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REVIEW ARTICLE

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FT-ICR-MS-based metabolomics: A deep dive into plant metabolism

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Abstract

Metabolomics involves the identification and quantification of metabolites to unravel the chemical footprints behind cellular regulatory processes and to decipher metabolic networks, opening new insights to understand the correlation between genes and metabolites. In plants, it is estimated the existence of hundreds of thousands of metabolites and the majority is still unknown. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is a powerful analytical technique to tackle such challenges. The resolving power and sensitivity of this ultrahigh mass accuracy mass analyzer is such that a complex mixture, such as plant extracts, can be analyzed and thousands of metabolite signals can be detected simultaneously and distinguished based on the naturally abundant elemental isotopes. In this review, FT-ICR-MS-based plant metabolomics studies are described, emphasizing FT-ICR-MS increasing applications in plant science through targeted and untargeted approaches, allowing for a better understanding of plant development, responses to biotic and abiotic stresses, and the discovery of new natural nutraceutical compounds. Improved metabolite extraction protocols compatible with FT-ICR-MS, metabolite analysis methods and metabolite identification platforms are also explored as well as new *in silico* approaches. Most recent advances in MS imaging are also discussed.

KEYWORDS

FT-ICR-MS, MALDI-FT-ICR-MS imaging, metabolite profiling, plant metabolism, untargeted and targeted approaches

1 | INTRODUCTION

Metabolomics targets an enormous number of compounds of unknown structure (Fiehn, 2002; Schauer & Fernie, 2006; Sumner et al., 2003). This is an extreme challenge in analytical chemistry when a high number of unknown natural compounds with different properties need to be addressed simultaneously (Ohta et al., 2007).

But what is in fact "metabolomics" and the "metabolome"? The term "metabolomics" originates from metabolic profiling, a definition that dates from the early 1970s by researchers at the Baylor College of Pharmacy (Devaux et al., 1971; Horning & Horning, 1971). Years later, in 1998, Oliver and coworkers proposed the concept of "metabolome" (Oliver et al., 1998). Thereafter, many plant chemists conducted research in this area. In 1999, another concept was proposed by Nicholson and co-workers: "metabonomics," defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological

stimuli or genetic modification" (Nicholson et al., 1999). Next, in 2001, "metabolomics" concept arose defining the "comprehensive and quantitative analysis of all metabolites in a biological system" (Fiehn, 2001). In short, in a biological sample, the metabolome comprises the total metabolite pool of an organism, a tissue and a cell, at a given moment, which can be unrevealed to characterize genetic background and responses to environmental challenges. Within "OMICs" research areas, metabolomics includes the identification and quantification of small molecule compounds, as well as the understanding of the chemical patterns involved in the regulation of the cellular processes in different biological species (Razzaq et al., 2019). Studies in metabolomics are crucial to explore environment–gene interactions, phenotyping, biomarkers identification, and drug detection (Razzaq et al., 2019).

Within the different biological systems, plants are the group that contains the highest diversity of metabolites with thousands of compounds already identified and many still unknown (S. Wang et al., 2019). Hence, it is not only important to develop and improve new analytical techniques and protocols, but also to exploit already existing metabolomic platforms to discover more of the unknown plant metabolome, to explain complex biological pathways and to explore hidden regulatory networks controlling plant growth and development (Castro-Moretti et al., 2020; Chen et al., 2019; Foito & Stewart, 2018; Razzaq et al., 2019; S. Wang et al., 2019).

In plant metabolomics, several techniques have been applied so far, from nuclear magnetic resonance (NMR) (Aranìbar et al., 2001; Choi et al., 2004; Crockford et al., 2006; Viant et al., 2003) to mass spectrometry (MS) (Gowda & Djukovic, 2014). Although nuclear magnetic resonance (NMR) is extremely reproducible and allows absolute quantification of detected signals, it lacks sensitivity as only a limited number of compounds are identified in complex mixtures and the resolution are not comparable to MS techniques. The choice of MS for metabolomics studies has innumerous advantages, namely a higher coverage through the use of separation steps, such as liquid chromatography (LC) or gas chromatography (GC) and capillary electrophoresis, which provide robust platforms for metabolomic studies (Tomita & Nishioka, 2005). Regarding ionization methods in MS, the preferred ionization chemistry tends to be electrospray ionization (ESI) and/or matrix-assisted laser desorption/ionization (MALDI). Both ESI and MALDI are soft ionization methods, which means individual naturally occurring metabolites can be ionized with great sensitivity without fragmentation of the molecules (Gross, 2017; Hiraoka, 2013). Furthermore, these methods enable very sensitive measurements allowing the detection of small levels of biological metabolites (in the pico- or femtomolar concentrations) (Tomita & Nishioka, 2005).

2 | HIGH RESOLUTION PLANT METABOLOMICS: THE CASE OF FT-ICR-MS

For metabolomics analysis, mass spectrometers with high mass resolution, ability to achieve measurements with ppm errors, and ability to differentiate metabolites at the ppb to ppm level, is a prerequisite. These characteristics can only be achieved by using high resolution mass spectrometers, such as time-of-flight (TOF) and Fourier transform (FT) mass spectrometers, including FT ion cyclotron resonance (FT-ICR) and Orbitrap. These mass spectrometers have proven to be the most valuable for analyzing complex mixtures, not only for their mass accuracy and resolution but also due to the fact that direct infusion of the samples without chromatographic separation or derivatization reactions may be achieved (Allwood et al., 2011; Barrow et al., 2005; Haijes et al., 2019).

The majority of the plant-based metabolomics published studies so far use the Orbitrap or TOF equipment (around 700 studies published since 2011—Pubmed, August 11, 2021). The main reason is that most recent TOF equipment's can achieve mass resolution values of 30,000–40,000 (Andrews et al., 2011; Pelander et al., 2011) and the resolution power is not affected by chromatography acquisitions rates (Ghaste et al., 2016; Glauser et al., 2013; Hopfgartner, 2011; S.-G. Park et al., 2021).

On the other hand, FT-ICR and Orbitrap outperform any other commonly used mass spectrometer in terms of absolute resolving power. Orbitrap mass spectrometers are especially useful in shotgun metabolomics as they allow rapid tandem MS spectra acquisition, high mass resolution (up to 240,000) and optional MS^n fragmentation (Ghaste et al., 2016; Herzog et al., 2011; Schuhmann et al., 2011, 2012). Also, these mass spectrometers are capable of rapid polarity switching with high mass accuracy, which simplifies and accelerates the analysis and improves the metabolome coverage (Ghaste et al., 2016; Glauser et al., 2013; S.-G. Park et al., 2021; Schuhmann et al., 2012).

Compared to TOF and Orbitrap, FT-ICR-MS had a slower start in metabolomics. FT-ICR-MS is an equipment with one of the highest resolution power and sensitivity. Its technical development began in the late 1920s, when Ernest O. Lawrence invented the cyclotron, which uses electrical and magnetic fields to accelerate protons to high velocities in a spiral-shaped path before they collide with their target (Comisarow & Marshall, 1996; Lawrence & Livingston, 1932). A few years later, it was also demonstrated that in ICR, the

angular frequency of the circular motion of ions species is independent of the radius they are traveling on (Gross, 2017). This principle was used by John A. Hipple to construct the first ICR mass spectrometer (L.G. Smith, 1951; Sommer et al., 1951). However, the major breakthrough in this technique happened in 1974 when FT was applied to ICR by Alan Marshall and Melvin Comisarow (Comisarow & Marshall, 1974a, 1974b).

Since then, the performance of FT-ICR-MS instruments has steadily improved to reach unprecedented levels of resolving power and mass accuracy (Hendrickson et al., 2015; Kanawati & Schmitt-Kopplin, 2019; D.F. Smith et al., 2018; van Agthoven et al., 2011).

In most mass spectrometers, the sample is introduced in the equipment as a solid, liquid, or gas, depending on the type of ion source used. Then, the ion species of the sample enter the mass analyzer, are detected and a mass-to-charge ratio (*m/z*) for each ion specie is obtained. In the case of FT-ICR-MS analyzers, ion species are usually generated externally in a separate ion source and then injected into a container known as the "ICR cell" (Kanawati & Schmitt-Kopplin, 2019). This cell is a Penning ion trap in which ion species are confined by a strong magnetic field, typically generated using a superconducting magnet, and possess not only excitation but also detection plates (Hiraoka, 2013; Junot et al., 2010).

In the cell, the ion species must have a coherent ion motion (Junot et al., 2010). To achieve that, the ion cloud present in the cell is excited, though the excitation plates. Due to the magnetic field, the ion species are forced on circular orbits by action of the Lorentz force. Once excited, the ions coherently circulate inside the ICR cell, perpendicular to the magnetic field, with an orbital frequency (cyclotron frequency) depending on their respective *m/z* ratios: ion species of equal *m/z* coherently circulate inside the ICR cell. The trajectories of the ion species induce an image current in the detection plates of the cell, which is amplified and stored as a time domain signal, and is composed of a set of frequencies corresponding to the motion of each ion species of a given *m/z* ratio. These currents are then transformed into frequency domain using a Fourier transform, from which the corresponding *m/z* values are calculated and mass spectra are reconstituted (Barrow et al., 2005; Gross, 2017; Hiraoka, 2013; Junot et al., 2010). The Fourier transform is a mathematical operation that transforms one complex-valued function of a real variable into another. In the ICR, the domain of the ions original function is time and after Fourier transformation is the frequency domain (Gross, 2017). Also, to allow a higher resolving power for small molecules a particular ion trap with dynamic harmonization was developed for FT-ICR mass spectrometers (Kostyukevich et al., 2012).

Nowadays, FT-ICR-MS has become one of the best-performing mass analyzers in terms of resolving power (10^5 to $>10^6$), mass accuracy (typically 0.1–2 ppm) and sensitivity (Barrow et al., 2005; Gross, 2017; Hiraoka, 2013). The resolving power and sensitivity of FT-ICR-MS is such that it is possible to detect the naturally abundant elemental isotopes (e.g., ^{13}C, ^{41}K, ^{15}N, ^{18}O, ^{34}S, and ^{37}Cl) and have the isotopic distribution for certain signals. This allows the calculation of highly accurate elemental compositions for the unknown signals, facilitating the selection of potential metabolite candidates before their confirmation by comparisons to analytical standards (Allwood et al., 2011). Such characteristics allow unequivocal mass assignment and the resolution of ion species which currently are not distinguishable with other types of mass spectrometers. As an example, this analytical technique has been used for the study of complex organic matter samples, such as crude oils, and is capable of evaluating around 50,000 molecular formulas in each analysis (Folli et al., 2020; Hughey et al., 2002; D.F. Smith et al., 2018). Another advantage is the wide range of ionization source types that can be applied to FT-ICR-MS: ESI; atmospheric pressure chemical ionization; atmospheric pressure photoionization; MALDI; electron impact and chemical ionization (EI and CI, respectively), allowing a broader application to different kinds of samples (Allwood et al., 2011). Furthermore, it is possible to select the mode of introducing the sample in the FT-ICR-MS.

Although, most of the FT-ICR-MS–based untargeted metabolomics studies use the direct infusion of the sample extracts, FT-ICR-MS has the capability to be coupled to several chromatographic techniques for compound identification and quantification (Barrow et al., 2005; Schrader & Klein, 2004).

Direct infusion mass spectrometry (DIMS) is an attractive approach with several advantages. Data acquisition only takes a few minutes with high throughput experiments and data processing is simpler than LC or GC-MS–based approaches (Junot et al., 2014). Still, this imposes several disadvantages as well. Some studies reported that DIMS based approaches are prone to severe matrix effects and the biological material concentration that is introduced into the MS, needs to be optimized, to have high sensitivity and a correct metabolite detection (Madalinski et al., 2008). If not, tandem MS experiments may be affected due to incorrect selection of the precursor ions. Also, using this approach, ion species of biological relevance could be masked by isomers or isobaric compounds, outlining another drawback (Junot et al., 2014). In addition, one should not disregard the space constraints of the ions directly infused in the FT-ICR cell. This space is limited and therefore the

capacity for ion trapping is also limited. As a consequence, the direct infusion of complex samples in FT-ICR, such as plant extracts, may generate ion suppression, lower mass accuracy and decrease in measured frequencies of the trapped ions (Hohenester et al., 2020; Leach et al., 2012).

Some authors suggest that DIMS could be used as a first screening filter used before chromatographic and spectral methods (Beckmann et al., 2008). In the case of chromatographic separation, it is important to have in mind that the achievable mass resolving power with FT-ICR-MS is proportional to ICR signal transient length (S.-G. Park et al., 2021). In other words, longer transient lengths can yield higher resolving power. This becomes a limitation of FT-ICR-MS coupled to chromatographic separation techniques (S.-G. Park et al., 2021). Nevertheless, this limitation is being addressed by increasing the strength of the magnetic field (Blair et al., 2017; He et al., 2019; D.F. Smith et al., 2018; Walker et al., 2017), using multiple parallel mass analyzers (S.-G. Park et al., 2016, 2017) and/or several frequency detectors (Cho et al., 2017; Nagornov et al., 2014; S.-G. Park et al., 2020; Shaw et al., 2018). For instance, FT-ICR-MS coupled with ultra performance liquid chromatography (UPLC) was used together with stable isotope (^{13}C) allowing background contamination removal with a true positive identification of compounds with biological origin; also it empowered structural isomers discrimination (Giavalisco et al., 2009). The choice between DIMS or LC-MS should take in consideration the high throughput capability and the optimal metabolome coverage.

The application of FT-ICR-MS to plant metabolomics started around the early 2000s and roughly 90 studies have been published until now, the majority being published in the last 10 years. To uncover new metabolites and metabolic pathways, FT-ICR-MS plant metabolomics studies have been focused mainly on untargeted metabolomics approaches (Table 1). A crude plant extract is analyzed and through the different signal patterns of metabolites it is possible to correlate this information with metabolic pathways and other OMICs approaches, allowing for better understanding of plant regulatory systems. Targeted approaches have also been used in plant metabolomics studies using FT-ICR-MS (Table 2) allowing the detection of metabolites belonging to a specific class, metabolic pathway or already known compounds, ranging from medicinal to agrochemicals, increasing plants market value and industrial uses. The application of FT-ICR-MS to plant metabolism goes beyond full plant extracts, and some studies have been published on the investigation of intact plant cells and cell compartments (Tables 3 and 4). Different plant samples' extraction protocols compatible with FT-ICR-MS have been developed and guidelines for FT-ICR-MS application in plant science have been published (Allwood et al., 2011; Barrow et al., 2005; Junot et al., 2010) (Figure 1).

3 | UNCOVERING THE PLANT METABOLOME THROUGH UNTARGETED FT-ICR-MS

Untargeted metabolomics approaches aim for comprehensive analysis of all the measurable analytes in a sample, being the biological significance of each metabolite determined during data analysis and metabolite identification (Roberts et al., 2012). Therefore, the chemical identity of each metabolite in the study is not known a priori. The main aim is to maximize the number of metabolites detected and therefore provide the opportunity to observe unexpected changes. The selection of the right powerful analytical technique, capable of detecting and discriminate hundreds to thousands of metabolites in a complex sample, such as FT-ICR-MS is a step in the right direction to increasing the metabolome coverage (Table 1).

The beginning of untargeted plant metabolomics studies by FT-ICR-MS dates back to 2002 with Aharoni and coworkers applying a high-throughput FT-ICR-MS-based method to detect metabolic modulation in strawberry fruit development and tobacco flowers overexpressing a strawberry MYB transcription factor (Aharoni et al., 2002). Methanol and acetonitrile extracts were used and over 1000 m/z values were detected in those extracts, using a direct infusion of the sample in the FT-ICR-MS. Results have shown not only changes in the levels of a large range of m/z values corresponding to known fruit metabolites, but also revealed novel information on the metabolic transition from immature to ripe fruit. Also, specific m/z values discriminated between transgenic and control plants, among which the cyanidin-3-rhamnoglucoside seemed to have a particular role (Aharoni et al., 2002).

Aharoni and coworkers' pioneer work demonstrated the feasibility and utility of FT-ICR-MS approaches for an untargeted and rapid metabolic plant "fingerprinting," starting a new era for plant metabolomics.

Since then, several FT-ICR-MS–based plant studies have emerged in which different plants, plant tissues, and plant cell compartments were analyzed, metabolite extractions were optimized, and even FT-ICR-MS metabolomics were combined with other OMICs approaches to better reveal gene-to-metabolite networks (Table 1).

One of the main plants analyzed using FT-ICR-MS, with an untargeted approach, has been *Arabidopsis thaliana* (Giavalisco et al., 2008, 2009; Hansen et al., 2019;

TABLE 1 Untargeted FT-ICR-MS plant metabolomics studies (alphabetical order by plant species name)

Plant (species)	Experimental conditions		References
Allium sativum (Garlic) species	DIMS	4.7 T FT-ICR-MS (ESI source; CID)	Maccelli et al. (2020)
Ananas comosus var. *comosus* (Pineapple)	DIMS	9.4 T FT-ICR-MS (ESI source; CID)	Ogawa et al. (2018)
Arabidopsis thaliana	DIMS	7 T FT-ICR-MS (ESI and APCI sources)	Hirai et al. (2004)
	DIMS	FT-ICR-MS (ESI and APCI sources)	Tohge et al. (2005)
	DIMS	7 T FT-ICR-MS (ESI source; SORI-CID)	Oikawa et al. (2006)
	DIMS	7 T FT-ICR-MS (ESI source; SORI-CID)	Ohta et al. (2007)
	DIMS	LTQ-FT-ICR-MS	Giavalisco et al. (2008)
	UPLC	LTQ-FT-ICR-MS	Giavalisco et al. (2009)
	DIMS	7 T FT-ICR-MS (ESI source)	Satou et al. (2014)
	DIMS	7 T FT-ICR-MS (ESI source)	Hansen et al. (2019)
Bellis perennis	UPLC	LTQ-FT-ICR-MS	Scherling et al. (2010)
Celtis iguanaea	DIMS	9.4 T FT-ICR-MS (ESI source)	Martins et al. (2014)
Chrysanthellum americanum	DIMS	12 T FT-ICR-MS (ESI source)	Cao-Ngoc et al. (2020)
Crataegus (Hawthorn)	DIMS	12 T FT-ICR-MS (ESI source)	Cao-Ngoc et al., (2020)
Dimocarpus longan (Longan)	DIMS	9.4 T FT-ICR-MS (ESI source)	Chen et al. (2014)
Eugenia calycina (Red pitanga)	DIMS	9.4 T FT-ICR-MS (ESI source)	Ferreira et al. (2014)
Fragaria x *ananassa*, cv. *Elsanta* (Strawberry fruit)	DIMS	7 T FT-ICR-MS (ESI and APCI sources)	Aharoni et al. (2002)
Knautia arvensis	UPLC	LTQ-FT-ICR-MS	Scherling et al. (2010)
Leontodon autumnalis	UPLC	LTQ-FT-ICR-MS	Scherling et al. (2010)
Lotus corniculatus	UPLC	LTQ-FT-ICR-MS	Scherling et al. (2010)
Mangifera indica (Mango)	DIMS	9.4 T FT-ICR-MS (ESI source; CID)	Oliveira et al. (2016)
Medicago truncatula	LC	FT-ICR MS (ESI source)	Pollier et al. (2013)
Medicago x *varia*	UPLC	LTQ-FT-ICR-MS	Scherling et al. (2010)
Nicotiana tabacum (Tobacco)	DIMS	7 T FT-ICR-MS (ESI and APCI sources)	Aharoni et al. (2002)
	DIMS	7 T FT-ICR-MS (ESI and APCI sources)	Mungur et al. (2005)
Ophiorrhiza pumila (Rubiaceae species)	DIMS	FT-ICR-MS (ESI and APCI sources)	Yamazaki et al. (2013)
Panax ginseng (Korean ginseng)	DIMS	15 T FT-ICR-MS (ESI source);	Park et al. (2013)
	HPLC	15 T FT-ICR-MS (ESI source; CID)	
Populus x *canescens* (Poplar)	DIMS	12 T FT-ICR-MS (ESI source)	Behnke et al. (2010)
		12 T FT-ICR-MS (ESI source)	Way et al. (2013)
	DIMS	12 T FT-ICR-MS (ESI source)	Janz et al. (2010)
Populus euphratica (Poplar species)	DIMS	12 T FT-ICR-MS (ESI source)	Janz et al. (2010)
Populus × *canescens* syn. *P. alba* × *P. tremula* (Poplar species)	DIMS	12 T FT-ICR-MS (ESI source)	Kaling et al. (2015)
Ribes nigrum (blackcurrant)	DIMS	12 T FT-ICR-MS (ESI source)	Cao-Ngoc et al. (2020)
Solanum lycopersicum cultivars (Tomato)	DIMS	4.7 T FT-ICR-MS (ESI source; CID);	Ingallina et al. (2020)
		7 T FT-ICR-MS (ESI source)	
Solanum tuberosum var. Kennebek (Potato)	DIMS	7 T FT-ICR-MS (ESI source)	Aliferis & Jabaji (2012)
Thymus vulgaris (Thyme)	DIMS	7 T FT-ICR-MS (ESI source)	Shahbazy et al. (2020)

(Continues)

TABLE 1 (Continued)

Plant (species)	Experimental conditions		References
Vitis vinifera (Grapevine)	DIMS	9.4 T FT-ICR-MS (ESI source; SORI-CID)	Becker et al. (2013)
	DIMS	7 T FT-ICR-MS (ESI source)	Maia et al. (2016)
	DIMS	12 T FT-ICR-MS (ESI source)	Adrian et al. (2017)
	DIMS	7 T FT-ICR-MS (ESI source)	Maia et al. (2018)
	DIMS	7 T FT-ICR-MS (ESI source)	Maia et al. (2019a)
	DIMS	7 T FT-ICR-MS (ESI source)	Maia et al. (2019b)
	DIMS	7 T FT-ICR-MS (ESI source)	Nascimento et al. (2019)
Vitis vinifera (Grapevine) and *Vitis* species	DIMS	7 T FT-ICR-MS (ESI source)	Maia et al. (2020)
	DIMS	7 T FT-ICR-MS (ESI source)	Maia et al. (2021)
Zea mays (Maize varieties: Aristis, Tietar and PR33P66)	DIMS	12 T FT-ICR-MS (ESI source)	Leon et al. (2009)

Abbreviations: APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; DIMS, direct-infusion mass spectrometry; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LTQ, linear trap quadrupole; T, Tesla; UPLC, ultra performance liquid chromatography.

Hirai et al., 2004; Ohta et al., 2007; Oikawa et al., 2006; Tohge et al., 2005). This model plant organism was one of the first to have its genome sequenced (Arabidopsis Genome Initiative, 2000) and is now one of the best model plants to study gene-to-metabolite correlation through the integrated analysis of gene expression (transcriptomics) and metabolite accumulation (metabolomics) (Bino et al., 2004; Fiehn, 2002; Kopka et al., 2004; Scherling et al., 2010; Sumner et al., 2003).

Hirai and coworkers were the first to study the *Arabidopsis thaliana* metabolome using FT-ICR-MS (Hirai et al., 2004). In their work, transcriptomics was combined with FT-ICR-MS metabolomics to investigate the gene-to-metabolite networks controlling nitrogen and sulfur, and secondary metabolism. They explored the plant whole-cellular processes under sulfur and nitrogen deficiency and understood that plants adapted to nutrient deficiency had a steady-state transcriptome and metabolome. The study opened new insights for a more precise investigation of gene-to-metabolite networks, aiming for functional genomics and better biotechnological application (Hirai et al., 2004). FT-ICR-MS was also applied to study *A. thaliana* metabolites and metabolic pathways after exposure to different concentrations of glyphosate (Ohta et al., 2007) and herbicidal chemical classes (Oikawa et al., 2006), as well as to study the light/dark regulation (Nakamura et al., 2007) and clarify the cytochrome P450 functions (Kai et al., 2009) in cell cultures.

Also, metabolomics studies in transgenic *A. thaliana* plants over-expressing or with loss of function of genes, helped to elucidate and correlate the impact of select genes with the overall metabolism. The combination of FT-ICR-MS metabolomics analysis with other OMICs was also used in *A. thaliana* mutants, to identify key metabolites involved in signaling pathways and to understand their biological roles (Hansen et al., 2019; Tohge et al., 2005). A recent study in *A. thaliana* wild-type and loss-of-function mutant of FER (*feronia*) identified a total of 68 and 52 compounds in positive and negative mode, respectively, with significant differences between wild-type and mutant plants. Arabidopsides (oxylipins) were found to be significantly enriched in the mutant (Hansen et al., 2019).

FT-ICR-MS has also been applied to study the metabolism of other model plants, such as *Nicotiana tabacum* and *Medicago truncatula*, and of different economically important crops and fruits, to gain deeper insights into the physiological responses of plant species (Table 1).

The biological activity of plant metabolic extracts towards several bacterial and fungal strains, their antioxidant effects and use in the treatment of specific medical conditions was also evaluated by FT-ICR-MS (Chen et al., 2014; Ferreira et al., 2014; Martins et al., 2014). FT-ICR-MS metabolomics studies were also applied to valorize several plants as a source of health and nutritional bioactive components (Maccelli et al., 2020; Maia et al., 2019a). As an example, the chemical diversity of eight different hydroalcoholic extracts of white and red crop *Allium sativum* and wild *Allium triquetrum*, *Allium roseum*, and *Allium ampeloprasum*, all originating from the Mediterranean Basin, were evaluated by FT-ICR-MS and 850 and 450 *m/z* values were detected, respectively, by ESI^+ and ESI^-. The annotation of all these *m/z* values covered all of the main classes of primary and secondary metabolites, including amino acids, alkaloids, organic and fatty acids, nucleotides, vitamins, organosulfur

TABLE 2 Targeted FT-ICR-MS plant metabolomics studies (alphabetical order by plant species name)

Plant (species)	Experimental conditions		References
Acanthopanax senticosus Harms	DIMS	7 T FT-ICR-MS (ESI source; SORI-CID)	Zhou et al. (2012)
Allium cepa (Onion)	LC	7 T FT-ICR-MS (ESI source)	Nakabayashi et al. (2013)
	LC	7 T FT-ICR-MS (ESI source)	Nakabayashi et al. (2016)
Allium fistulosum (Green onion)	LC	7 T FT-ICR-MS (ESI source)	Nakabayashi et al. (2016)
Allium sativum (Garlic)	LC	7 T FT-ICR-MS (ESI source)	Nakabayashi et al. (2016)
Arabidopsis thaliana plants	DIMS	9.4 T FT-ICR-MS (ESI source)	Qin et al. (2011)
Artocarpus altilis		FT-ICR-MS (ESI source)	Huong et al. (2012)
Asparagus officinalis cv. Purple Passion	DIMS	9.4 T FT-ICR-MS (ESI source)	Sakaguchi et al. (2008)
Camellia sinensis (Black tea)	DIMS	9.4 T FT-ICR-MS (ESI source)	Kuhnert et al. (2010)
Cerbera manghas	DIMS	FT-ICR-MS (ESI source)	Zhang et al. (2010)
Fallopia convolvulus	DIMS	FT-ICR-MS (ESI source)	Brennan et al. (2013)
Ginkgo biloba	DIMS	HPLC-LTQ-FT-ICR-MS (ESI source; CID)	Beck and Stengel (2016)
Ibervillea sonorae	DIMS	FT-ICR-MS	Vidal-Gutiérrez et al. (2021)
Medicago truncatula	UPLC	FT-ICR-MS (ESI source; CID)	Pollier et al. (2011)
Morus alba (Mulberries)	DIMS	15 T FT-ICR-MS (ESI source)	Y.J. Park et al. (2017)
	DIMS	7 T FT-ICR-MS (ESI source; CID)	Xiao et al. (2017)
Panax ginseng (Red ginseng)	DIMS	7 T FT-ICR-MS (ESI source; CID)	Du et al. (2012)
Piper methysticum (Kava)	DIMS	4.7 T FT-ICR-MS (ESI source; SORI-CID; IRMPD)	Warburton & Bristow (2006)
Polygonum multiflorum	HPLC	LTQ-(7 T)-FT-ICR-MS (ESI source; CID)	Yang et al. (2019)
Salvia miltiorrhiza Bunge (Tanshen)	DIMS	7 T FT-ICR-MS (ESI source; SORI-CID)	H. Li et al. (2008)
Schisandra chinensis	DIMS	7 T FT-ICR-MS (ESI source; CID)	Huang et al. (2007)
	DIMS	7 T FT-ICR-MS (ESI source; CID)	Huang et al. (2008)
Schisandra sphenanthera (Fruits)	DIMS	7 T FT-ICR-MS (ESI source; CID)	Huang et al. (2008)
Solanum lycopersicum cultivars (Tomato)	HPLC	LTQ-FT-ICR-MS (ESI source; CID)	Iijima et al. (2013)
Sorghum bicolor and *Neptunia lutea*	DIMS	15 T FT-ICR-MS (ESI source); 15 T FT-ICR-MS (MALDI source)	Reeves et al. (2020)
Stemona tuberosa		7 T FT-ICR-MS	Khamko et al. (2013)
Vaccinium myrtillus (Bilberries)	DIMS	7 T FT-ICR-MS (ESI source; CID)	Xiao et al. (2017)
Vaccinium oxycoccos (Cranberries)	DIMS	7 T FT-ICR-MS (ESI source; CID)	Xiao et al. (2017)
Vaccinium uliginosum (Blueberries)	DIMS	7 T FT-ICR-MS (ESI source; CID)	Xiao et al. (2017)

Abbreviations: CID, collision-induced dissociation; DIMS, direct-infusion mass spectrometry; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IRMPD, infrared multiple photon dissociation; LC, liquid chromatography; LTQ, linear trap quadrupole; MALDI, matrix-assisted laser desorption/ionization; SORI, sustained off-resonance irradiation; T, Tesla; UPLC, ultra performance liquid chromatography; UV, ultraviolet detection.

compounds, and flavonoids (Maccelli et al., 2020). In grapevine, FT-ICR-MS allowed the characterization of the leaves of *Vitis vinifera* cv. Pinot noir and their valorization as a source of diverse biologically active phytochemical compounds (Maia et al., 2019a)

One of the major applications of metabolomics studies is the study of plant metabolite responses to different stimuli in vitro that mimics the overall stresses that plants are exposed to every day in the field. Exposure to these environmental stresses reduces and limits the productivity of any plant. Abiotic constraints include radiation, salinity, flood, drought, extremes in temperature, heavy metals, among others. Biotic stressors account for attacks by various pathogens such as fungi, bacteria, oomycetes, nematodes, and herbivores. Plants have developed various mechanisms to overcome these

TABLE 3 FT-ICR-MS plant products metabolomics studies (alphabetical order by plant species name)

Plant (species)	Product	Experimental conditions	References
Acer (Maple) and *Quercus alba* (White oak)	Veneers	DIMS 12 T FT-ICR-MS (ESI source)	He et al. (2019)
Coffea arabica (Arabica) and *Coffea canephora* var. *robusta* (Robusta)	Coffee	DIMS LTQ-(7.2 T)FT-ICR-MS (ESI source)	Garrett et al. (2012)
Cocos nucifera (Coconut)	Water	DIMS 9.4 T FT-ICR-MS (ESI source)	Costa et al. (2015)
Medicago sativa (Alfalfa), *Phaseolus vulgaris* (Bean), *Hordeum vulgare* (Barley), *Zea mays* (Maize), *Triticum aestivum* (Wheat), *Lolium perenne* (Ryegrass) and *Cucurbita maxima* (Pumpkin)	Root exudates	DIMS 15 T FT-ICR-MS (ESI source)	Miao et al. (2020)
Quercus (Oak barrel), grain varieties and *Saccharum officinarum* (Sugarcane)	Whisky, Rum and wood barrel samples	DIMS 12 T FT-ICR-MS (ESI source)	Roullier-Gall et al. (2018)
Satureja montana	Essential oil	DIMS 4.7 T FT-ICR-MS (ESI and APCI sources; CID)	Vitanza et al. (2019)
Triticum aestivum (Hard white wheat)	Bran samples	DIMS FT-ICR-MS (ESI and APCI sources)	Matus-Cádiz et al. (2008)
Vitis species	Red and white wines	DIMS 12 T FT-ICR-MS (ESI source)	Gougeon et al. (2009)
	Red and white wines	DIMS 12 T FT-ICR-MS (ESI source)	Roullier-Gall et al. (2014)
	White wines	DIMS FT-ICR-MS	Romanet et al. (2021)

Abbreviations: APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; DIMS, direct-infusion mass spectrometry; ESI, electrospray ionization; LTQ, linear trap quadrupole; T, Tesla.

TABLE 4 FT-ICR-MS plant cells and cell compartments metabolomics studies

Plant (species)	Cell/cell compartment	Experimental conditions		References
Arabidopsis thaliana	Cells	DIMS	7 T FT-ICR-MS (ESI source)	Kai et al. (2009)
	Cells	DIMS	7 T FT-ICR-MS (ESI source; SORI-CID)	Nakamura et al. (2007)
	Intact vacuoles	DIMS	12 T FT-ICR-MS (ESI source)	Ohnishi et al. (2018)
Vitis vinifera (Grapevine)	Apoplast	DIMS	7 T FT-ICR-MS (ESI source)	Figueiredo et al. (2021)

Abbreviations: CID, collision-induced dissociation; DIMS, direct-infusion mass spectrometry; ESI, electrospray ionization; SORI, austained off-resonance irradiation; T, Tesla

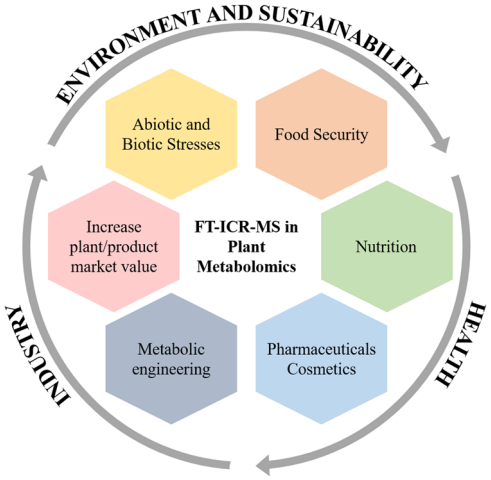

FIGURE 1 The role of FT-ICR-MS applied to plant metabolomics in different fields. FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry [Color figure can be viewed at wileyonlinelibrary.com]

stresses. Signaling metabolic pathways play an important role and act as a connecting link between sensing the stress and generating an appropriate biochemical and physiological response (Gull et al., 2019). Several reviews have been published regarding plant metabolism in response to biotic and abiotic stresses (Arbona & Gómez-Cadenas, 2016; Genga et al., 2011; Jorge et al., 2015; Piasecka et al., 2019) and considering the application of FT-ICR-MS, several works have also been published (Adrian et al., 2017; Kaling et al., 2015; Maia et al., 2020; Nascimento et al., 2019; Shahbazy et al., 2020) (Table 1).

Shahbazya and coworkers studied the distinctive metabolites and metabolic pathways of thyme (*Thymus vulgaris*) plants, responding to drought stress, and highlighted a possible correlation for the accumulation of carbohydrates and amino acids with osmotic protection as an adaptive stress response mechanism. In this study, it was demonstrated that galactose metabolism is the most significant in thyme (Shahbazy et al., 2020). Janz and coworkers studied the metabolomics changes of two poplar species (*Populus euphratica* [tolerant] and *Populus* × *canescens* [sensitive]) in response to salinity stress (Janz et al., 2010) and, in the same plant, Kaling and coworkers elucidated the influence of UV-B radiation on overall metabolite patterns in transgenic poplar plants (Kaling et al., 2015). In both studies, hundreds of *m/z* values were found to be discriminant and revealed an up or downregulation of various metabolic pathways, depending on the experimental conditions, such as, flavonoids, anthocyanins, osmotic adjustment, reactive oxygen species, and others.

The study of the effects of *Rhizoctonia solani*, the causal agent of Rhizoctonia disease, was studied by FT-ICR-MS on the global metabolic network of potato sprouts (*Solanum tuberosum*, var. Kennebek) by Aliferis and Jabaji (2012). An upregulation of mevalonic acid and deoxy-xylulose pathways leading to the biosynthesis of sesquiterpene alkaloids was reported. Fluctuations on the content of amino, carboxylic and fatty acids in infected potato sprouts were also detected (Aliferis & Jabaji, 2012).

Grapevine metabolomics has also been widely explored by FT-ICR-MS. The first study was performed on a grapevine population, obtained by different crosses, to investigate the metabolism of infected and non-infected leaves with *Plasmopara viticola*, the causal agent of downy mildew disease (Becker et al., 2013). The comparison of MS profiles obtained from control and infected leaves of different levels of resistant grapevines highlighted several classes of metabolites involved in the discrimination between infected and noninfected leaves. Moreover, the high mass accuracy provided by FT-ICR-MS, allowed a precise analysis and critical discrimination between all signals which led to the identification of 19 possible markers between inoculated and healthy samples. Several studies have followed, the chemical diversity of different *Vitis* species, including grapevine (*Vitis vinifera*) was explored, and the metabolites present in grapevine with and without pathogen interaction were identified (Adrian et al., 2017; Maia et al., 2019b; Maia et al., 2019c; Maia et al., 2020, 2021; Nascimento et al., 2019).

Plant development associated metabolism is another focus of untargeted FT-ICR-MS studies (Aharoni et al., 2002; Ogawa et al., 2018; Oliveira et al., 2016). Pineapple (*Ananas comosus* var. *comosus*) and mango

(*Mangifera indica*) are some of the most cultivated plants in tropical areas and highly exported to other countries. The maturation of these fruits indicates the best stage for harvesting and determines the correct time for fruit consumption. In both works, the metabolism of different maturation stages of these plants was studied, and the results pointed to primary (mainly sugars) and secondary (mainly phenolic compounds) metabolites as the most abundant in the third stage of maturation (Ogawa et al., 2018; Oliveira et al., 2016). More recently, Ingallina and coworkers thoroughly analyzed and compared the metabolic profile of two tomato cultivars (Torpedino di Fondi and San Marzano) in different ripening stages by FT-ICR-MS untargeted analysis combined with NMR spectroscopy (Ingallina et al., 2020). The different tomato extracts were analyzed in both positive and negative ionization modes, detecting up to 1646 different molecular formulas in only one extract. Some metabolites were shared by all extracts and others were found to possibly be considered marker compounds, being detected only in one extract (Ingallina et al., 2020). In grapevine leaves direct infusion FT-ICR metabolomics was done with the focus to improve metabolome coverage, through the use of different solvents in sequential elutions from the solid phase extraction, allowed the extraction of polar and non-polar compounds, covering all major metabolic classes in plants (Maia et al., 2016).

To cope with the increase in metabolomics biological studies, there is an ever-growing need for faster and more comprehensive analysis methods. As metabolites vary widely in both concentration and chemical behavior, there is still no single analytical procedure allowing the unbiased and comprehensive structural elucidation and determination of all metabolites present in a given biological system (Kueger et al., 2012). But the ever-increasing resolving power and the improved mass accuracy by commercially available FT-ICR-MS, significantly boosts untargeted metabolomics studies. Moreover, the capability of having a higher metabolome coverage, using direct infusion, metabolites are analyzed in a high-throughput way, providing a rapid analysis of complex metabolite samples, eliminating the time-consuming separation approaches.

4 | TARGETING METABOLIC PLANT COMPOUNDS BY FT-ICR-MS

Targeted metabolomics can be described as the measurement of defined groups of chemically characterized and biochemically annotated metabolites with established biological importance at the start of the study before data acquisition is performed. Targeted methods have a greater selectivity and sensitivity than untargeted methods, but targeted studies can only be performed if an authentic chemical standard of the metabolite is available or if the fragmentation pattern of the compound is known (Roberts et al., 2012).

Most FT-ICR-MS-targeted-based plant metabolomics studies are related to the detection of plant compounds or classes with nutritional, medicinal or pharmaceutical value, identification, and characterization of plant compounds already known to have interesting health properties and for cultivation improvement and marketing (Table 2).

The significance of plants, in particular medicinal plants, in human health cannot be overlooked. Plants have been used since ancient times as resources of molecules with medicinal properties, due to the presence of naturally occurring compounds, and today many of the modern pharmaceuticals are still produced indirectly from plant extracts or from specific plant compounds. Hence, there is an urge to further investigate the "black box" of plant metabolites with medicinal potential to uncover which compounds possess the desired properties and biological activities for human body.

The FT-ICR-MS was first used to specifically characterize plant compounds with health beneficial properties by Warburton and Bristow (Warburton & Bristow, 2006). Six kavalactones present in kava (*Piper methysticum*) roots were investigated by FT-ICR-MS (Warburton & Bristow, 2006). This plant is known for its relaxing and calming properties and its extracts have been used in herbal medicine over the last 2000 years, with preparations of kava being commercialized as capsules and fluid extracts. However, some reports of liver toxicity have questioned the safety of kava-containing products and extracts. The utilization of FT-ICR-MS in kava root extracts allowed the determination of their elemental formula and structural confirmation, leading to the identification of kavalactones with high certainty.

Further studies with FT-ICR-MS have followed with the aim of, not only characterizing plant bioactive compounds, but also to improve the detection methods of these compounds (Table 2). Xin Huang and coworkers developed a method for *Schisandra chinensis*, whose ripe fruits are a famous tonic in traditional Chinese medicine, to detect and analyze its lignan constituents, the major bioactive compounds present in this plant with anti-hepatotoxic, anti-asthmatic and central nervous system protecting properties (Huang et al., 2007, 2008). Some plant compounds with decreased incidence or treatment of some diseases have also been investigated by FT-ICR-MS. *Acanthopanax senticosus* leaves were screened to identify α-glucosidase inhibitors which have potential antidiabetic applications (Zhou et al., 2012), and *Fallopia*

convolvulus was analyzed to identify compounds responsible for estrogenic activity (Brennan et al., 2013). Two compounds, emodin and rhodoeosein, were identified as being responsible for estrogenic activity (Brennan et al., 2013).

More recently, Vidal-Gutiérrez and coworkers identified and quantified by FT-ICR-MS cucurbitacins, the main group of compounds found in *Ibervillea sonorae*, which have demonstrated apoptotic and antitumoral activities in cervical cancer cells. One new cucurbitacin was also identified in this study (Vidal-Gutiérrez et al., 2021).

Other similar studies applied FT-ICR-MS for the detection of phenolic compounds, oligosaccharides, anthocyanidins and others (Huong et al., 2012; Iijima et al., 2013; Khamko et al., 2013; Kuhnert, 2010; H. Li et al., 2008; Y.J. Park et al., 2017; Pollier et al., 2011; Reeves et al., 2020; Sakaguchi et al., 2008; Xiao et al., 2017; Yang et al., 2019; Zhang et al., 2010) (Table 2).

Besides therapeutically active substances, plants also play a great role in supplying food for personal care of mankind. Hence, a detailed knowledge of their metabolites is crucial to understanding the relation between food composition and health properties to attract more customers which will increase plants market value. Also, marketable plants are subject to phytosanitary treatments, thus the study of the presence of these compounds in plants ready to consume is a must for food safety controls. Several studies focused on the evaluation of plants and crops for human consumption to enhance their value and/or attract more consumers (Kuhnert, 2010; Y.J. Park et al., 2017; Pollier et al., 2011; Reeves et al., 2020; Sakaguchi et al., 2008; Xiao et al., 2017). As an example, Ting Xiao and coworkers evaluated the polyphenolic profiles of different berries (blueberry, bilberry, mulberry and cranberry) by FT-ICR-MS (Xiao et al., 2017). The study revealed 39 polyphenols including: 26 anthocyanins, 9 flavonoids, and 4 phenolic acids were identified accurately. Their results provided not only a basis for further research on berries but also for the selection of certain berries as potential sources of anthocyanins.

5 | DISCOVERING VALUE-ADDED PLANT PRODUCTS THROUGH FT-ICR-MS

Plants produce a diverse repertoire of complex small-molecule compounds which can be used in pharmaceutical and industrial products (Fischer et al., 2015). But to move from proof-of-concept experiments to commercial production, it is important to shift the focus from the untargeted and target identification of molecules, and start building-up reliable plant products reference databases, useful to guarantee food authenticity and freshness, and to support consumers, further nutraceutical evaluations and industries. This will ensure quality, purity, and yield aspects that determine commercial feasibility. Some plant products with important industrial value are, for example, wood and cork, fibers, fatty oils, vegetable fats and essential oils, sugars and starches, papers, resins, among others.

Recently, interesting studies have been published in the metabolic profiling of plant products using FT-ICR-MS (Table 3). From the different studies published, it can be highlighted the utilization of FT-ICR-MS, to detect and quantify Arabica *(Coffea arabica*) coffee adulterations by Robusta (*Coffea canephora* var. *robusta*) coffee (Garrett et al., 2012). The admixture of Robusta coffee is illegal into high-quality Arabica coffee; thus, it is crucial for the coffee industry to investigate the coffee's quality in a fast and precise manner. Rafael Garrett and coworkers developed a method to quantify blends of Robusta and Arabica coffee as well as to investigate the identity of the major compounds responsible for the distinction between the coffee varieties using FT-ICR-MS together with other MS techniques (Garrett et al., 2012).

As already mentioned, FT-ICR-MS was also used to characterize the metabolome of *V. vinifera* cv. Pinot noir's leaves to assess their potential as a source of bioactive nutraceutical compounds (Maia et al., 2019a).

The chemical composition of wine has mostly been studied using targeted analyses of selected metabolites (Bi et al., 2018; Flamini & De Rosso, 2006; Pinu, 2018). However, the chemical diversity of wine composition can be unraveled through an untargeted approach if using an ultrahigh-resolution MS, like FT-ICR-MS, providing an instantaneous image of complex interacting processes. The analysis of barrel-aged wines by an untargeted metabolomics approach by FT-ICR-MS, revealed that 10-year-old wines still show a geographic metabolic signature of the forest location where oaks of the barrel in which they were aged have grew (Gougeon et al., 2009). Most recently, Romanet and coworkers applied FT-ICR-MS to explore the chemical diversity associated with the antioxidant capacity of white wines (Romanet et al., 2021). More than 350 molecular markers were found to be correlated with wines with higher antioxidant capacity (Romanet et al., 2021). Bottled white and red wines from different appellations in Burgundy were also analyzed by FT-ICR-MS to characterize wine complexity and identify markers that can separate wines (Roullier-Gall et al., 2014). Roullier-Gall and coworkers also analyzed 150 whisky samples from 49 different

distilleries in seven countries, ranging from 1-day new made spirit to 43 years of maturation with different types of barrel (Roullier-Gall et al., 2018). FT-ICR-MS analysis revealed some interesting results: the impact of the wood history on the distillate's composition during barrel aging. Whiskies could be differentiated according to the history of the barrel used for the maturation, regardless of the cereal source. Also, the comparison of barrel aged rums and whiskies revealed specific metabolic signatures (Roullier-Gall et al., 2018).

Coconut water was also analyzed by FT-ICR-MS to verify its quality after storage and characterize the chemical compounds produced during natural ageing (Costa et al., 2015).

The study of antimicrobial activities of marketable products are also extremely important for food security. Vitanza and coworkers characterized the metabolite profile of commercial essential oil of *Satureja montana* to evaluate its antimicrobial properties, both alone and combined with gentamicin towards gram-negative and gram-positive bacterial strains, through the combination of FT-ICR-MS and antibacterial activity experiments (Vitanza et al., 2019).

6 | A DEEPER ANALYSIS OF PLANT CELLS AND CELL COMPARTMENTS' METABOLITES BY FT-ICR-MS

Plants sense all the external stresses present in the environment, get stimulated and then generate appropriate cellular responses. The stimuli received from the sensors located on the cell surface or cytoplasm are transferred to the transcriptional machinery situated in the nucleus, with the help of various signal transduction pathways (Gull et al., 2019). This leads to major differential metabolic changes making the plant tolerant/prepared against the stress. Hence, studying plant cells and different cellular compartments allows a broader understanding of cell dynamics (Table 4). FT-ICR-MS was applied to profile the metabolome of *A. thaliana* cell cultures, overexpressing P450-genes to identify and characterize its pathway (Kai et al., 2009). Cytochromes P450 of higher plants play crucial roles in both primary and secondary metabolism processes, such as catalysis and synthesis of structurally diverse specialized metabolites important in essential ecological roles and constitute a valuable resource for the development of new drugs (Shang & Huang, 2019). An FT-ICR-MS based metabolomics scheme was successfully implemented to clarify the P450 functions and fatty acid hydroxylation activity of *A. thaliana CYP78A7* gene was reported (Kai et al., 2009). Also in *A. thaliana* cell cultures, metabolites behind light/dark regulation were investigated, leading to the identification of 40 and 8 ions, respectively in negative and positive ionization modes, to growth conditions (Nakamura et al., 2007). Moreover, it was suggested that accumulations of several phenylpropanoids, a disaccharide and a trisaccharide were prominent in the light condition (Nakamura et al., 2007).

Both these studies opened new insights, not only for the use of plant cell cultures to similar studies, but also to explore their compartments.

This approach was also applied to plant compartments such as the vacuoles and the extracellular space (i.e., apoplast). Nontarget analysis with FT-ICR-MS of *A. thaliana* culture-suspension cells identified 1,106 m/z signals only present in vacuoles (Ohnishi et al., 2018). The apoplastic fluid of grapevine leaves was also evaluated by FT-ICR-MS. A total of 1,100 and 1,657 putative metabolites were annotated for *V. vinifera* cv. Trincadeira and *V. vinifera* cv. Regent, being 514 common to both grapevine genotypes (Figueiredo et al., 2021).

Although there is still much to be discovered about metabolic functions and pathways in cells and cell compartments, these studies demonstrate the advantage of using an ultrahigh-resolution and mass accuracy technique. The exact identification of metabolites and its correlation is a step forward in the comprehension of cellular mechanisms.

7 | MALDI-FT-ICR-MS: SPATIAL DISTRIBUTION OF PLANT METABOLITES

Besides the identification of metabolites in the overall system or in specific plant compartments, it is also important to understand the spatial distribution of metabolites and their respective accumulation pattern. Mass spectrometry imaging (MSI) has proven to be a very powerful tool, with several advantages, being the most important the ability to simultaneously determine the exact location and distribution of specific or multiple metabolites in a single experiment in a complex biological material, typically plant tissue sections (Bjarnholt et al., 2014; Boughton et al., 2016).

There are several laser desorption ionization (LDI) techniques available nowadays (Boughton et al., 2016), being the matrix assisted laser desorption ionization (MALDI) MS, by far the most commonly used form of LDI. In MALDI the analyte/tissue is cocrystallized with a chemical matrix, which absorbs the laser energy and releases the analytes into the gas-phase in a process leading to ionization (Bjarnholt et al., 2014; Boughton

et al., 2016). The addition of the matrix has several advantages. Not only does it allow to specify the type of compounds to analyze but also, since the energy of the laser light is absorbed by the matrix and not directly by the analytes, MALDI is considered a soft ionization as well as ESI, enabling researchers to detect very small levels of detectable biological analytes, making it an excellent technique for metabolomics studies.

In a MSI experiment, pixels are established by virtually defining an array of discrete spots over the sample area. The laser is fired several times in each pixel before moving to the next spot. For each coordinate individual mass spectra are collected representing all the ionizable molecules on that spot. The combination of all spectra allows the reconstruction of an image with the intensity values of the ionized molecules, representing the spatial distribution of all molecules along the sample, which can be compared with an optical image of the sample (Barkauskas et al., 2009; Boughton et al., 2016). MALDI-MSI has been adopted for the direct visualization of plant tissues and the investigation of plant biology as well. The studies range from studying the mechanisms of plant responses to both abiotic and biotic stresses and symbiotic relationships, to fundamental ecophysiological important processes (reviewed by Qin et al., 2018). An important point to have in consideration when performing plant tissues analysis is sample preparation, which continues to be the major bottleneck of this technique. All the major concerns when performing MSI in plants have been reviewed in Bjarnholt et al. (2014), Bodzon-Kulakowska and Suder (2016), Boughton et al. (2016), and Grassl et al. (2011). A detailed characterization of complex plant tissues by MALDIMSI requires an instrument that is capable of high mass resolving power, mass accuracy, and dynamic range. FT-ICR-MS is the technique for such analysis as it offers the highest mass spectral performance for MALDI-MSI experiments (Bowman et al., 2020). Coupling of MALDI to FT-ICR for MSI analysis, has several limitations. MSI experiments of large samples, at very high spatial resolutions, need higher measurement times, which are limited by the length of FT-ICR acquisition times (Buck et al., 2016). As it was previously mentioned, this subject can be addressed by increasing the magnetic field strength. Also, the complexity of the biological sample, due to a vast array of concentrations from different biomolecules with different chemistries and molecular sizes present, may generate an ion suppression effect, making the MSI analysis less sensitive (Boughton et al., 2016). The addition of matrix to a sample generates high-abundance low-weight ion species which leads to significant interfering signals, being also a limitation. Therefore, upon MSI experiments with high-performance analyzers, such as FT-ICR, there is a significant balance between acquisition speed, spatial resolution and sensitivity (Boughton et al., 2016; Buck et al., 2016). Although several plant metabolic studies appeared using MALDI-MSI (reviewed in Qin et al., 2018), few MSI studies combined MALDI and FT-ICR-MS (Alcantara et al., 2020; Gorzolka et al., 2014; Sarabia et al., 2018; Takahashi et al., 2015) (Table 5).

MALDI-FT-ICR-MSI was applied to barley seeds and roots to study the spatial distribution and profiles of metabolites, their characterization and quantify their change (Gorzolka et al., 2014). Barley is one of the model organisms to investigate the cereal germination process that involves complex interactions between different organs that lead to the growth of the plant.

MALDI-FT-ICR-MSI was applied to germinated barley seeds for the detailed localization of metabolites in longitudinal and transversal seed sections (Gorzolka et al., 2014). Several compounds responsible for the prevention of pathogen infestation in seeds, as well as distinct localization patterns within seed organs were identified (Gorzolka et al., 2014). Also, the high-mass resolution of MALDI-FT-ICR-MSI was also used in barley roots to reveal the detailed spatial distribution of metabolites, such as lipids, in response to an abiotic stress, salinity stress (Sarabia et al., 2018).

In another study, an improved method of MALDI-FT-ICR-MSI was developed to achieve a higher-resolution and higher mass accuracy and applied to analyze the distribution of small metabolites in *A. thaliana* roots (Takahashi et al., 2015). Most recently, Dos Santos and coworkers optimized and studied the spatial distribution of alkaloids in *Erythroxylum coca* leaves (Dos Santos et al., 2021). Three matrices were tested and 2,5-dihydroxybenzoic acid (DHB) was selected as the best matrix. Different tissue thicknesses were also evaluated, to study the inner part of the leaf tissue, and alkaloids and flavonoid molecules were detected.

MALDI-FT-ICR-MSI can also be used for industrial purposes. Heavy metal soil contaminations are very problematic and cause severe negative impacts on human health. Development of cost-effective methods of heavy metals extraction, such as Hg and Au, may offer significant benefits through remediation of contaminated land and extraction of valuable resources. In a recent study, Hg and Au localization in cassava roots were explored for these heavy metals phytoextraction. The results of Alcantara and coworkers using MALDI-FT-ICR-MSI indicated that exposure to Hg and Au did not disturb the plant tissues, being the plants healthy and alive at the time of harvest (Alcantara et al., 2020).

All these studies open new insights for plant metabolomics studies. Also, it is anticipated that

TABLE 5 MALDI-FT-ICR-MSI plant metabolomics studies (alphabetical order by plant species name)

Plant (species)	Experimental conditions	Reference
Arabidopsis thaliana tissues (seedlings and roots)	9.4 T FT-ICR-MS (MALDI and LDI sources)	Takahashi et al. (2015)
Cannabis leaves and *Jatropha curca*	9.4 T FT-ICR-MS (MALDI and LDI sources)	dos Santos et al. (2019)
Erythroxylum coca leaves	FT-ICR-MS (MALDI source)	Dos Santos et al. (2021)
Hordeum vulgare cv. *Optic* (Barley) seeds	FT-ICR-MS (MALDI source)	Gorzolka et al. (2014)
Hordeum vulgare cv. Hindmarsh (Uniform barley)	7 T XR-FT-ICR-MS (MALDI source)	Sarabia et al. (2018)
Manihot esculenta Crantz. (Cassava)	7 T FT-ICR-MS (MALDI source)	Alcantara et al. (2020)

Abbreviations: LDI, laser desorption/ionization; MALDI, matrix-assisted laser desorption/ionization; T, Tesla.

MALDI-FT-ICR-MSI approaches will bring a new level of understanding to metabolomics as scientists will be encouraged to consider spatial heterogeneity of metabolites in descriptions of metabolic pathway regulation.

8 | TANDEM MS AND *in silico* PREDICTION TOOLS IN PLANT METABOLOMICS

In MS experiments, the selection and isolation of specific ion species from the mixture and their fragmentation or ion–molecule reactions, allows their thorough characterization. This approach is denominated tandem mass spectrometry or MS/MS (Gross, 2017). The output of MS/MS is a mass spectrum of all the fragments generated by the isolated and fragmented analyte. This approach, by the isolation of a single analyte precursor to obtain a mass spectrum containing only its fragments, provides MS metabolomics studies with structural information of the molecules under analysis, allowing an unequivocal identification of the compounds.

Another important point to have in consideration in MS/MS, are the currently available chemical spectral libraries of raw and validated identified compounds in public databases (Tada et al., 2019). These platforms are essential to identify which metabolites are present in a sample. The fragmentation pattern of the unknown analytes present in the sample can be matched with tandem MS spectra of reference standards and other already identified compounds, allowing an accurate and unambiguous metabolite identification, which still remains the major bottleneck in metabolomics data interpretation (Ara et al., 2021; Cao et al., 2021; Chaleckis et al., 2019; Guijas et al., 2018; Horai et al., 2010; Scheubert et al., 2017; M. Wang et al., 2016; Wohlgemuth et al., 2016).

One of the alternative approaches used to annotate an unknown fragmentation mass spectrum is using *in silico* predictions, one of the key focuses in computational MS research (Agahi et al., 2020; Djoumbou-Feunang et al., 2019; Krettler & Thallinger, 2021; Ruttkies et al., 2016).

Different studies have been published presenting new methods that facilitate the identification of small molecules from tandem MS experiments, even without spectral reference data or a large set of fragmentation rules (Dührkop et al., 2015; Hufsky et al., 2014; Krettler & Thallinger, 2021; Rogers et al., 2009; Ruttkies et al., 2016; Wolf et al., 2010). Also, web-based facilities have been created to help the analysis of raw or processed metabolomics mass spectrometric data, displaying the metabolites identified, changes in their experimental abundance and the metabolic pathways in which they occur (Leader et al., 2011).

One of the challenges of *in silico* annotation remains the multiple candidate structures predicted for each fragmentation spectrum meaning that the user still must visually inspect the predictions from a candidate list. Thus, defining new algorithms with improved quality and annotation rates is crucial (Böcker, 2017; L. Li et al., 2013; Silva et al., 2018).

Albeit recent technological equipment improvements, the complexity and diversity of plants surpasses these advances. The complete understanding of intricate metabolic pathways, step by step, is mainly incomplete to this diversity of specialized metabolites. To tackle this challenge, different approaches have been used through the years to study metabolites in plants, such as the identification of genes related to metabolites production and functions, utilization of protein sequences to predict enzymatic functions on specific points at the metabolic pathway and gene co-expression networks (Adio et al., 2011; Chae et al., 2014; Karp et al., 2011; Menikarachchi et al., 2013; Moore et al., 2020; Saito et al., 2008; Schläpfer et al., 2017; Tohge et al., 2005; Wisecaver et al., 2017). Despite innovative experimental approaches, all the metabolites detected need to be identified. Thus, high precision MS data needs to be annotated so that the results can be displayed in specific databases. These databases contain linked information of genomes, biological pathways, diseases, drugs and chemical

substances, allowing the depth comprehension of the compounds analyzed (Booth et al., 2013; Misra, 2021).

However, there is still a wide gap between the known and unknown as all these experimental approaches have high error rates and depend on the plant material and type of analysis. Thus, multiple network analysis tools have been developed to deal with these flaws and *in silico* metabolomics studies is appearing as an alternative approach (Desmet et al., 2021). In the last few years, computational advances, and the availability of libraries with fragmentation patterns information, made it possible to perform classification and predict plant chemical structures based on computational methods (Cao et al., 2021; Moore et al., 2020; Peters et al., 2021; Ruttkies et al., 2016; Scheubert et al., 2017; Toubiana et al., 2019). As a result, several databases and tools have been established (Cottret et al., 2010; de Groot et al., 2009; Ellis et al., 2008; L. Li et al., 2013; Wicker et al., 2016; Yousofshahi et al., 2015). These platforms allow the prediction of the metabolism and creation of networks by computationally generating the enzymatic products of a particular compound (Desmet et al., 2021). *in silico* algorithms also allow the creation of predicted compound databases, helping and guiding laboratory experiments (Zhu et al., 2016).

A recent study by Toubiana and coworkers combined network analysis and machine learning, to predict metabolic pathways from tomato metabolomics data (Toubiana et al., 2019). Also, with bryophytes, Peters and coworkers presented an automated *in silico* compound classification framework to annotate metabolites using an untargeted data from MS experiments (Peters et al., 2021).

Albeit these approaches seem to bring a new perspective for plant metabolomics studies, there are still some limitations. The majority of these *in silico* approaches were designed to perform analysis in model plants, e.g., *A. thaliana*, being unclear how these methods work in other species and, therefore, even though similarity-based approaches may be used to surpass the first problem, it is challenging to transfer annotation information across species without having high error rates (Moore et al., 2020; Yu et al., 2004). Nevertheless, a recent study employed machine learning strategies, where knowledge from *A. thaliana* was transferred to predict specialized metabolism genes functions of cultivated tomatoes (Moore et al., 2020).

9 | PERSPECTIVES

Since the beginning of XXI century, numerous works on plant metabolomics have used FT-ICR-MS. This technique has had quite an impact on this OMICs approach, since it allows, not only the detection of a huge number of small size compounds (metabolites), but also compounds present in small concentrations, impossible to detect with other available techniques. The potential of FT-ICR-MS to study in plant science is immense. However, there is still a long way to fully uncover and understand the complete metabolome of plants.

Multiparallel approaches that combine FT-ICR-MS with other OMICs techniques and improved protocols, that expand the coverage of metabolite analysis, are a valuable tool to advance the comprehension of the complex regulatory networks of plants and improve sustainable productions.

The future of plant metabolomics also depends on technological advances. One of the major challenges in the undeniable identification and structural characterization of metabolites is the presence of many structural isomers, present in the mixtures. Although FT-ICR-MS is the mass spectrometer with the highest mass accuracy and resolution available in the market, the impossibility to separate isomers is still a limitation. Hence, the development of new techniques to overcome this matter should also be taken into account.

Another important challenge in plant metabolomics studies is the lack of a general plant metabolites' database, compiling, for example, the plant material and equipment used in the analysis, with the ability to search by metabolite classes or plant species, which is possible for other biological systems. Since, FT-ICR-MS provides an enormous amount of data, the creation of new analytical platforms and software, will allow researchers to identify in a high-throughput manner metabolic changes, to compare raw data, facilitating the study of plants metabolic fingerprint.

Regarding industrial uses of FT-ICR-MS, the majority of applications of this technique are in the sector of chemicals and crude analysis. FT-ICR-MS can be expanded to food, pharmaceutical, and cosmetics industries that rely on plant compounds, taking advantage of FT-ICR-MS sensitivity, thus increasing plant market values, add-value to plant products already in the market and improving sustainable productions.

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ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
CI	chemical ionization
DHB	2,5-dihydroxybenzoic acid
EI	electron ionization
ESI	electrospray ionization
FT-ICR	Fourier transform ion cyclotron resonance
ICR-MS	ion cyclotron resonance mass spectrometry
LDI	laser desorption ionization
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MSI	mass spectrometry imaging
NMR	nuclear magnetic resonance
UPLC	ultra performance liquid chromatography

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