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MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY: A RELIABLE TOOL FOR RAPID DETECTION AND IDENTIFICATION OF PLANT PATHOGENS

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as an efficient and cost-effective analytical approach for the detection and identification of plant pathogens, addressing key limitations of conventional diagnostic techniques such as culture-based methods, microscopy, serological assays, and polymerase chain reaction, which are often time-intensive and laborious. Considering the substantial yield losses caused by fungal, bacterial, and viral diseases, the development of rapid and reliable diagnostic tools is critical for effective plant disease management and food security. MALDI-TOF MS operates through laser-induced ionization of analytes in the presence of an energy-absorbing matrix, typically α-cyano-4-hydroxycinnamic acid, followed by acceleration and separation of ions based on their mass-to-charge ratio, generating peptide mass fingerprints that are matched against reference databases for precise identification at the species or subspecies level. This technique has demonstrated applicability in identifying diverse phytopathogens, including *Fusarium*, *Phytophthora*, *Colletotrichum*, *Clavibacter*, *Xanthomonas*, *Dickeya*, and *Potato virus Y*, offering advantages such as rapid analysis, high throughput, and low reagent consumption, despite challenges related to database limitations and sample preparation variability.

Keywords **:** MALDI-TOF MS, Plant pathogens, Peptide mass fingerprinting, Sample preparation, Fungal identification, Bacterial diagnostics.

Introduction

Plant diseases caused by fungi, bacteria, viruses, and other pathogens are among the biggest challenges facing global agriculture today. They not only cause severe yield losses but also pose a serious threat to global food security. To prevent major outbreaks and protect crops, farmers and researchers need tools that can quickly and accurately detect plant pathogens. Early detection enables timely treatment and better disease management, which is critical to sustaining agricultural productivity.

For decades, traditional diagnostic methods such as culture-based tests, microscopy, serological techniques, and molecular tools like PCR (Anhalt and Fenselau, 1975) have been the backbone of plant pathogen detection. These methods are well established and remain important, but each has limitations (Table 1). Culture-based methods, while simple, are often slow and can't be used for microbes that don't grow easily in the lab. Microscopy gives valuable insights into a pathogen's appearance but requires expert knowledge and can miss low-level infections (Akimowicz and Bucka-Kolendo, 2020). Serological tests like ELISA are fast and high-throughput, yet they sometimes give false positives due to cross-reactions. PCR and qPCR are extremely sensitive and specific, but they can be costly, labour-intensive, and prone to

contamination (Van Veen *et al.*, 2010; Böhme *et al.*, 2011). Meanwhile, DNA sequencing and metagenomic approaches deliver unmatched accuracy but are too time-consuming and resource-heavy for routine, large-scale use (Anhalt and Fenselau, 1975; Marklein *et al.*, 2009).

In recent years, Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a powerful alternative that bridges the gap between classical microbiology and modern molecular diagnostics. MALDI-TOF MS works by analyzing the unique peptide or protein "fingerprints" of microorganisms, mainly ribosomal proteins (Nakamura *et al.*, 2017; Han *et al.*, 2021), using a soft ionization process that preserves the integrity of the molecules. This allows researchers to accurately determine their mass without complicated sample preparation or sequencing.

When compared to conventional methods, MALDI-TOF MS stands out for its speed, efficiency, and sustainability (Li *et al.*, 2022). It can deliver precise results within minutes, uses minimal reagents, supports high-throughput processing (Van Veen *et al.*, 2010), and has a lower environmental footprint qualities that make it especially appealing for agricultural applications. While the technique has already transformed clinical microbiology, it is now gaining traction in plant pathology for its ability to identify pathogens directly from cultures or even infected plant tissues. Its compatibility with chemometric analyses and expanding reference databases only adds to its diagnostic power. As agriculture faces growing pressures from increasing global food demand and emerging plant diseases intensified by climate change, tools like MALDI-TOF MS represent a promising step forward. By enabling rapid, reliable, and cost-effective detection of plant pathogens (Murray, 2012), this technology has the potential to become a cornerstone of sustainable plant disease management. This review explores the history, working principle, key components, and diverse applications of MALDI-TOF MS, with a particular focus on its role in plant pathogen diagnostics. We also discuss its strengths, current challenges, and future prospects, highlighting how it could shape the next generation of agricultural disease detection.

Table 1 : Comparison between different techniques used for detection of plant pathogens

Techniques	Advantages	Limitations	References
Culture-based methods	Simple, low-cost	Time-consuming, pure culture needed, some microbes can't be cultured	Shree *et al.*, 2024
Microscopy	Fast, shows morphology, fluorescent tagging possible	Low sensitivity, needs expertise	Akimowicz and Bucka-Kolendo, 2020
Serological Methods (ELISA)	Fast, high-throughput, suitable for viruses	Cross-reactions, lower sensitivity	Shree *et al.*, 2024; Venbrux *et al.*, 2023
PCR	Highly sensitive, detects unculturable microbes	Expensive, risk of contamination	Anhalt and Fenselau, 1975; Marklein *et al.*, 2009; Venbrux *et al.*, 2023
qPCR	Quantitative, Multiplex possible	More expensive than conventional PCR, requires standard curves and controls	Böhme *et al.*, 2011
LAMP	Rapid, field-deployable, visual output	Complex primer design, may lack specificity	Becherer *et al.*, 2020
DNA Sequencing	Accurate, identifies unknown pathogens	Expensive, time consuming	Anhalt and Fenselau, 1975; Marklein *et al.*, 2009; Venbrux *et al.*, 2023
Metagenomics	Detects whole microbial community, culture independent	High cost, complex data analysis	Anhalt and Fenselau, 1975; Marklein *et al.*, 2009
Biosensors	Fast, portable, suits for field use	Less sensitive, still developing	Madufor *et al.*, 2017; Venbrux *et al.*, 2023
Remote Sensing / Imaging	Non-destructive, large area coverage	Not specific, needs calibration	Oerke, 2020

Evolution of MALDI-TOF MS

The development of MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) is the result of decades of breakthroughs in mass spectrometry and ionization techniques. Some key milestones include: i) electron beam ionization (early 20th century): the earliest mass spectrometry methods used high-energy electron beams to ionize molecules, a process that often shattered them into fragments. While this "hard ionization" worked well for small molecules, it was far too destructive for delicate biomolecules like proteins and peptides (Gross, 2004). Although MS was discovered in the early 1900s, its application was restricted to the chemical sciences (Singhal *et al.,* 2015); ii) soft ionization breakthrough by Hillenkamp and Karas (1985): a turning point came in 1985, when German scientists Michael Karas and Franz Hillenkamp introduced a gentler approach. They used a UV laser and an organic matrix co-crystallizing alanine with tryptophan and applied a 266 nm laser pulse. This method produced ions with minimal fragmentation, preserving the integrity of larger molecules (Fenn *et al.,* 1989). Their innovation, later called MALDI, opened the door to analyzing proteins and peptides that had previously been too fragile for mass spectrometry (Karas and Hillenkamp, 1985; Fuh *et al.,* 2017); iii) Koichi Tanaka's contribution (1987): Japanese researcher Koichi Tanaka advanced the field further by successfully ionizing large biomolecules using a fine metal powder matrix and a nitrogen laser (Elbehiry and Abalkhail, 2025). His achievement demonstrated the power of soft ionization for studying biological macromolecules a breakthrough that earned him a share of the 2002 Nobel Prize in Chemistry (Calderaro and Chezzi, 2024); iv) commercialization of MALDI-MS (early 1990s): following ongoing refinements by Karas and Hillenkamp, MALDI-MS instruments were commercialized in the early 1990s. This step made the technology widely available to researchers and accelerated its adoption in fields like proteomics, biomolecular research, and clinical diagnostics (Drzeżdżon *et al.,* 2019); v) introduction of the Time-of-Flight (ToF) technique (1950s): the Time-of-Flight mass analyzer, developed in the 1950s (Price, 1994), works by accelerating ions through a vacuum toward a detector and measuring how long they take to arrive (Degand *et al.,* 2008). While the concept was promising, early ToF instruments suffered from low resolution, which limited their practical use; vi) integration of MALDI with ToF (1990s): combining MALDI with Time-of-Flight analysis in the 1990s transformed biomolecular mass spectrometry. The resulting MALDI-TOF-MS platform offered unmatched speed, sensitivity, and precision. In reflectron mode, it provided particularly accurate mass measurements, enabling detailed analysis of proteins, peptides, and even synthetic polymers (Li *et al.,* 2022); vii) and current status of MALDI-TOF MS (2000s-2020s): MALDI-TOF MS is well-established in clinical microbiology and is quickly growing in plant pathology, food microbiology, and environmental microbiology as a rapid, economical method for identifying microbes, differentiating strains, and monitoring outbreaks, though its wider use is still constrained by database coverage and standardization issues (Zaluga *et al.,* 2011). It is also used for detection of antimicrobial resistance and to study the differentially expressed proteins (Calderaro and Chezzi, 2024).

Working Principle of MALDI-TOF MS

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is a two-stage analytical technique that enables the precise measurement of molecular masses, particularly for large and fragile biomolecules such as peptides, proteins, and polymers (Marklein *et al.,* 2009; Yates III, 2011). The system comprises two key components: i) ionization source (MALDI); ii) and mass analyzer (TOF). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is a powerful analytical technique designed to measure the mass of large, delicate biomolecules with remarkable accuracy and ratio of their mass to charge (Singhal *et al.,* 2015).

The MALDI process starts with a "soft" ionization method that protects fragile molecules from breaking apart (Hettick *et al.,* 2004). In this step, the sample (analyte) is mixed with a UV-absorbing organic matrix and placed on a metal target plate. When a pulsed UV laser strikes the sample, the matrix absorbs the laser's energy and transfers it to the analyte. This gentle transfer causes the molecules to both desorb (lift off the surface) and ionize without shattering. These newly formed ions are then accelerated into the Time-of-Flight (TOF) mass analyzer (Akimowicz and Bucka-Kolendo, 2020). Inside it, these are propelled through a vacuum drift tube by an electric field. Because all the ions start with the same amount of kinetic energy, their speeds and therefore the time they take to reach the detector depend on their mass-to-charge (m/z) ratios (Degand *et al.,* 2008): lighter ions race ahead and arrive first, while heavier ones lag (Siuzdak *et al.,* 2004). The instrument records these flight times to calculate the m/z values and generate a mass spectrum (Viboud *et al.,* 2024), which represents the molecular "fingerprints" of the sample (Akimowicz

and Bucka-Kolendo, 2020). Many MALDI-TOF systems include a reflectron, a type of ion mirror, which fine-tunes the resolution by correcting small differences in ion energy (Kaklamanos *et al.*, 2020). In short, MALDI-TOF MS combines sensitivity, speed, and non-destructive analysis, making it an invaluable tool for high-throughput studies of complex biological samples (Kluz *et al.*, 2025).

Components of MALDI-TOF MS

A MALDI-TOF mass spectrometer consists of several key components, each playing a crucial role in generating accurate mass measurements. The target or sample plate is a flat metal plate, usually stainless steel, on which the sample is prepared; the analyte (molecule of interest) is mixed with a specific matrix and deposited onto this plate prior to analysis (Marklein *et al.*, 2009). The laser source, commonly a nitrogen laser emitting at a wavelength of 337 nm, provides the energy required for ionization of the sample (Andreadi *et al.*, 2025). The matrix, a UV-absorbing small organic acid, is mixed with the analyte and co-crystallizes on the target plate, embedding analyte molecules within matrix crystals in a process known as co-crystallization (Akimowicz and Bucka-Kolendo, 2020). This matrix protects fragile biomolecules during ionization. Common matrices include CHCA (α-cyano-4-hydroxycinnamic acid), which is best suited for smaller peptides (<2,500 Da) and is considered a "hard" matrix that may cause greater ion fragmentation but forms small, uniform crystals providing excellent resolution (Fukuyama, 2015; Jang and Kim, 2018; Yoo *et al.*, 2020; Kobylis *et al.*, 2021); SA (sinapinic acid), preferred for larger peptides and proteins (>2,500 Da), functioning as a "soft" matrix that minimizes fragmentation and is ideal for intact protein analysis (Fukuyama, 2015; Jang and Kim, 2018; Yoo *et al.*, 2020; Kobylis *et al.*, 2021); and DHB (dihydroxybenzoic acid), used for glycoproteins, glycans, and certain peptides, which tolerates contaminants effectively but tends to form large, needle-like crystals that may reduce resolution and introduce variability across the sample spot (Fukuyama, 2015; Jang and Kim, 2018; Yoo *et al.*, 2020; Kobylis *et al.*, 2021). The ion source is the region where the laser interacts with the sample, resulting in analyte ionization (Han *et al.*, 2021). In the acceleration region, ions are propelled by an electric field, ensuring they acquire the same kinetic energy before entering the flight tube. The flight tube is a long, vacuum-sealed chamber through which ions travel toward the detector; lighter ions move faster and therefore reach the detector earlier than heavier ones. The detector captures the arriving ions and converts them into electronic signals, which are subsequently used to generate the mass spectrum. Many modern instruments also incorporate a reflectron, or ion mirror, which enhances resolution by correcting for minor differences in the initial kinetic energies of ions (Han *et al.*, 2021). Finally, a sophisticated data system records, processes, and analyzes the acquired data to produce the final mass spectrum.

Steps in MALDI-TOF MS for Identification of Microorganisms

(i) Sample preparation

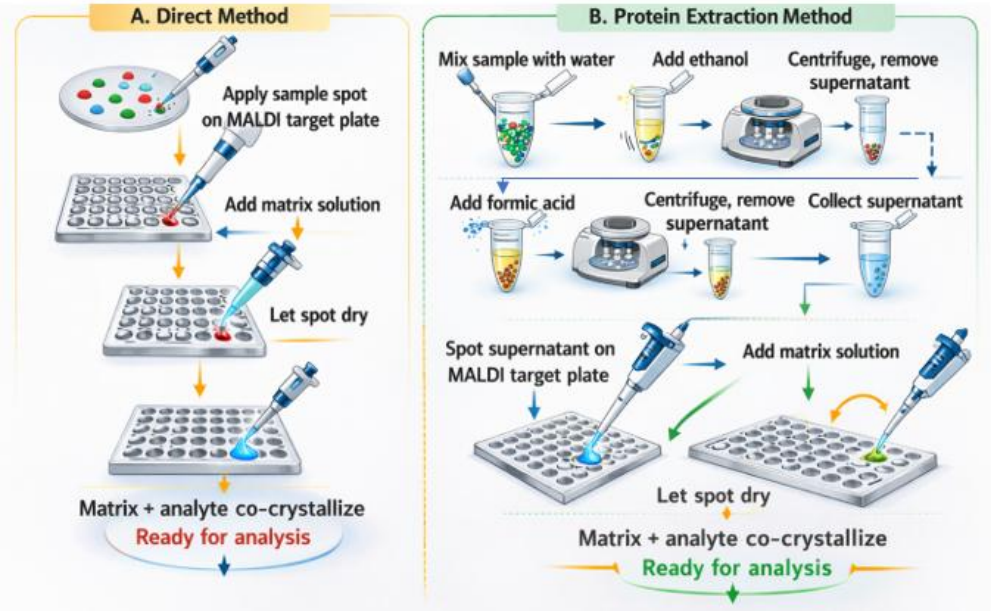

Fig. 1 : Sample preparation methods for MALDI-TOF MS

Direct method (Fig. 1A): simplest and fastest approach for sample preparation in MALDI-TOF MS, typically used for Gram-negative bacteria (Degand *et al.,* 2008), when the sample is relatively clean and requires minimal processing (Smole *et al.,* 2002). In this method, the sample (analyte) is directly applied onto the MALDI target plate, followed by the addition of a suitable matrix solution, such as CHCA (α-Cyano-4-hydroxycinnamic acid) or DHB (2,5-Dihydroxybenzoic acid) (Croxatto *et al.,* 2012; Akimowicz and Bucka-Kolendo, 2020). The matrix and analyte co-crystallize as the spot dries, forming a solid mixture. Once dried, the sample is ready for laser ionization and mass spectrometric analysis (Darji *et al.,* 2024). This method is especially useful for rapid screening or when handling well-characterized or simple samples like bacterial culture (Croxatto *et al.,* 2012).

Protein extraction method (Fig. 1B): the extraction in a tube before matrix addition method is a more thorough sample preparation technique used to enhance purity, concentration, and signal quality (Hodge *et al.,* 2013), particularly for complex or microbial samples like Gram-positive bacteria, cultured fungi and diseased plant samples (Croxatto *et al.,* 2012). The process begins by mixing the sample with water to suspend the material, followed by the addition of ethanol and centrifugation to precipitate proteins and remove impurities. After discarding the supernatant, the pellet is dried and treated with formic acid, which lyses cells and extracts proteins (Mellmann *et al.,* 2008; Marklein *et al.,* 2009; Patel, 2024). A second centrifugation step is performed to collect the supernatant, which now contains the analytes of interest, then spotted onto the MALDI target plate. Finally, the spot is overlaid with a matrix solution and allowed to dry, forming co-crystals suitable for MALDI-TOF MS analysis. The target plate is then placed in the MALDI-TOF mass spectrometer, where it is subjected to short pulses from a laser (typically a nitrogen laser at 337 nm wavelength) (Hillenkamp and Karas, 2000).

(ii) Laser Desorption/Ionization

Laser Desorption/Ionization is the key step in MALDI-TOF mass spectrometry that makes it possible to turn a solid sample into ionized molecules ready for analysis. The process begins with a prepared sample that has been mixed and co-crystallized with a matrix on the MALDI target plate (Fig. 2). When a pulsed UV laser is fired at the sample spot, it doesn't directly hit the analyte molecules. Instead, the matrix absorbs the laser's energy (Li *et al.,* 2022). This is by design the matrix is chosen specifically for its ability to strongly absorb the laser wavelength. Almost instantly, the matrix undergoes rapid desorption, transitioning from solid to gas in a tiny "micro-explosion" (Li *et al.,* 2022). This sudden burst of energy lifts both the matrix and the analyte molecules into the gas phase. At the same time, ionization takes place. The matrix helps transfer charge to the analyte, usually by donating a proton, so most analyte molecules leave the surface carrying a single positive charge. This type of ionization is considered "soft" because it happens without breaking the analyte into fragments (Andreadi *et al.,* 2025). As a result, the molecules remain intact, which is critical for accurate mass measurement. In short, laser desorption/ionization does two things at once: it vaporizes the analyte and gives it a charge, all in a controlled way. This ensures that the molecules can be detected and measured by the TOF analyzer without losing their structural integrity.

(iii) Time-of-Flight Mass Analysis

In a TOF (Time-of-Flight) mass spectrometer, the journey of a sample begins in the ionization zone (Fig. 2). Here, a laser pulse strikes the sample, which is embedded in a matrix material. The matrix absorbs the laser's energy and helps release (desorb) and ionize the sample molecules (Croxatto *et al.,* 2012) (Fig. 2). This process creates positively charged ions that are now free and ready for analysis. Next comes the acceleration zone, where a high-voltage electric field is applied (Fig. 2). This field gives all the ions the same amount of kinetic energy, propelling them toward the drift region (Yoo *et al.,* 2020). However, because mass affects velocity, lighter ions accelerate faster, while heavier ions move more slowly (Degand *et al.,* 2008). Even small differences in how the ions form can slightly affect their speeds. The ions then enter the drift zone (Fig. 2), a long vaccum flight tube. Here, no additional energy is applied, so the ions continue traveling at the speeds they reached during acceleration. As they move forward, they naturally separate according to their mass-to-charge ratio (m/z). The lighter, faster ions reach the end of the tube first, while the heavier ones trail behind. Finally, in the ion detector zone, the ions strike the detector surface (Fig. 2). Each impact produces a measurable signal, and the instrument precisely records how long each ion took to travel from ionization to detection, this is its time-of-flight. By analyzing these flight times, the system calculates the corresponding m/z values, allowing scientists to identify and characterize the molecules in the sample.

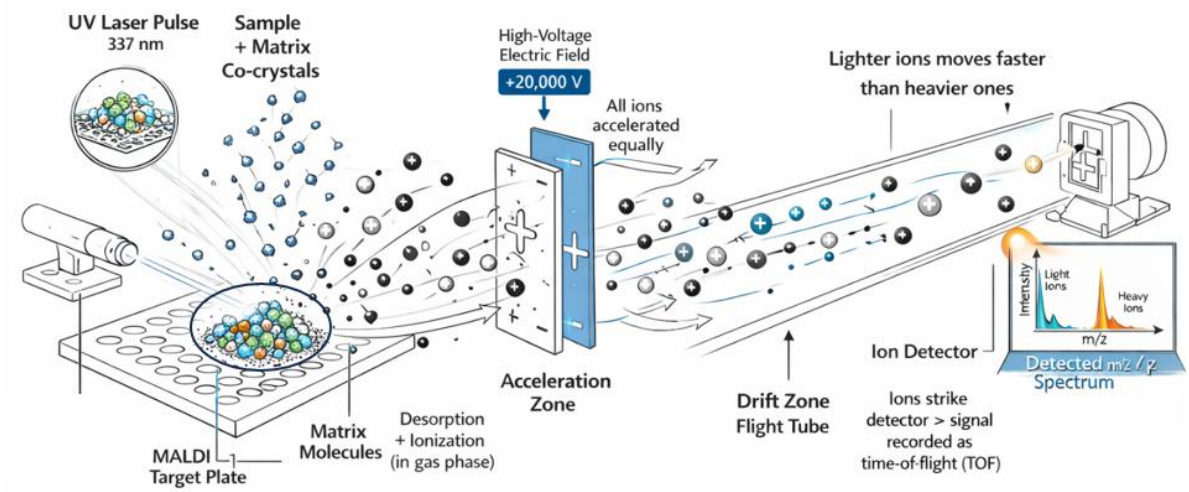

Fig. 2 : Laser desorption/ionization and time-of-flight analysis

(iv) Spectral interpretation and identification

In MALDI-TOF MS, spectral interpretation and identification depend heavily on the detector's performance. The detector records two key pieces of information: Time of Flight (TOF), which represents how long it takes each ion to travel from the ion source to the detector, and signal intensity, which reflects the number of ions with a specific mass-to-charge ratio (m/z) arriving at a given time. These measurements are subsequently converted into a mass spectrum and processed by specialized software, which extracts a peak list comprising the m/z values and their corresponding intensities from the sample spectrum.

To identify the organism or the substance being analyzed, the resulting peak list is compared to the database of known microbial spectra. The software assigns a match score between 0 and 3.0. The score indicates the degree to which the spectral "fingerprint" of the organism being analyzed matches the spectral "fingerprints" in the database. A higher score closer to 3.0 indicates strong and confident identification. Even higher scores can identify the species (Marklein *et al.,* 2009). Scores between 1.75 and 2.0 indicate moderate identification, which could require further verification. Even these scores can identify the genus (Marklein *et al.,* 2009). Scores less than 1.75 suggest that the spectrum was not very good or the organism was not represented in the database (Mellmann *et al.,* 2008). The scoring method ensures the accuracy and confidence level in the identification of microorganisms based on their unique peptide mass fingerprints (Mellmann *et al.,* 2008; Marklein *et al.,* 2009).

Peptide Mass Fingerprint (PMF)

Peptide Mass Fingerprint (PMF) refers to the unique pattern of mass-to-charge (m/z) values obtained when proteins or peptides from a biological sample (Calderaro and Chezzi, 2024), such as fungal mycelia, spores, or bacterial cells are analyzed using MALDI-TOF MS (Croxatto *et al.,* 2012). During the MALDI process, proteins in the sample are ionized and accelerated through the time-of-flight analyzer, producing a mass spectrum that represents the relative abundance and m/z of different peptide ions. This pattern of peaks is based on the masses of specific proteins and is characteristic of each microorganism (Welker, 2011). Because these profiles differ significantly between species (and often even between strains), the resulting PMF acts like a biochemical fingerprint, allowing for the identification and classification of microorganisms by comparing the sample's spectrum to reference databases (Demirev *et al.,* 1999; Welker, 2011; Murray, 2012). The strength of PMF lies in its ability to provide rapid, accurate, and culture-based identification without the need for extensive biochemical or molecular testing (Van Veen *et al.,* 2010).

The peptide mass fingerprints are generated mainly from ribosomal proteins with unique patterns for each microorganism (Nakamura *et al.,* 2017). Because these ribosomal proteins are most abundant protein in all microbial cells; are conserved within a species; they have enough variation between species to allow for accurate identification; are structurally stable, making them less likely to degrade during sample preparation; their molecular weight is in the range of 2–20 kDa, which is optimal detection range of

MALDI-TOF MS (Nakamura *et al.,* 2017; Han *et al.*, 2021). These PMFs are stored in reference databases (like Bruker Biotyper) and used to match unknown organisms.

Application of MALDI-TOF MS in Plant Pathology

Identification of phytopathogenic fungi using MALDI-TOF MS

Rapid identification of fungi causing plant diseases enables early and precise disease management, reducing crop losses and preventing large-scale outbreaks. It also supports targeted fungicide use, lowers economic costs, and strengthens food security and biosecurity measures. An early study by Welham *et al*. (2000) demonstrated that MALDI-MS could be adapted to characterize intact fungal spores directly. The study generated repeatable spectral fingerprints in the 2–13 kDa range by analysing species including *Penicillium, Scytalidium dimidiatum*, and *Trichophyton rubrum*, exposing unique profiles for each species. This work demonstrated the potential of MALDI-based fingerprinting for quick characterisation of plant and therapeutically relevant fungi, laying the groundwork for its application beyond bacterial identification to fungal identification.

In a polyphasic taxonomic investigation, Al-Hatmi *et al*. (2016) revealed that *Fusarium ficicrescens* constitutes a new species within the *Fusarium fujikuroi* species complex (FFSC), which includes many plant pathogens and mycotoxin producers. Despite initially being misidentified morphologically as *Fusarium andiyazi*, multilocus sequencing (TEF1, BT2, and RPB2), AFLP profiling, and MALDI-TOF MS protein fingerprinting consistently identified them as a distinct, well-supported clade closely related to *F. lactis, F. ramigenum*, and *F. napiforme*. MALDI-TOF MS produced a distinct spectral cluster for *F. ficicrescens*, effectively distinguishing it from closely related and morphologically similar FFSC members, with elevated log score values affirming species-level differentiation. The high agreement among DNA barcoding, AFLP, and MALDI-TOF MS underscores the utility of proteomic profiling as a quick and dependable supplementary method for identifying and delineating closely related plant pathogenic fungi within species groups.

The efficiency of MALDI-TOF MS in identifying and differentiating species within the *Fusarium fujikuroi* species complex a group that causes significant crop illnesses and mycotoxin contamination was shown by Wigmann *et al.* (2019). Using 49 phylogenetically validated species to build a reference spectral database, the approach successfully identified non-reference isolates at the species level with 94.61% accuracy. The study demonstrated that MALDI-TOF MS provides a quick, dependable, and efficient substitute for multilocus sequencing (MLST) in the identification of closely related plant pathogenic fungi.

Barthélemy *et al*. (2020) employed MALDI-TOF MS protein fingerprinting to categorize 42 endophytic strains of *Colletotrichum*, demonstrating that ITS sequencing by itself was insufficient to distinctly differentiate closely related species. MALDI-TOF MS categorized the isolates into 18 Operational Chemically Units (OCUs) according to unique protein profiles, more accurately representing their metabolic diversity. The integration of LC-HRMS/MS molecular networking facilitated dereplication and directed the extraction of bioactive metabolites like cyclopeptides, cytochalasins, and novel colletamides. The research emphasizes MALDI-TOF MS as a swift and efficient method for distinguishing closely related fungal strains associated with plants and prioritizing bioactive isolates.

A comprehensive study by Božik *et al.* (2021) demonstrated the application of MALDI-TOF MS for rapid identification of species within the genus *Phytophthora*, a group of destructive plant pathogens responsible for severe agricultural losses worldwide. Twenty-four genetically validated species (42 isolates) were first confirmed using ITS and COI sequencing, after which more than 3,500 high-quality spectra were generated to construct a curated reference library comprising 144 main spectrum profiles (MSPs) using the Bruker MALDI Biotyper platform. The updated in-house database enabled correct identification of 90.6% of isolates at the species level and 100% at the genus level, despite the absence of *Phytophthora* representatives in commercial libraries. Notably, closely related species such as *P. palmivora* and *P. infestans* were reliably differentiated, highlighting the discriminative power of MALDI-TOF MS. The study emphasized that MALDI-TOF MS provides a rapid, cost-effective, and less labor-intensive alternative to DNA sequencing and underscored the importance of developing open-access spectral databases to expand its application in plant pathogen diagnostics.

In a practical workflow investigation, Barker *et al*. (2022) enhanced MALDI-TOF MS identification for *Aspergillus*, *Fusarium*, and *Mucorales* order members by assessing database choice, culture medium, and incubation duration. With the Bruker Filamentous Fungi v3 database, accurate identification attained 85.7% at the species level for *Aspergillus*, 94.4% at the species-complex level for Fusarium, and 90% at the species level for *Mucorales*, whereas the MSI-2

database enhanced species resolution especially for hidden *Aspergillus* species. Id-Fungi plates (IDFP) showed comparable or slightly superior performance to Sabouraud dextrose agar and allowed for cleaner biomass recovery, with optimal identification reached at 24 hours for *Fusarium* and *Mucorales*, and 48 hours for *Aspergillus*. This research emphasizes that uniform extraction methods, suitable database choices, and regulated incubation environments greatly improve the dependability and efficiency of MALDI-TOF MS in identifying filamentous fungi, particularly those that are important plant pathogens in agriculture.

A recent investigation by Rolland *et al*. (2024) upgraded the VITEK MS database to better identify fungi linked to food through MALDI-TOF MS. This augmented database featured 380 strains encompassing 133 species across 51 genera, addressing key plant pathogenic complexes like *Colletotrichum* (*acutatum* and *gloeosporioides* complexes), *Fusarium dimerum* complex, *Aspergillus* series *Nigri*, and *Mucor circinelloides* complex. Through this enhancement, the system attained significant accuracy, successfully identifying more than 96% of strains in cross-validation and over 90% in external assessments. Significantly, it successfully differentiated closely related and cryptic species in key phytopathogenic groups, showcasing the robust resolving capacity of MALDI-TOF MS. The authors stressed that ongoing database enhancement and refined spectral analysis methods are crucial for enhancing species-level identification in plant pathology and food safety contexts.

Another recent study by Kurera *et al.* (2025) demonstrated the utility of MALDI-TOF MS for rapid identification of *Fusarium* species responsible for head blight in Canada. Using a custom peptide mass fingerprint (PMF) reference library, the method was validated on over 800 field isolates and showed 95% agreement with species-specific qPCR for predominantly *F. graminearum* samples and 86% agreement for oat and barley isolates mainly consisting of *F. poae*. The study confirmed that MALDI-TOF MS is sufficiently sensitive to identify *Fusarium* isolates at the species complex level and, in many cases, at the species level, highlighting its potential as a rapid and high-throughput alternative to DNA-based diagnostics in plant pathology.

Identification of phytopathogenic bacteria using MALDI-TOF-MS

Accurate identification of plant pathogenic bacteria is essential for effective disease management and quarantine enforcement, yet traditional methods such as biochemical assays, serological tests, and 16S rRNA analysis are often time-consuming, prone to cross-reactions, and insufficient for distinguishing closely related taxa (e.g., *Clavibacter*) (Zaluga *et al.*, 2011). Research conducted by Zaluga and colleagues showed that MALDI-TOF MS in combination with gyrB sequence analysis offers precise and dependable identification of plant pathogenic species within the genus *Clavibacter*. Examination of 165 strains encompassing all five subspecies of *Clavibacter michiganensis* revealed that gyrB sequencing distinctly differentiated subspecies through unique nucleotide signatures, whereas MALDI-TOF MS produced consistent, subspecies-specific protein mass profiles with identifiable biomarker peaks. Both techniques effectively distinguished quarantine-related subspecies from similar genera like *Curtobacterium*, and validating MALDI-TOF MS as a quick, reliable, and economical method for the routine identification of plant pathogenic *Clavibacter* strains.

Research by Niculau *et al*. (2022) emphasized the effectiveness of the MALDI-TOF MS method for the identification of citrus canker induced by *Xanthomonas citri* subsp. *citri* (*Xcc*). The PCR technique, emphasizing the 747 bp *atpD* gene, successfully identified the pathogen in symptomatic leaves; however, the researchers showed that the MALDI-TOF MS method serves as a quicker and more economical alternative. The most effective bacterial identification outcomes were achieved with 48-hour cultures, which generated distinct and reliable peaks, notably at m/z 4664, 5859, 7418, and 9330, thereby assisting in the precise identification of the genus and species. Through the efficient application of chemometric techniques such as MSP dendrograms and PCA, the researchers successfully differentiated the infected leaf tissue samples from the healthy and herbicide-affected samples, aided by the ions exceeding m/z 7000. The variation in the mass spectrometric spectra between the pure bacterial cultures and the plant tissue samples also contributed to the technique's precision. It is important to mention that the method also demonstrated high reproducibility, with the cost per sample, conducted in triplicate, being around US$0.12, while results were accessible within three hours, thus highlighting its significant potential as a diagnostic tool for identifying citrus canker and other plant bacterial infections.

A phyloproteomic study conducted by Motyka-Pomagruk *et al*. (2023) examined the intraspecies variation of *Dickeya solani*, a virulent soft-rot pathogen affecting potatoes in the *Pectobacteriaceae* family. Analyzing 20 strains from various geographic locations and years of isolation with MALDI-TOF MS, the study revealed that although the species displayed general genetic uniformity, subtle yet consistent

variations in protein mass spectra enabled the identification of four separate clades. Crucially, all *D. solani* isolates created a distinctly separated cluster from other *Dickeya* species, validating the robust discriminatory ability of MALDI-TOF MS at the species level. The proteomic clustering was additionally reinforced by the phenotypic characterization of traits associated with virulence, such as plant tissue maceration, enzyme production, and growth dynamics, even though the majority of strains showed consistently high virulence potential. The results emphasize that MALDI-TOF MS serves as a quick and dependable method for the routine species-level identification of *D. solani* isolates from environmental sources while also being able to identify subtle intraspecies variation, thus enhancing its significance in diagnostics of plant pathogenic bacteria and epidemiological research.

A recent investigation by Sangeetha *et al*. (2025) showcased the practical importance of MALDI-TOF MS in managing plant diseases by identifying endophytic bacteria that act against the sugarcane red rot pathogen, *Colletotrichum falcatum.* Through whole-cell MALDI-TOF MS (MALDI BioTyper system), 15 bacterial endophytes obtained from sugarcane roots were quickly and precisely identified, mainly associated with the genera *Bacillus*, *Enterobacter*, and *Klebsiella*. Of these, *Bacillus subtilis* SOE 7 exhibited the most potent antifungal effect, reducing pathogen mycelial growth by over 62% in vitro, with MALDI-TOF MS verifying its species-level identity with a high-confidence score (2.149). Biochemical and HPLC assessments further indicated the generation of antifungal lipopeptides like surfactin and iturin, clarifying its antagonistic impact. Field evaluation confirmed these results, as treatment with *B. subtilis* SOE 7 markedly lowered red rot occurrence while enhancing yield and quality metrics like brix and sucrose levels. In summary, the research emphasizes that MALDI-TOF MS facilitates quick identification of microbes, aiding in the choice and confirmation of effective biocontrol agents for the sustainable management of plant pathogenic fungi.

Plant virus detection using MALDI-TOF-MS

Detection of plant viruses is particularly important due to their high genetic variability, which leads to the rapid emergence of new strains with altered virulence, host range, or resistance-breaking ability (Roossinck, 2008; Elena *et al.,* 2014). The recent study by Dederko *et al*. (2025) proved the potential for the simultaneous detection and differentiation of *Potato virus Y* strains, a highly important virus for agriculture on a global scale, by employing the technique of MALDI-TOF MS. In the study, the three major strains of PVY, i.e., PVY^{O}, PVY^{NTN}, and PVY^{N-Wi}, were analyzed for their genetic material, protein extracts, and whole virus particles. All three strains had unique spectral fingerprints, with PVY^{N-Wi} and PVY^{O} showing rich spectral characteristics, especially for the low-mass protein (LP; 2-20 kDa) region. Certain strain-specific ion features were identified. Based on spectral profiles, Principal Component Analysis (PCA) showed a distinct clustering of strains, with 45% laser power being shown to be ideal for spectral balance. While RT-qPCR demonstrated a linear detection range between 6000 and 0.6 pg of viral RNA, the technique allowed detection down to 0.001 mg/mL of viral protein. This work demonstrates the potential of MALDI-TOF MS as a quick, supplementary tool to molecular diagnostics in plant pathology and is one of the first attempts to use it for plant virus strain identification.

Detection of phytoplasma using MALDI-TOF MS

MALDI-TOF Mass Spectrometry has been effectively utilised for the rapid identification of various plant pathogens, including bacteria, fungi, and oomycetes; however, its application in direct identification of phytoplasma is still very limited. The inability to culture phytoplasmas and their low concentration in plant phloem tissues primarily account for this limitation, hindering the development of reliable MALDI spectral databases. Accordingly, most phytoplasma diagnostics still use PCR-based molecular methods. A study of proteomic and phosphoproteomic analysis of *Vitis vinifera* infected with the phytoplasma which causes Flavescence dorée revealed multiple proteins associated with stress response, photosynthesis, and antioxidant metabolism. The research indicated that 48 proteins showed varying abundance and phosphorylation levels in infected grapevines compared with healthy vines. During the infection, enzymes linked to oxidative stress, like glutathione S-transferase and isocitrate dehydrogenase, were exhibiting increased activity. However, levels returned to normal in recovering plants. These results highlight the significance of protein phosphorylation in modulating host defence mechanisms during phytoplasma infection and subsequent recovery. Proteomic studies offer deeper insights into host-pathogen interactions at the protein level, complementing pathogen identification techniques like MALDI-TOF MS (Margaria *et al.,* 2013). Subsequent research concentrating on protein fingerprinting from infected plant tissues or insect vectors may facilitate MALDI-TOF-based detection of phytoplasmas (Chun *et al.,* 2022).

Table 2 : Plant pathogens identified using MALDI-TOF MS (2011–2025)

Year	Pathogen(s) identified	Host plant/ Sample	Key findings	References
2011	*Clavibacter michiganensis* subspecies	Crop plants (quarantine pathogens)	MALDI-TOF MS produced unique spectral fingerprints enabling accurate subspecies-level identification	Załuga *et al.*, 2011
2015	Bacteria, fungi, viruses	Various	MALDI-TOF MS established as a rapid, low-cost method for microbial identification across pathogen types	Singhal *et al.*, 2015
2017	Yeasts and filamentous fungi (phytopathogens)	Agricultural systems	MALDI-TOF MS shown as a reliable tool for fungal pathogen identification, though database limitations exist	Drissner and Freimoser, 2017
2017	Arbuscular mycorrhizal fungi (AMF)	Soil/plant roots	Successfully differentiated species and strains using proteomic fingerprints	Crossay *et al.*, 2017
2018	Melanized fungi (plant-associated)	Environmental isolates	Identification accuracy significantly improved after expanding MALDI databases	Paul *et al.*, 2018
2019	Filamentous fungi (incl. phytopathogens)	Environmental/plant isolates	MALDI-TOF achieved ~92% correct identification at species level	Peng *et al.*, 2019
2021	Environmental and plant-associated bacteria (e.g., *Pseudomonas*, *Bacillus*)	Soil, water, crops	MALDI-TOF widely applied for identifying plant-associated and pathogenic bacteria	Ashfaq *et al.*, 2021
2023	Agricultural bacteria (incl. plant pathogens)	Irrigation water, vegetables	MALDI-TOF identified multiple plant-associated pathogens at genus/species level	Surányi *et al.*, 2023
2023	*Lasiodiplodia theobromae*	Grapevine	MALDI-TOF used to identify antifungal metabolites targeting plant pathogens	Saucedo-Bazalar *et al.*, 2023
2024	Bacteria, fungi, viruses	Multiple	MALDI-TOF confirmed as routine, rapid diagnostic tool with high reliability	Calderaro and Chezzi, 2024
2025	30 potato pathogens (fungi, bacteria, viruses, viroids)	Potato	MALDI-TOF (with PCR) enabled simultaneous detection of 30 phytopathogens	Lei *et al.*, 2025
2025	Yeasts and filamentous fungi (incl. plant-related species)	Environmental/clinical	Expanded libraries improved identification coverage up to ~88%	Ban *et al.*, 2025

The above table (Table 2) complements the detailed examples (e.g., Fusarium, Clavibacter, Potato virus Y) by offering a broader timeline, highlighting trends like improving accuracy and database expansions.

Advancements in MALDI-TOF MS Technology

Expression studies by proteomic analysis using MALDI-TOF MS

The antifungal mechanism of 1-(4-bromophenyl)-5-phenyl-1H-1,2,3-triazole (BPT) against *Colletotrichum gloeosporioides*, a primary cause of mango anthracnose, was examined in a proteomics-based study by Wang *et al.* (2025), who discovered target proteins and differentially expressed proteins using MALDI-TOF MS in conjunction with chemical proteomics and bioinformatics. They identified cytochrome P450 enzymes, including sterol 14α-demethylase (CYP51), as important interaction targets. This study illustrates the value of MALDI-TOF MS in both fungal identification and proteomic-level elucidation of antifungal processes.

Discovery of biomarker for antimicrobial resistance using MALDI-TOF MS

A review by Vrioni *et al*. (2018) highlights the increasing use of MALDI-TOF MS for identifying antimicrobial resistance (AMR) biomarkers alongside standard pathogen detection in clinical laboratories. The technology allows for quick identification of resistance via spectral analysis, antibiotic hydrolysis tests, isotope labelling, and growth-based methods, providing a quicker and more economical method than traditional susceptibility testing, though additional validation is required. The expanding application of MALDI-TOF MS for antimicrobial resistance

detection in clinical microbiology suggests strong potential for its future incorporation into plant pathology, particularly for rapid identification of resistance traits in phytopathogenic bacteria.

Analytical utility and practical limitations of MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a significant instrument for identifying microbes because of its quick processing time, great precision, limited sample preparation, low reagent usage, and ability to handle many samples at once, rendering it economical and eco-friendly for use in clinical, food, environmental, and plant microbiology (Zaluga *et al.*, 2011; Jang and Kim, 2018; Niculau *et al.*, 2022; Calderaro and Chezzi, 2024). Nonetheless, its effectiveness may be affected by the quality of samples and methods of preparation, differences in culture media and incubation environments, and challenges in distinguishing closely related or genetically similar species, whereas accurate identification heavily relies on the existence of extensive and meticulously curated spectral databases (Jang and Kim, 2018; Vrioni *et al.*, 2018; Motyka-Pomagruk *et al.*, 2020; Calderaro and Chezzi, 2024).

Conclusion

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) is emerging as an important tool in plant pathology because it allows rapid and accurate identification of pathogens by analyzing distinctive protein or peptide mass fingerprints. Compared with traditional culturing or sequencing, the method greatly reduces diagnostic time while supporting high-throughput analyses, which is particularly useful for large-scale agricultural monitoring. It also has the advantage of being more environmentally sustainable, since it relies on fewer reagents than conventional techniques, and it serves as a practical link between older phenotypic methods and advanced molecular approaches such as PCR and DNA sequencing. Despite these benefits, MALDI TOF MS still faces certain limitations, including difficulties in distinguishing very closely related species and a strong reliance on the depth and quality of reference databases to achieve reliable results. Even so, its speed, accuracy, cost-effectiveness, and potential to integrate with other technologies highlight its promise as a sustainable and next-generation approach for efficient plant disease diagnosis in agriculture.

Future Directions

The future of MALDI-TOF MS in plant pathology will be shaped by a series of strategic advancements that are likely to improve its scope and application in diagnosing and identifying plant pathogens. First and foremost, there is a need to expand and improve existing spectral libraries by incorporating a broader range of plant pathogens and their closely related species and strains in order to improve their accurate identification and minimize misclassifications. Moreover, the integration of mass spectrometry-based proteomic analysis and genomics, coupled with artificial intelligence tools, will offer a new avenue of growth and expansion of MALDI-TOF MS technology in plant pathology. In particular, it may offer possibilities for predictive diagnostics of plant pathogens by inferring phenotypic characteristics such as virulence and antimicrobial resistance from spectral data and genomic sequences. In addition, the development of portable MALDI-TOF MS technology will offer new opportunities and prospects for pathogen detection and diagnosis directly from agricultural fields and crops in real-time and *in situ*. Furthermore, developments in the application of multiplex detection methods may enable the simultaneous detection of several pathogens in complex samples and the simultaneous detection of resistance-associated markers, which is particularly important in the context of the 'field environment,' where co-infections are common. Overall, these developments suggest that MALDI-TOF MS is likely to become an increasingly robust, accessible, and predictive tool in the future in the context of plant disease diagnostics.

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Abbreviations

AMF: Arbuscular mycorrhizal fungi
BPT: 1-4-bromophenyl-5-phenyl-1H-1,2,3-triazole
CHCA: α-cyano-4-hydroxycinnamic acid
DHB: 2,5-Dihydroxybenzoic acid
ELISA: Enzyme-linked immunosorbent assay
FFSC: Fusarium fujikuroi species complex
HPLC: High-performance liquid chromatography
ITS: Internal transcribed spacer
LAMP: Loop-mediated isothermal amplification
LC-HRMS/MS: Liquid chromatography-high resolution mass spectrometry/mass spectrometry
MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MLST: Multilocus sequence typing
MSP: Main spectrum profile
OCU: Operational Chemotaxonomic Unit
PCA: Principal Component Analysis

PCR: Polymerase chain reaction
PMF: Peptide mass fingerprint
qPCR: Quantitative polymerase chain reaction
RT-qPCR: Reverse transcription quantitative polymerase chain reaction
SA: Sinapinic acid
TEF1: Translation elongation factor 1
TOF: Time-of-Flight
Xcc: *Xanthomonas citri* subsp. *citri*

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