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MINI-REVIEW ARTICLE

Mass Spectrometry Imaging to map metabolites in plant-microbe interactions: grapevine as a case study

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ABSTRACT

Every year, viticulture faces several disease outbreaks caused by pathogens with different life cycles and modes of action, and due to climate changes, the appearance of new pathogens is a concern for viticulture. To cope with the different pathogenic microorganisms, the plant must recognise the invading agent and arm itself with an arsenal of defence reactions, including the accumulation of antimicrobial metabolites.

The modulation of plant metabolites is one of the first responses to biotic factors. Their rapid synthesis can greatly contribute to strengthening plant defence allowing it to adapt, minimise pathogen colonisation and survive. Despite the scientific community's efforts to characterise the grapevine defence responses to pathogens, the molecules involved in pathogen control and their specific localisation remain to be deciphered.

Mass Spectrometry Imaging (MSI) analytical techniques enable one to visualise and map the spatial distribution of numerous biological molecules within plant tissues allowing a better understanding of their biosynthesis, localisation and function.

In this review, we explore the applications of MSI techniques to the study of plant/microbe interactions focusing on grapevine studies. This review opens new doors to gain a comprehensive understanding of the dynamics and variations of metabolite profiles in grapevine organs, at different developmental stages and under various stress conditions. This knowledge is crucial for elucidating the role of specific metabolites in grapevine defence mechanisms, and for identifying specific regions of high or low metabolite production, which can contribute to targeted breeding to improve disease resistance traits and have an impact on grapevine productivity and quality.

KEYWORDS: Chemical imaging, Analytical advances, Metabolomics, Metabolite location, Grapevine-Pathogen Interaction, Grapevine defence

INTRODUCTION

Within the different Kingdoms, plants constitute the group that is believed to contain the greatest diversity of metabolites (Fang *et al*., 2019; Wang *et al*., 2019; Desmet *et al*., 2021). A few years ago, researchers estimated that plants synthesised around 200,000 metabolites (Dixon and Strack, 2003; Ernst *et al*., 2014), however, currently it is believed that the chemical diversity of metabolites in plants is much higher and it seems that this number can reach millions of metabolites (Fang *et al*., 2019; Horn and Chapman, 2023). Thus, it is clear that there is still a long way to go to fully understand the metabolite diversity in these organisms.

Metabolites of low molecular weight are of particular importance when trying to understand plants' responses to biotic stresses, as these metabolites are known to be involved in early host defence reactions and in the activation of various signalling cascades (Yu *et al*., 2022). Despite the recent achievements in characterising the grapevine defence responses to pathogens at the metabolic level (Razzaq *et al*., 2019; Castro-Moretti *et al*., 2020; Gupta *et al*., 2022; Muñoz-Hoyos and Stam, 2023; Serag *et al*., 2023), information on spatiotemporal modulation of most defence metabolites is still missing. Some of the most intriguing and challenging issues are undoubtedly knowing which metabolites are modulated, where are these metabolites accumulated, what are their localisation patterns at the site of infection by the pathogen and which metabolites are involved in pathogen restrain.

To fully understand and characterise plants' defence responses, researchers have applied and adapted microscopy techniques, staining and tagged biomolecular interactions with probes to obtain some visual insights into the localisation of a given group of metabolites. However, all of these techniques have some major drawbacks. As an example, most protocols are sample-specific and the analyses are mainly targeted to a specific compound or a class of compounds (reviewed by Hall *et al*., 2002; Schauer and Fernie, 2006; Khoshravesh *et al*., 2022).

In recent years, Mass Spectrometry Imaging (MSI) techniques have emerged and revolutionised the way that researchers can simultaneously visualise the spatial distribution of numerous metabolites in various tissues. Briefly, these techniques raster a defined area of the samples with a localised ion source device, such as a laser, which hits several times specific coordinates (pixels) with a specific frequency. For each location, ions are generated and measured, leading to the obtention of a unique mass spectrum specific to each pixel analysed. Then, all mass spectra are combined and, based on the intensity obtained for each ion in each position, an ion image is reconstructed representing the spatial distribution of the *m/z* values. This image can be then compared to an optical image of the sample, providing information on the actual location of the metabolites.

Several extensive reviews on plant MSI have been published in the last decade, focusing on plant tissue sample preparation, different mass analysis techniques, ionisation sources and data analysis (Bjarnholt *et al*., 2014; Boughton *et al*., 2016; Ho *et al*., 2017; Ajith *et al*., 2022; Horn and Chapman, 2023; Parker *et al*., 2023). However, when focusing on plant-microbe interactions there is a gap, and up to our knowledge, none were emphasised on the application of MSI techniques to study grapevine-microbe interactions.

This review is divided into two main sections. The first section is focused on the application of MSI techniques to investigate metabolic patterns and localisation in different plants and their interactions with microbes, with the exception of grapevine. All the works performed in grapevine by MSI are detailed in the second section, with a specific focus on grapevine-pathogen interactions.

METABOLIC SNAPSHOT OF PLANT-MICROBE INTERACTIONS THROUGH MSI

The study of plant-microbe interactions is not recent. Despite, countless published works reporting the profiling, identification, quantification and cellular dynamics of metabolites during different interactions, there is still no clear information regarding the localisation of metabolites of interest, the sites of action of the anti-microbial metabolites and whether the metabolites are plant or microbe specific.

To answer these questions, MSI has been used and, due to the dramatic results obtained by different techniques, MSI has been considered as an emerging and useful technique in the study of plant-microbe interactions (Tenenboim and Brotman, 2016; Dong and Aharoni, 2022). The application of MSI approaches to plant-microbe interactions started in the early 2010s, and around 20 studies have been published to date (Table 1 and Table 2).

Ye *et al*. (2013), as well as Gemperline *et al*. (2015), studied the symbiotic interaction between *Medicago truncatula–Sinorhizobium meliloti* to characterise the different metabolites produced and exchanged during nitrogen fixation. The use of matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI MSI) enabled the detection of a wide range of metabolic classes, from carbohydrates and flavonoids to amino acids and lipids (Ye *et al*., 2013). Using MSI, the authors have shown that these metabolites displayed distinct distribution patterns in roots and nodules upon the interaction. Different specific metabolites were identified in the nodule region, while others were only present in the roots. Interestingly, the authors also reported a difference in the distribution among compounds from the flavonoids class. The identified flavonoid formononetin and its glucoconjugated form displayed a greater abundance in the roots, whereas the afrormosin-7-*O*-glucoside-6"-*O*-malonate accumulated more in the nodule region. The authors suggested that the distinct distributions were not only due to the diversity of flavonoid molecules but also to their functions (Ye *et al*., 2013).

TABLE 1. MSI studies of plant-microbe interactions (except grapevine-microbe interactions).

Plant	Sample	Kingdom	Pathogen	MSI Technique	Reference
Medicago truncatula	Roots	Bacteria	*Sinorhizobium meliloti*	MALDI-TOF/TOF MS	Ye *et al.* (2013)
Tomato (*Solanum lycopersicum*), *Arabidopsis thaliana* and Tobacco	Roots	Bacteria	*Bacillus amyloliquefaciens*	MALDI-TOF/TOF MS	Debois *et al.* (2014)
Tomato (*Solanum lycopersicum*) *Arabidopsis thaliana*	Leaves and roots	Bacteria	*Bacillus amyloliquefaciens*	MALDI-TOF/TOF MS	Debois *et al.* (2015)
Tomato (*Solanum lycopersicum*)	Leaves	Fungi	*Cladosporium fulvum*	LAESI Q-TOF MS	Etalo *et al.* (2015)
Medicago truncatula	Roots	Bacteria	*Sinorhizobium meliloti*	MALDI-Q	Gemperline *et al.* (2015)
Basil (*Ocimum sp.*)	Leaves	Fungi	*Fusarium oxysporum f. sp. basilici*	DESI LTQ MS	Hemalatha *et al.* (2015)
Rice (*Oryza sativa*)	Leaves	Bacteria	*Xanthomonas oryzae pv oryzae*	(NALDI) MALDI LTQ-Orbitrap	Klein *et al.* (2015)
Pea (*Pisum sativum*)	Pods	Fungi	*Fusarium solani f. sp. phaseoli*	MALDI Q-TOF MS	Seneviratne *et al.* (2015)
Sweet orange (*Citrus sinensis* (L.) Osbeck) grafted in Rangpur lime (*Citrus limonia Osbeck*) rootstocks	Leaves and stems	Bacteria	*Xylella fastidiosa*	MALDI-TOF/TOF MS	Soares et al. (2015)
Potato (*Solanum tuberosum* L.)	Potato sprouts	Fungi	*Pythium ultimum*	DESI LTQ MS	Tata et al. (2015)
Arabidopsis thaliana	Leaves	Bacteria	*Sphingomonas melonis, Pseudomonas syringae pv. Tomato and Methylobacterium extorquens*	MALDI LTQ-Orbitrap	Ryffel et al. (2016)
Tomato (Santa Cruz cultivar)	Roots	Nematode	*Meloidogyne incognita*	MALDI-TOF/TOF MS	Barbosa et al. (2018)
Wheat (cultivar Florence-Aurore)	Roots	Fungi	*Fusarium graminearum*	AP-SMALDI MS	Bhandari et al. (2018)
Soybean (*Glycine max* (L.) Merr.)	Roots	Bacteria	*Bradyrhizobium japonicum*	LAESI-Q-TOF MS	Agtuca et al. (2020)
Sweet orange (*Citrus sinensis* (L.) Osbeck) grafted in Rangpur lime (*Citrus limonia Osbeck*) rootstocks	Leaves	Bacteria	*Candidatus Liberibacter asiaticus*	DESI Q Orbitrap	de Moraes Pontes et al. (2020)
Barley	Leaves	Fungi	*Magnaporthe oryzae*	IR-MALDESI Q Orbitrap	Kalmar et al. (2020)
Soybean (*Glycine max* (L.) Merr.)	Germinating seeds	Fungi	*Aspergillus oryzae*	MALDI-TOF MS	Abe et al. (2021)
Rice (*Oryza sativa*)	Leaves	Fungi	*Magnaporthe oryzae*	MALDI-TOF MS	Komkleow et al. (2021)
Wheat (*Triticum durum* and *Triticum aestivum*)	Kernels	Fungi	*Fusarium spp.*	AP-SMALDI Q Orbitrap	Righetti et al. (2022)

TABLE 2. MSI studies in grapevine-microbe interaction.

Grapevine genotype	Sample	Pathogen	MSI Technique	Reference
Cabernet Sauvignon	Leaves	*P. viticola*	LDI-TOF MS	Hamm *et al.* (2010)
Cabernet Sauvignon	Leaves	*P. viticola*	MALDI-TOF MS	Becker *et al.* (2014)
Hybrid genotypes susceptible to *P. viticola* (*Muscadinia rotundifolia* x *Vitis vinifera* cultivars)	Leaves	*P. viticola*	MALDI-TOF MS	Becker *et al.* (2017)
Trincadeira	Leaves	*P. viticola*	MALDI FT-ICR MS	Maia *et al.* (2022)
Chardonnay	Leaves	*B. cinerea*	MALDI FT-ICR MS	Maia *et al.* (2023)
Red Globe	Dual solid culture medium	*Lasiodiplodia theobromae*	MALDI-TOF MS	Saucedo-Bazalar *et al.* (2023)

Gemperline *et al*. (2015) detected metabolic differences relevant to nitrogen fixation when comparing wild-type (Jemalong A17) and mutant (*dnf1-1*) Medicago with *S. meliloti* wild-type (Rm1021) and mutant [VO2683 (*fixJ2.3:Tn5-233*)]. Although different mutants were tested, the combination of the wild-types of both organisms proved to be the best model for examining nitrogen fixation, as several metabolites known to be important in nitrogen fixation, were only mapped in the Jemalong A17-Rm1021 combination.

In 2014, Debois and co-workers applied MALDI MSI to study the interaction of tomato, *Arabidopsis* and tobacco with *Bacillus amyloliquefaciens* (Debois *et al*., 2014). The authors sought to understand the variety of antibiotics that can be secreted by this beneficial bacterium during root colonisation. Using MALDI-MSI, it has been possible to spatially monitor the changes in the antibiome secreted by this bacterium and the biofilm formed on the roots. The results demonstrated that the secretome was mainly composed of lipopeptides, particularly surfactins, and their accumulation patterns were quite similar regardless of the plant. One year later, the same group pinpointed that although surfactin homologues mainly accumulated around the roots, their spatial patterns varied depending on the length of the fatty acids bound to the peptide moiety (Debois *et al*., 2015). The study also revealed that the different surfactin homologues were not produced at the same time during the first hours (24, 48 and 72 h) of tomato and *Arabidopsis* interaction with *B. amyloliquefaciens* (Debois *et al*., 2015).

Over the following years, different studies were carried out on different plant species (e.g., pea, Medicago, potato, rice, basil) and on various plant organs (leaves, stems and roots) during challenges with different bacteria and fungi (Table 1).

To contrast with MSI studies conducted on plant-mutualistic interactions, in pea pods, the MALDI-MSI analysis has revealed that upon infection with *Fusarium solani* f. sp. *phaseoli*, pinoresinol monoglucoside and (+)-pisatin accumulated and localised in the epidermal cells of the endocarp, acting as phytoalexins (Seneviratne *et al*., 2015). Also using MALDI-MSI, Soares and colleagues studied the localisation of hesperidin, known for its toxic properties against *X. fastidiosa*, in transversal sections of *Citrus* petioles and leaves (Soares *et al*., 2015). The results showed a high accumulation of hesperidin in the infected tissues compared to the healthy ones. MALDI MS/MS was performed to unambiguously confirm the presence and distribution of hesperidin in infected tissues. Hesperidin was detected in both petioles and leaves, throughout tissues, and mainly in the vessel regions. Interestingly, since hesperidin was also detected in healthy tissues, but with low intensity, the authors proposed that hesperidin acted as a phytoanticipin in *Citrus* (Soares *et al*., 2015).

The interaction between *Arabidopsis thaliana* leaves and different bacteria: *Sphingomonas melonis*, *Pseudomonas syringae* pv. Tomato and *Methylobacterium extorquens* were also studied to get new insights into the consequences of bacterial colonisation and the resulting changes at the metabolite level (Ryffel *et al*., 2016). The major modifications observed were linked to an increase in the accumulation of disaccharides (mainly sucrose) and adenosine diphosphate in Arabidopsis leaves by *S. melonis* and *P. syringae* and to a lesser extent by *M. extorquens*. Through MALDI-MSI experiments, the authors were also able to identify changes in phytoalexin biosynthesis and arginine metabolism, with a particular production of agmatine.

In tomato root galls induced by the nematode *Meloidogyne incognita*, MALDI enabled the detection and mapping of lipid molecules, specifically glycerophospholipids, suggesting their important role in the process of gall infection, nematode survival and development (Barbosa *et al*., 2018). MALDI-MSI was also recently used to identify the sites of phytoalexin accumulation in germinating soybeans (seed and roots sections) inoculated with *Aspergillus oryzae* (Abe *et al*., 2021) and in rice leaves

infected with *Magnaporthe oryzae* (Komkleow *et al*., 2021). In soybeans, glyceollins were produced only on seed coats and germinated roots, though in rice leaves, five phytoalexins were detected only at the infection site.

Upon application of MSI to study plant-microbe interaction, one important point to consider is sample preparation. It is quite common that a sample preparation protocol, optimised for a given plant species, is not suitable for other plant species. This was highlighted by Klein *et al*. (2015). Although the "imprinting method" was adequate for the study of soybean leaves, it was not appropriate for the analysis of rice leaves. Klein *et al*. (2015) developed and applied a new "fracturing method" compatible with MSI. During the interaction of rice with *Xanthomonas oryzae* pv *oryzae*, a decrease in intensity of phosphocholine, sucrose, chlorophyll-a fragment and monogalactosyldiacylglycerol was observed, especially at the site of infection, in susceptible rice leaves compared to resistant ones. A high abundance of rice phytoalexins momilactones (momilactone-A and momilactone-B) and phytocassanes (phytocassane-A, D, or E, phytocassane-B and phytocassane-C) was also observed at the bacteria infection site of resistant leaves.

Although the MALDI technique by itself appears to be the preferred method for studying plant-microbe interactions, some researchers have used a combination of techniques with MALDI, such as matrix-assisted laser desorption electrospray ionisation (MALDESI) (Kalmar *et al*., 2020), or took another route and have employed other MSI techniques as desorption electrospray ionisation mass spectrometry (DESI), laser-ablation electrospray ionisation (LAESI), or scanning microprobe matrix-assisted laser desorption/ionisation (SMALDI) (Etalo *et al*., 2015; Hemalatha *et al*., 2015; Tata *et al*., 2015; Agtuca *et al*., 2020; Bhandari *et al*., 2018; de Moraes Pontes *et al*., 2020; Righetti *et al*., 2022).

MALDESI is a hybrid approach which combines MALDI and electrospray ionisation (ESI). It was used with an infrared laser to study the metabolic interaction of barley infected by *Magnaporthe oryzae* (Kalmar *et al*., 2020). With this technique authors imaged the course of infection for 8 days and through the application of an advanced polarity switching method, it was possible to detect compounds which ionise in both ionisation modes. A defence metabolite of barley, serotonin, was identified as well as different metabolites belonging to the melanin pathway. More importantly, the authors suggested that such method could be used to characterise compounds associated only with barley and/or *M. oryzae* during infection.

DESI was first employed to study the responses of young and mature basil leaves to the pathogen *Fusarium* (Hemalatha *et al*., 2015). Several metabolite ions were detected in both young and mature infected basil leaves, namely fusaric acid, one of the primary toxic metabolites of any *Fusarium* pathogenic species, only detected in infected samples. More importantly, this pathogenic metabolite was detected in asymptomatic infected leaves, demonstrating the feasibility of using DESI MSI as a simple and rapid method of detection of the infection. The analysis of potato sprouts by DESI showed that, during infection, there is a clear modulation of the accumulation of different potato toxins, mainly from the class of glycoalkaloids. Some of these glycoalkaloids increased while others decreased. Interestingly, although only compounds from the same class were investigated, glycoalkaloids were shown to be differentially distributed in the infected sprout (Tata *et al*., 2015). DESI was also applied to *Citrus sinensis* grafted onto infected *Citrus limonia* rootstocks (infected by grafting with budsticks carrying the bacteria *Candidatus* Liberibacter asiaticus) (de Moraes Pontes *et al*., 2020). Three types of leaves were tested: healthy, asymptomatic and symptomatic. Based on DESI-MSI analysis, several metabolites were detected which enabled the authors to distinguish leaves in these three stages. More importantly, some biomarkers already described for this disease (nobiletin, phenylalanine, sucrose and quinic acid) were detected only in the infected leaves, indicating that DESI can be used for a rapid and real-time diagnosis, needing no sample preparation.

Etalo *et al.* (Etalo *et al*., 2015) and Agtuca *et al.* (Agtuca *et al*., 2020) used LAESI MSI to analyse, respectively, tomato leaves infected with *Cladosporium fulvum* and soybean roots inoculated with *Bradyrhizobium japonicum*. Etalo and co-authors observed a low accumulation (almost undetectable) of the antifungal glycoalkaloid α-tomatine in infected tomato leaves compared to healthy ones. The combination of LAESI with LC-MS showed an increase in tomatine and confirmed previous results which demonstrated that *Cladosporium fulvum* converts the antifungal glycoalkaloid α-tomatine into its non-toxic form, tomatine. Agtuca and co-workers tested different nitrogen-fixing strains of *B. japonicum* and tracked the distribution of different metabolites in roots. The spatial distribution of two metabolic markers of infection, soyasaponin βg and heme B, was determined. With both the wild-type and the *B. japonicum nifH* (mutant bacteria), soyasaponin βg was detected in the outer layers of roots, whereas, for heme B, a high abundance in the infection zone was only observed for the wild-type. Next, the authors carried out a broader metabolomics analysis using the MSI data to search whether it was possible to correlate the spatial distribution of metabolites and their respective metabolic classes. Authors suggested that the metabolic differences observed in the inner part of the roots, mainly more visible upon the interaction with the wild-type bacteria, were associated to metabolites essential for nitrogen-fixing symbiosis and organogenesis.

Atmospheric-pressure SMALDI-MS imaging was combined with optical microscopy to investigate the interaction between wheat and *Fusarium graminearum* (Bhandari *et al*., 2018). The root-shoot junction was studied after 10, 14 and 21 days after inoculation. With this work, authors showed new insights into key metabolites involved in wheat defence responses, which were induced independently of the plant organ or the developmental stage. More interestingly, using AP-SMALDI-MS imaging authors were able to observe the spatio-temporal metabolic dynamics, particularly at

plant-pathogen interaction zones. This defence dynamic was locally induced at interaction sites, suggesting that defence metabolites are strategically induced by the host to control pathogen colonisation. These results indicated that the accumulation of defence metabolites is only induced by necessity in the host plant and that they accumulated locally and temporally at the infection sites. Most recently, SMALDI MSI has also been used to visualise the tissue distribution of certain metabolites, already reported to play a role in mediating the wheat (*Triticum durum* and *Triticum aestivum*) response to Fusarium Head Blight disease. Although different classes of metabolites were mapped, the lipid class was the one that presented the most significant changes with different spatial distributions according to its subclasses (Righetti *et al*., 2022). Diacylglycerols (DGs) were only found in infected kernels and localised in the outer layers of the seeds, while monogalactosyl-diacylglycerol (MGDG) and digalactosyl-DG (DGDG) showed an opposite trend, being only present in the non-infected kernels. The accumulation of MGDG and DGDG was explained by the *Fusarium* infection route, while for DGs the authors suggested two reasons for this accumulation: either (1) DGs are being used by the pathogen, since DGs have already been reported to be essential for the growth and development of *Fusarium* spp., or (2) DGs serve as plant defence compounds being used as building blocks of major galactolipids in chloroplasts and endoplasmic reticulum.

MSI APPLIED TO GRAPEVINE-MICROBE INTERACTIONS

Cultivated on over 7.4 million hectares, *Vitis vinifera* L., a cornerstone crop of temperate climates, faces a growing struggle against diseases. Most of the premium grapevine cultivars used in winegrowing, such as the European *Vitis vinifera* cultivars, are affected by an array of pathogens throughout the growing season, leading to substantial production losses. Downy mildew, powdery mildew, and grey mould are some of the most significant threats. However, due to climate outbreaks of these established diseases are common and new diseases are emerging (e.g. Pierce's disease). These microbes with different life cycles (biotrophic, necrotrophic and hemibiotrophic) and infection strategies, affect the vegetative grapevine cycle, grape production and harvesting.

A better understanding of the complex relation between grapevine and pathogens is crucial to define sustainable control measures. Although many advances have been given in the last decades, our current knowledge on the modulation of metabolite biosynthesis and catalysis, particularly on metabolite localisation is scarce.

To decipher these responses, researchers have focused on analysing pools of infected plant samples at given time-points and pathogens themselves through targeted and/or untargeted approaches. In recent years, certain metabolites have been identified as possible biomarkers of grapevine tolerance to pathogens and others were even associated to specific diseases (Jeandet *et al*., 2002; Batovska *et al*., 2008; Batovska *et al*., 2009; Chitarrini *et al*., 2017; Viret *et al*., 2018; Ciubotaru *et al*., 2021; Jeandet *et al*., 2021; Ciubotaru *et al*., 2023). Nevertheless, the specific location of these metabolites still remains unknown. To uncover the spatial distribution of metabolites in grapevine upon pathogen challenge, mass spectrometry imaging has been used (Table 2).

MSI studies on grapevine started in 2010 with Hamm and coworkers (Hamm *et al*., 2010). These authors used laser desorption/ionisation time-of-flight mass spectrometry (LDI-ToFMS) to study the surface of *V. vinifera* 'Cabernet Sauvignon' leaves infected with *P. viticola* to detect stilbene compounds (phytoalexins). By LDI analysis and though authors did not use any sort of sample preparation or matrix, they were able to easily generate several MS images with good spatial resolution on a small portion of the leaf. Besides the detection of both resveratrol and pterostilbene, this approach made it possible for the first time to identify the localisation of stilbene phytoalexins in grapevine leaves. These compounds were detected close to *P. viticola* lesions (Hamm *et al*., 2010).

A few years later, the same interaction was studied using MALDI (Becker *et al*., 2014), with a focus on stilbene phytoalexins. Through the application of a technique which depends on the application of a matrix on the samples, in this study the MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was used, authors reported the detection not only of monomeric phytoalexins such as resveratrol and piceid but also more complex dimeric phytoalexins like viniferins (e.g., ε-viniferin, ω-viniferin and δ-viniferin). In fact, the authors mentioned that by using MALDI, the sensitivity of the detection of the dimeric viniferins increased. After examining the spatial distribution of these compounds in the infected leaves, it was concluded that the distribution pattern of phytoalexins was specific to each stilbene or each class of stilbenes. Viniferins were mainly detected around the veins of the leaf, while resveratrol and piceid were scattered on the leaf surface. Taking into account that the stilbene phytoalexin monomers, resveratrol and piceid, are much less toxic against *P. viticola* than viniferins (Pezet *et al*., 2003; Pezet *et al*., 2004), these results led the authors to speculate that these differences were linked to the differential antimicrobial activities of those compounds towards *P. viticola*. The same group investigated in more detail the spatial localisation of these stilbenes (Becker *et al*., 2017). The distribution of stilbene phytoalexins was non-homogeneous as previously reported, demonstrating the repeatability of this technique. Moreover, through the integration of data from MALDI and fluorescence microscopy, it was possible to highlight that the accumulation of stilbene phytoalexins was regularly spaced and distributed across the entire leaf surface. These results were associated with a higher stilbene content in the stomata guard cells, through which *P. viticola* enters and initiates the infection process.

More recently, MALDI was also used to study *Vitis vinifera* 'Trincadeira' grapevine leaves infected with *P. viticola* (Maia *et al*., 2022). This work has underlined the challenges of using field leaves in sample preparation for MSI, as

trichomes hindered matrix deposition and, consequently influenced MSI analysis. Results revealed that sucrose mainly accumulated in infected samples compared to control and that this accumulation was more intense in the leaf veins. This distribution was associated with the development of *P. viticola* infection structures, primary hyphae and mycelium, which grew enclosed within the veins of the leaves.

In addition to *P. viticola*, other grapevine-pathogen interactions were also studied by MSI. Maia and co-workers reported the detection of eight *m/z* values corresponding to deprotonated grapevine stilbene phytoalexins on the interaction between grapevine leaves with *B. cinerea* (Maia *et al*., 2023). Both monomeric stilbenes, such as resveratrol, piceatannol and piceid, and oligomeric forms such as α-viniferin, miyabenol and vaticanol, were detected. The localisation of stilbene phytoalexins was investigated and, in agreement with the results of other studies, stilbene compounds were found to accumulate in areas close to the sites of infection of the pathogen, although the pattern of accumulation of stilbenes differs from that already described. By investigating the spatial distribution of two time-points following *B. cinerea* infection, the study revealed for the first time that the accumulation patterns of the stilbene phytoalexins can vary during the infection period. Moreover, the specific accumulation patterns of each phytoalexin varied depending on their chemical complexity, i.e. monomeric forms were located around the infection sites and in the main veins of the leaf, while the oligomeric forms were confined to the main site of infection (Maia *et al*., 2023).

The control of *Lasiodiplodia theobromae*, a grapevine trunk pathogen, by the endophytic *Bacillus* strains, has also been reported (Saucedo-Bazalar *et al*., 2023). Unlike other studies, this one did not use any plant organ. In fact, the authors evaluated the biocontrol potential of two endophytic bacteria (*Bacillus velezensis* M1 and *Bacillus amyloliquefaciens* M2) through the confrontation of the pathogen with these two microorganisms in a solid culture medium followed by MALDI analysis. This approach enabled to visualise the inhibition and degradation of the mycelium of *L. theobromae* at the interface between the two microorganisms, this behaviour possibly being linked to the greater accumulation of antifungal lipopeptides (fengycin and mycobacillin) in the interaction zone.

CONCLUSIONS AND FUTURE APPLICATIONS OF MSI TO GRAPEVINE

The distribution of metabolites, in the different plant tissues, can be highly heterogeneous. This heterogeneity is quite important to consider when attempting to fully understand the intricate metabolic responses of plants to pathogens. Metabolites have rapid turnover rates and express quick responses to any type of changes (abiotic, biotic, internal or external) allowing researchers to have a real snapshot of the metabolic state of a biological system. Furthermore, since they constitute the end products of cellular processes they represent the physiological state of plants at a given time. It is therefore essential to continue the optimisation of the sample preparation protocols, the improvement of MSI instruments, to test already existing methods and the development of analysis software, to capture these dynamic changes in real-time.

Bringing together all the studies mentioned in the previous sections of this paper, it is clear that MSI techniques can provide a multi-dimensional analysis of any plant tissue. In addition to providing the metabolic profile of the samples in a relatively high-throughput manner, they allow the localisation of the detected metabolites with a high spatial resolution. Therefore, the application of these techniques to the study of the interactions between grapevine and its pathogens is essential to get information on the actual localisation of its defence metabolites.

It is clear from the various studies discussed throughout this review that one of the main advantages that stands out by using MSI techniques to study different pathosystems is the discovery of novel plant metabolites with anti-microbial properties against pathogens. As it was shown, these techniques can be applied to the identification of new phytoalexin oligomers and other antimicrobial compounds. Moreover, these techniques provide valuable insights on the activation of already described defence pathways as well as facilitating the discovery of new metabolic pathways.

Another point to emphasise is that these techniques enable the determination of the chemical formulas of these metabolites with great confidence, allowing researchers to propose chemical reaction mechanisms and to understand signalling pathways implemented in response to pathogens.

However, despite the major progress that has been made from a biological point of view, many questions remain unanswered. The majority of the described studies have pointed out that anti-microbial metabolites mainly accumulate around the pathogen's infection sites. Nevertheless, their spatial distributions depend on different factors: the time elapsed after infection; the development of the infectious structures of the pathogen; the pathogen life cycle; the mode of infection of the pathogen; the chemical class of the compounds studied and their chemical complexity. In view of the information gathered, it is clear that the spatial distribution of these compounds is very diverse and that comparative studies on their localisation must take all this diversity into account.

Also, the application of MSI to grapevine has been limited to its interaction with *P. viticola*, *B. cinerea* and *Lasiodiplodia theobromae* and mainly to the determination of stilbene phytoalexins in the infected tissues. Exploring the interaction of grapevine with other pathogens, such as *Erysiphe necator* and *Xylella fastidiosa*, merits broader knowledge on common and pathogen-specific metabolites; defence metabolites specific to the life cycle of the pathogens; the different patterns of spatial accumulation of specific metabolites in response to these pathogens. Additionally, the majority of the studies published so far have been focused on the analysis of grapevine leaves. Investigating other plant organs, such as stems and grapes, would allow to understand common and

organ-specific anti-microbial metabolites. Furthermore, it is known that the interaction of plants with microbes can have two completely different directions: mutualistic or pathogen interactions. Studying the interaction of grapevine tissues with beneficial micro-organisms, where both systems benefit from each other, would open the way to the identification of metabolites that could help grapevine to overcome certain types of stresses.

From an analytical point of view, improvements in MSI techniques are needed to take full advantage of the information these techniques can provide. Each MSI technique is limited by the preparation of samples, the number of metabolites that can be detected simultaneously in a single sample and the capacity of these metabolites to be ionised. To date, the techniques that have been used to study grapevine-pathogen interactions, are MALDI and LDI. Applying, for example, DESI to study grapevine-pathogen interactions could be interesting since this technique does not require *a priori* any sample preparation and causes limited damage to the sample during analysis, since it uses a charged solvent spray to desorb and ionise the analytes, allowing reuse of the sample after analysis. Combining information obtained from different sample preparation protocols and various ionisation sources could allow researchers to get maximum metabolomic coverage and complementary results.

Furthermore, efforts should be made to improve the confidence level of MS signals' annotation and the development of software's that can handle very complex data and compare different datasets in a user-friendly manner.

Globally, gathering knowledge not only about novel grapevine defence metabolites but also about the sites of their production can help determine the degree of tolerance/susceptibility of grapevine varieties or species to pathogens, accelerate targeted screening assays in breeding programs and define new approaches to control grapevine diseases (such as biopesticides). This will have an impact on grapevine productivity and quality and will lead to a more sustainable viticulture.

TRIBUTE TO PROFESSOR DOCTOR PHILIPPE JEANDET

Aziz Aziz, Marisa Maia, Andreia Figueiredo and Vincent Carré would like to dedicate this manuscript to our colleague and friend, Professor Philippe Jeandet.

Philippe Jeandet was a dedicated Scientist and Professor. His research activities, passion and perseverance led him to publish over 300 articles. He was the world's leading contributor to the discovery of stilbene phytoalexins, promoting their role and benefits, from the chemist's and biochemist's perspectives, particularly in the field of vines and wine. Philippe Jeandet's work is impressive and a source of inspiration for all researchers.

In addition, his daily enthusiasm, positivism and sincerity were some of his undeniable human qualities.

We are incredibly grateful to have had the privilege of working with him and will miss him forever.

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