties at –20°C.

Matrix application

The choice of MALDI matrix and the way it is applied is another important step in MS imaging. The MALDI matrix is a chemical compound that enables the desorption/ionization process of the analysed substance. The matrix allows for ion generation, which is essential since the mass spectrometer only detects charged particles. It has the ability to absorb strong laser power emission and therefore to protect the analytes. Matrix also prevents cluster formation which could impact sensitivity of the measurement. Usually, the matrix is a small organic acid, such as 2,5-dihydroxybenzoic acid (DHB, 154 Da) or α-cyano-4-hydroxycinnamic acid (CHCA, 189 Da) (Spraker *et al.*, 2020). Less popular matrices, such as TiO_2, gold or silver nanoparticles, are used to improve the spectral quality, crystallization and vacuum stability (Shrivas *et al.*, 2011). The MALDI matrix application method and its crystallization are extremely important steps in the mass spectrometry analysis because they have a direct impact on the amount of metabolites found in tissue (Gemperline *et al.*, 2016). The most popular method is to apply matrix by using an airbrush. It is widely used because of a relatively quick and easy distribution of the matrix, however, it requires a lot of skill from the operator who applies the matrix to the tissue. It is difficult to obtain reproducible results with this method, so the size of the matrix crystals is not always the same. The MALDI matrix can be also applied on the tissues by using an automatic sprayer system that applies layers of specific thickness, which gives this method more reproducibility (Baluya *et al.*, 2007; Gemperline *et al.*, 2014). Finally, matrix application based on sublimation is becoming more popular, thanks to its low diffusion (Hankin *et al.*, 2007). The choice of matrix and the way of application should be undertaken based on spatial resolution and molecular mass range for a given analysis.

MSI data acquisition

After applying matrix to the sample and co-crystallization of the analyte together with the matrix, the plate on which the sample is located is introduced into the spectrometer in a specific x, y plane. Pulsed laser irradiates the sample, releasing both – the matrix and analyte ions. The ability to move in the x, y plane allows the laser to cross the sample and obtain mass spectra from each of the previously defined points. After completing the 2D raster, it is possible to observe ion images for each (selected) mass (m/z value) in the spectrum, and the software will display the relative amount of each ion as a colour map signal intensity in the raster area (Gemperline *et al.*, 2016; Züllig & Köfeler, 2020). The matrix absorbs a significant part of the laser energy, ensuring gentle ionization of the analytes, which allows ionizing larger particles (m/z above 100 kDa) without their disintegration (Xue *et al.*, 2019). Unfortunately, there is a possibility that the matrix itself, which produces ions, can interfere with or mask the analysed ions with the same molecular weights as the matrix molecules. This type of problems can be solved by using high-resolution spectrometers for analysis or by using other matrices whose molecular weight will not coincide with the molecular weight of the analytes (Shariatgorji *et al.*, 2015; Shrivas *et al.*, 2011).

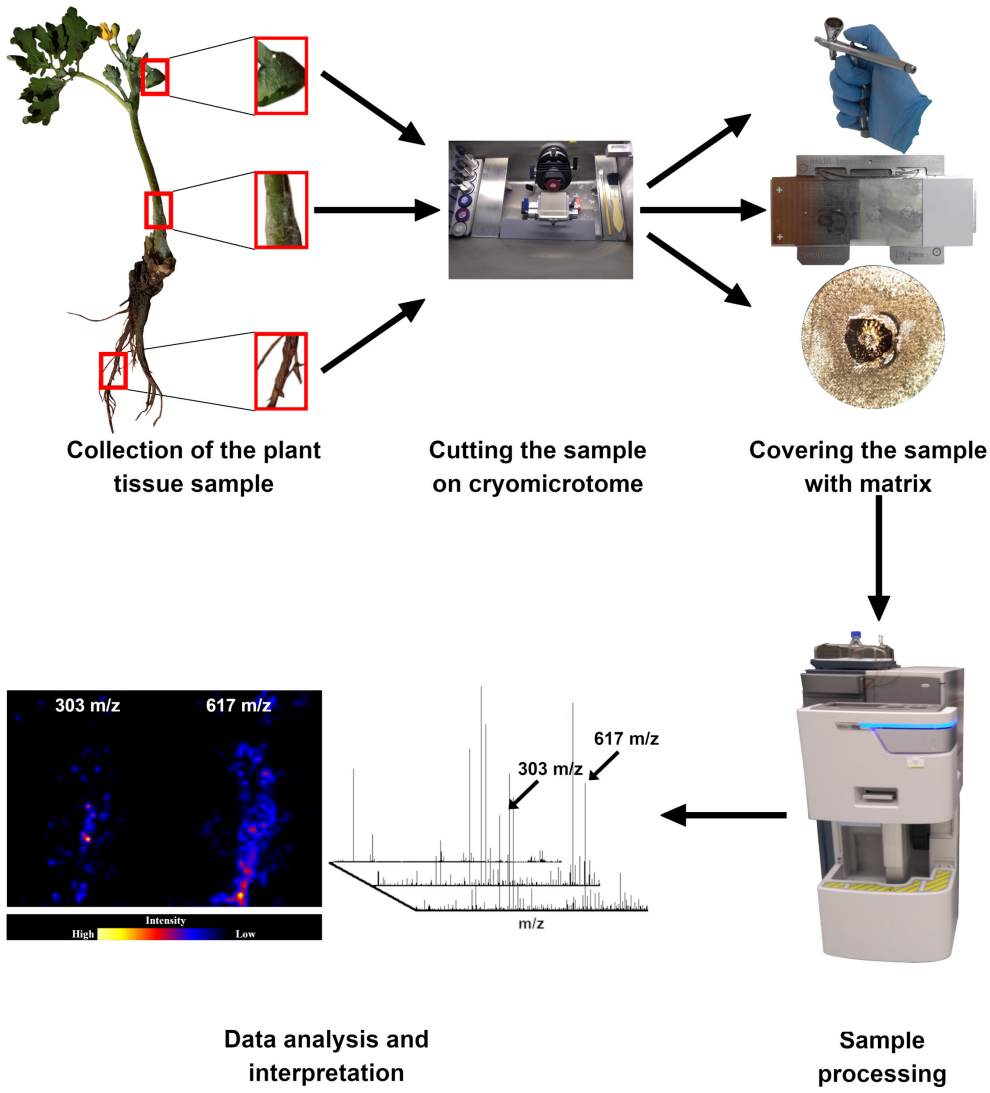

Figure 2. The scheme presents MALDI MSI workflow.

Acquisition of MS images is enabled by ion separation according to their mass to charge ratio (m/z). The most common types of analysers used in MSI are time of flight (TOF) analysers, where the m/z value is determined by the time it takes for ions to pass from the ionization source through the analyser tube to the detector (Yasunaga *et al.*, 2018). TOF analysers assure high acquisition speed and sensitivity, as well as good mass resolution, and therefore they overtop different types of analysers in MALDI MS imaging. However, their resolution is usually up to 20 000 and the time difference for ions reaching the detector can be smaller than 10^{-7}s, therefore using efficient and fast electronic systems is required to reach suitable resolution capacity.

Data analysis

Following the MALDI MSI experiment, identification of the metabolites is performed. Raw spectral data obtained during analysis are processed by the MSI software. After selection of specific m/z of compounds of interest, they can be separated from the matrix ions and visualized by spectral images. During processing of the data they are normalized, smoothed and the baseline correlation is determined (Norris *et al.*, 2007). The MALDI MSI workflow is presented in Fig. 2.

MALDI MSI APPLICATIONS TO INVESTIGATE PLANT METABOLITES

MALDI MSI is a non-selective technique for visualization of metabolites. This feature enables visualization of all of the ionized metabolites in one run. MALDI MSI gives an inside view of the wide variety of plants' primary and secondary metabolites.

Despite the fact that preparation of plant samples might be problematic, almost all organs might be studied using MALDI MSI. This includes: leaves, flower buds, stems, roots, root nodules and fruits (Aziz *et al.*, 2017; Becker *et al.*, 2014; Nakamura *et al.*, 2017; Sarsby *et al.*, 2012; Taira & Ikeda, 2010; Velickowic *et al.*, 2018).

Primary metabolites' visualization

One of the plants' primary metabolites that can be studied with this technique are proteins. Although there are not many reports on the plant proteins, there were several proteins visualized using MALDI MSI, e.g. the allergenic non-specific lipid transfer protein in tomato, or the allergenic non-specific lipid transfer protein in peach (Bencivenni *et al.*, 2014; Cavatorta *et al.*, 2009).

MALDI MSI is a technique of choice for investigation of the plants' lipidomics. It allows to study distribu-

tion of many lipid groups, such as phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid) and triacylglycerols (Berisha *et al*., 2014; Horn *et al*., 2012; Horn & Chapman, 2014).

MALDI MSI also enables visualization of carbohydrates, such as hexoses and hexose polymers (Robinson *et al*., 2007; Ye *et al*., 2013).

Secondary metabolites' visualization

Plant hormones are substances that regulate growth and development of plants. Cytokinin and abscisic acid are the plant hormones that have been visualized so far (Klein *et al*., 2015; Shiono *et al*., 2017). However, some of the plant hormones have not been visualized yet. These include: ethylene, auxins, gibberellins and jasmonates. The reason why plant hormones are so rarely reported, is that they occur at very low concentrations in the plant tissue and they may be lost in the noise peaks. However, if some methods for MALDI MSI plant hormones' imaging would be developed, one could have a greater insight into plant metabolism and aim new fertilizer inventions at plant hormones.

Flavonoids are plant secondary metabolites that exhibit an anti-microbial, anti-insect and antioxidant properties in plants. They also act as attractants for pollinators and for the root nodule forming bacteria (Panche *et al*., 2016). Moreover, flavonoids exhibit a wide variety of positive effects for humans, ranging from anti-inflammatory to cardio-protective effects (Tungmunnithum *et al*., 2018). Many flavonoids can be visualized by using MALDI MSI, e.g. formononetin, chyroseriol or velutin (Kuo *et al*., 2019; Ye *et al*., 2013).

Phenolic acids are metabolites that play a role in the plant defense systems and exhibit antioxidant properties (Kulbat, 2016). The latter also refers to humans – they have high antioxidative properties (Lin *et al*., 2016). They can be easily detected, and they also exhibit ionization properties – some of them are used as MALDI matrices (e.g. DHB, CHCA, sinapinic acid).

Alkaloids are a group of substances that mostly exhibit toxic or psychoactive properties in animals (Diaz, 2015). They are usually produced in order to protect the plants from herbivores. Despite toxic properties of alkaloids, some of them are used at low concentrations as medicines, e.g. vinblastine is used as part of an anticancer therapy (Smith *et al*., 2001). Alkaloids are quite easily visualized with MALDI MSI due to their alkaline properties. Some of them were already studied, e.g. magnoflorine or acetyltropine (He *et al*., 2019; Marques *et al*., 2018).

3D plant tissue imaging

Recent studies revealed that it is possible to create 3D images visualizing lipidomics in plant tissues with the use of advanced computational techniques (Sturtevant *et al*., 2017). Despite the fact that this experiment was done only for lipids, it can be also done for other plant metabolites. A limitation of such research is the time required for analysis.

MALDI MSI LIMITATIONS

Despite many advantages of MALDI MSI, it also has some limitations. One of them is the use of the thaw-mounting technique, which might result in slight displacement of metabolites and slight change in the metabolic profile of the samples. Moreover plant tissue is very effortful to work with because of its heterogeneity throughout the sample. Differences in thickness and density of plant tissues could be troublesome in cryosectioning for an untrained user. Furthermore, biological samples (especially plant tissues) are characterized by a very complex chemical composition. Therefore, one of the major advantages of MALDI MSI – its lack of selectivity, is also one of its limitations. In this technique most of these compounds are detected throughout the whole sample, which results in spectra with a very high amount of signals. Analysis of such results requires some knowledge and experience. At the beginning of work with plant metabolites, MALDI MSI can be substituted by slightly easier techniques, such as HPLC or classical LC-MS. These methods provide an opportunity to identify metabolites, but unfortunately information on their distribution would be lost.

SUMMARY

MALDI MSI is a technique that provides great amount of information about metabolomics in plant tissues. Because of its non-selectivity, it provides the tools for complex studies of a wide variety of metabolites form different chemical groups. Information obtained by MALDI MSI studies can provide insights into metabolic changes in plants treated with different growth stimulators or provide information about the best way to use a given plant in order to obtain this plant's secondary metabolites.

Conflict of interest statement

The authors declare no conflict of interest.

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