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MALDI-TOF mass spectroscopy of yeasts and filamentous fungi for research and diagnostics in the agricultural value chain

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Abstract

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; MALDI biotyping) has become a standard tool for the accurate, rapid, and economical identification of pathogens in the clinical diagnostics laboratory. The method is continuously being improved, and new applications for distinguishing strains, identifying metabolites or functional characteristics (e.g., antibiotic resistance), and detecting microbes directly in patient samples have been developed. Adopting these methods in other disciplines than clinical diagnostics, for example, in agriculture, food safety and quality testing, or ecology, will open up new opportunities for diagnostics and research. This review focuses on MALDI-TOF MS approaches for the identification of yeasts and filamentous fungi. In contrast to bacterial diagnostics, MALDI biotyping of fungi is more challenging and less established. We thus start by discussing the role of MALDI-TOF MS as a tool for species identification; in particular with respect to DNA-based identification methods. The review then highlights the value of custom-made reference spectra for MALDI biotyping and points out recent advancements of MALDI-TOF MS, mainly from the field of clinical diagnostics that may be adopted and used for fungal diagnostic challenges. The overview ends with a summary of MALDI-TOF MS studies of yeasts and filamentous fungi of agricultural relevance.

Keywords: Agriculture, MALDI-TOF MS, Biotyping, Diagnostics, Filamentous fungi, Yeast

Introduction

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; also called MALDI biotyping) has become increasingly popular for the identification of microorganisms and their functions and is now approved for the routine identification of bacteria and fungi in clinical diagnostic laboratories [1–3]. It is the goal of this review to highlight recent technological advancements of MALDI biotyping and to illustrate applications of this tool for research in agriculture and the agricultural value chain. This outlook focuses on MALDI-TOF MS applications in mycology, because these are usually more demanding than bacterial identifications [4, 5] and will thus greatly benefit from the recent advances in bacterial MALDI-TOF MS diagnostics. In this context, it is important to note that MALDI-TOF MS is not an alternative to DNA sequence-based species identification, but rather a complementary method. In order to make use and realize the potential of MALDI-TOF MS it may thus be helpful bringing to mind the advantages and disadvantages of this method; in particular as compared to DNA-based assays and techniques.

MALDI biotyping and DNA-based identification complement each other

The identification of fungi usually involves a DNA sequence-based analysis that determines the identity of a given isolate based on sequence similarity; often taking advantage of the universal fungal barcode sequence of the nuclear ribosomal internal transcribed spacer (ITS) [6]. Second and third generation sequencing techniques now allow the sequencing of thousands or millions of barcodes, or even entire genomes, in parallel and such

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high-throughput technologies are available to virtually every laboratory. These barcode (amplicon) and metagenome sequencing methods allow identifying hundreds or thousands of species simultaneously and are thus powerful tools to describe the composition of entire microbial communities (Fig. 1a) [7–9]. However, although massive parallel DNA sequencing generates a wealth of data, often more data are generated than needed and storage of the data and extraction of the required information is challenging and often limiting [10]. In addition, preparation and quality control of the template libraries for DNA sequencing is time consuming and costly (Table 1). In specific cases, for the repeated identification of a defined number of species, it may thus be best not to generate the largest amount of data, but rather to generate exactly the amount of data needed in the fastest and most economical way. It is in this realm, where MALDI biotyping shines (Fig. 1a; Table 1).

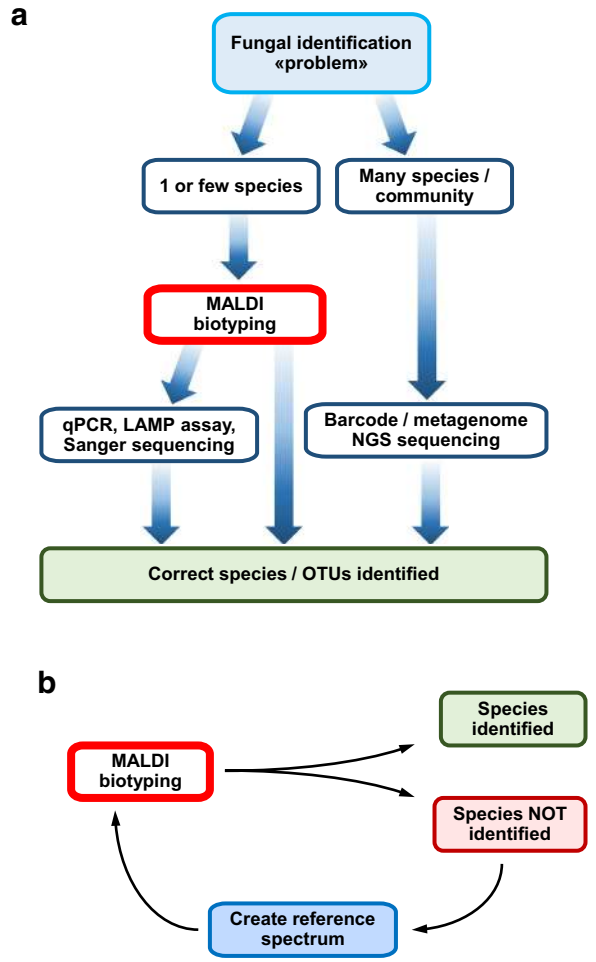

Fig. 1 MALDI biotyping and DNA-based identification complement each other. **a** MALDI biotyping is the method of choice for the identification of a defined number of species. **b** Generating MALDI-TOF MS reference spectra for fungal species identified by DNA-based methods allows the rapid and economical detection of this species by MALDI biotyping in the future

In contrast to DNA sequencing-based approaches, MALDI-TOF MS uses whole cells or crude, acidic extracts, and mass spectra for the identification of individual species [2, 11]. The simple sample preparation, short measurement times, easy preparation of reference spectra, and low costs per sample are important advantages of MALDI-TOF MS, as compared to specific, DNA-based assays that are often laborious to establish and usually require costly reagents (Table 1) [1, 12]. MALDI-TOF MS is also highly flexible and can be combined with additional extraction or processing steps in order to identify specific biomarkers, metabolites, or biochemical functions. Because of these advantages, MALDI biotyping has become a standard in routine clinical diagnostics and MALDI-TOF MS devices have become readily accessible, also for researchers in other disciplines than clinical diagnostics [4, 13, 14]. This opens up new possibilities for functional hypothesis-driven research.

Unless it is the goal to identify entire microbial communities, MALDI-TOF MS is the method of choice for species identification, because it is fast, economical, and many common bacteria and an increasing number of fungi can be identified. In those cases where MALDI-TOF MS does not reveal the identity of a particular organism, classical Sanger sequencing or DNA-based assays (e.g., qPCR or LAMP assays) are used for identification (Fig. 1a; Table 1). Once the organism is identified based on such an assay, the generation of new MALDI-TOF MS reference spectra will allow identifying this species or isolate in the future (Fig. 1b). The iterative procedure comprises DNA-based identification and MALDI-TOF MS reference spectrum generation for each new organism continually increases the number of species that can be identified and thus the benefit of a particular MALDI-TOF MS system (comprises robust software, reliable algorithms, and databases). In particular in large culturomics projects, an integration of MALDI-TOF MS in the identification pipeline results in a comprehensive database of reference spectra that allows identifying a plethora of species and even challenges, or rather complements, DNA sequencing-based analyses of microbiomes [15, 16].

Custom-made reference spectra improve MALDI-TOF MS-based identification

DNA-based identification methods benefit from large, public, DNA sequence repositories, and many openly accessible or downloadable analysis tools. In contrast, MALDI-TOF MS reference spectra, as well as analysis tools, are usually proprietary, only commercially available, and heavily focused on medical applications. Even

Table 1 MALDI-TOF- and DNA sequencing-based approaches complement each other

	MALDI biotyping	qRT-PCR/lamp assay	Sanger sequencing	NGS sequencing
Starting material	*Crude extracts or whole cells*	DNA extract from mixed sample	DNA extract from pure sample	DNA extract from community
Premises	Reference spectrum	Specific assay developed	Clone or PCR product available	*None/PCR product*
Equipment costs (machines)	High	*Low-medium*	High	High
Cost per sample	*Negligible*	Low-medium	Medium	High
Time for sample preparation	*Short*	Short-long	Long	Long
Time for analysis	*Short*	Medium	Medium	Medium-long
Data	Mass spectrum of one sample	Detection of one DNA fragment	DNA sequence of 400–1000 bp	*In total 50 Mbp–1000 Gbp*
Number of species/strains detected	Usually 1 per sample	1 per assay	1 per sample	*Up to many thousands*
Cultivation step required	Usually yes	*No*	Usually yes	*No*

Properties and comparison of MALDI biotyping and DNA sequencing-based approaches (of barcodes/PCR products or genomic DNA) methods for the identification of fungi. References: [100, 101]

The advantages of each method are shown in italics

organisms commonly found in environmental, agricultural, or food samples are thus often not recognized by standard, commercial MALDI-TOF MS systems. In addition, MALDI-TOF MS spectra are not universal, such as a DNA sequence, but depend on the crude cell extract or the physiological state of the fungal cells that are applied onto the target plate and used for recording mass spectra. Consequently, the age and growth conditions of a microbial culture, as well as the settings of the ionizing laser, flight tube, and mass detector, can influence a MALDI biotyping experiment.

Many of these problems can be overcome by generating custom-made, specific MALDI-TOF MS reference spectra for the particular application in question [5]. Since these reference spectra are generated with the same MALD-TOF MS device, settings, and sample preparation as the experimental samples, the scores obtained with these references are usually higher as compared to generic reference databases [5, 17–19]. Custom-made reference spectra thus allow ample flexibility with respect to different sample preparation protocols and experimental set-ups in a particular lab or for specific applications. For example, reference spectra for different cell densities of a particular organism may allow an estimation of cell densities [20]. It is of course also possible to generate reference spectra for fungi grown on different agar plates, different physiological states, different extraction protocols, or directly applied bacterial or yeast cells (as opposed to crude extracts).

The integration of custom-made reference spectra in a commercial MALDI-TOF MS system is only the first step towards a truly open platform for MALDI-TOF MS-based species identification. Although it is easy to generate reference spectra, a comprehensive public repository of MALDI-TOF MS reference spectra is lacking. Such a resource would greatly facilitate the exchange of reference spectra between research groups and thus allow identifying microorganisms for which no reference spectra have been generated in a particular lab [21]. Until now, only few MALDI-TOF MS spectra databases for microbial identification are publicly available. The FoodBIMS database comprises reference mass spectra of food-borne bacteria [22] (http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm). SpectraBank is a freely accessible database that also comprises mainly bacterial reference spectra [23], and the free Spectra site hosts an extended database of MALDI-TOF MS reference spectra for bacteria and fungi (which is made available by the Public Health Agency of Sweden (Folkhälsomyndigheten): http://spectra.folkhalsomyndigheten.se/spectra/welcome.action).

In addition to MALDI-TOF MS data repositories, instrument- and provider-independent analysis tools for microbial biotyping using mass spectra are urgently needed. SPECLUST is an application to perform cluster analyses of MALDI-TOF MS spectra and was for example used to separate the bacterium *Ralstonia solanacearum* into different species [24, 25]. More recently, Starostin et al. [26] developed a tool to use geometric distances between MALDI-TOF MS spectra represented in a multi-dimensional space to distinguish closely related *Bacillus* strains. Mass-up is a comprehensive, open-source tool for the processing and analysis of MALDI-TOF MS data [27]. Besides classification, it has also biomarker discovery, principle component analysis, and clustering functions implemented and was, for example, used to fingerprint bacterial isolates or classify peritoneal dialysis patients by mass spectrometry-based

profiling [28, 29]. For baseline correction, normalization, peak detection, and matching, it uses open-source packages such as MALDIquant [30] and MassSpec Wavelet [31], which are also available as separate R packages. Finally, BIOSPEAN (http://software.cr-hana.upol.cz/biospean/login.php) is a web-based application and database that was specifically developed for analyzing whole-cell MALDI-TOF MS data and includes peak picking, generation of MS databases, and data sharing among users [32].

The public deposition of MALDI-TOF MS spectra, together with information concerning culture conditions and experimental details is highly desirable and could largely expand the potential of MALDI-TOF MS for agricultural and food diagnostics, as well as ecological research. Standardized extraction buffers (an acidic extraction using formic acid and acetonitrile seems often used) and particularly matrix solutions (e.g., α-cyano-4-hydroxycinnamic acid, HCCA) would much improve comparability of results among different laboratories. However, since different types of cells and organisms require different extraction buffers and matrices for the best MALDI-TOF MS spectra, including this information in the reference spectra and in biotyping experiments seems necessary.

Clinical MALDI-TOF MS applications benefit fungal diagnosis and research

The low sample preparation costs and fast measurement time, in addition to its accuracy, are highly attractive properties of MALDI-TOF MS; in particular for clinical applications. MALDI biotyping of clinically relevant microorganisms is thus more advanced than of other microbes and new methods are first introduced and tested with clinical samples. Consequently, the majority of MALDI-TOF analyses of fungi so far have dealt with clinical isolates [33]. In particular, the rapid and economical identification of *Candida* species is an important medical application [34–39], but filamentous fungi present in clinical samples have been studied by MALDI biotyping as well [40–43]. Overall, MALDI-TOF MS is an accurate, reliable, and rapid method for the identification of human pathogenic fungi, and new applications for clinically relevant yeasts and filamentous fungi are continuously being developed [43–45]. A comparison of two commercially available systems, VITEK MS (bioMérieux) and MALDI Biotyper (Bruker Daltonics) with their associated databases, has shown similar identification efficiencies of clinically relevant yeasts for the two systems [46, 47], while other studies found differences between biotyping systems [48, 49]. The identification power is improved by including in-house generated reference spectra in the databases [17, 43, 46] (also see above). In addition, it was shown that the sample preparation method and quality of the database are crucial for accurate identification [38]. In general, identification rates for yeasts are above 90% and higher when using acidic extracts, as compared to direct transfer of whole cells [38, 50]. In many cases, clinical diagnostics of human pathogenic bacteria and fungi drives the development of new MALDI biotyping approaches that will also benefit fungal diagnosis and research in other areas. It therefore seems worthwhile discussing new MALDI-TOF MS techniques that are mainly used in the clinical setting in order to highlight the potential and outline opportunities for MALDI-TOF MS of yeasts and filamentous fungi.

Shortening the cultivation time for faster MALDI-TOF MS identification

In clinical diagnostics, as in agricultural and food safety diagnostic, time-to-result and cost are the most important criteria for selecting analytical methods and may, in the most extreme situation, literally be a matter of life or death. The cultivation step, protein extraction, and pure cultures, in many cases required for a successful MALDI-TOF MS identification, may thus prevent this method from being used [51]. Therefore, efforts are undertaken and considerable progress has been made to address these shortcomings. For the diagnosis of bacterial pathogens responsible for bloodstream infections the mean incubation time to identify Gram-positive bacteria was 5.9 h, which dropped to 3.1 h if a crude acidic extract was prepared [52]. In other studies, 97 to 69.5% of bacteria were correctly identified after a short incubation of positive blood cultures on solid media of only 3 to 5 h [53–56], which enabled identification on the same day as the positive blood sample was detected. By increasing the sample concentration (by reducing the sample spot size), creating reference spectra for different cell densities, and immunoaffinity enrichment of bacteria, it was possible to detect as few as 10 to 100 bacterial cells after a blood culture time of only 4 h [20]. These examples illustrate the vastly shortened time-to-result of clinical MALDI-TOF MS applications: the duration of the cultivation step has been reduced to just a few hours and is thus in the same range as DNA amplification steps. In other studies of positive urine or blood samples, or of cerebrospinal fluid, the cultivation step was omitted entirely, and pathogenic bacteria and yeasts were directly identified [57–59].

MALDI-TOF MS applications beyond species identification

MALDI-TOF MS is not only used for taxonomic identification, but also able to draw functional conclusions about clinically relevant properties of a particular isolate [60]. Since MALDI-TOF MS is, in principle, able to identify any ionizable compound [61], it can detect antibiotic resistance (e.g., via the identification of specific proteins

or degradation products of antibiotics), but has also been used to reveal recombinant proteins, plasmid insertions in bacteria, or other biomarkers and diagnostic peptides in bacteria [62–66].

Recently, MALDI-TOF MS has been employed to rapidly and simultaneously detect and identify the *Alternaria* mycotoxins alternariol, monomethyl ether, and tentoxin in cereal grains [67]. It may be promising adapting this method for the identification of mycotoxins in further food products in the near future. The identification of clinically relevant anaerobic bacteria has also greatly benefited from MALDI-TOF MS approaches. This slow growing and fastidious group of pathogens has been mainly diagnosed by biochemical tests, but MALDI-TOF MS approaches have successfully identified such anaerobic pathogens and could delineate antibiotic-resistant and antibiotic-susceptible strains within the same species [68–70]. In the case of pathogenic yeasts, the sibling species of the *Cryptococcus gattii/Cryptococcus neoformans* complex, which cannot be discriminated by routine biochemical techniques, have been distinguished by MALDI biotyping [71]. Similarly, MALDI-TOF MS could reliably separate closely related members of the genus *Saccharomyces* (*S. arboricola*, *S. bayanus*, *S. cariocanus*, *S. cerevisiae*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, and *S. pastorianus*) [72]. Finally, MALDI-TOF MS more rapidly identified reduced susceptibility to caspofungin or triazoles in *Candida* and *Aspergillus* species, as compared to classical determination of the minimum inhibitory concentration [39, 73, 74]. With yeast cells from positive blood cultures and prepared by using the Sepsityper kit (Bruker Daltonics), direct MALDI-TOF MS identified the *Candida* species in 62.5% of the samples and antifungals susceptibility results were obtained for 72.7% of the blood samples [35]. Alterations in protein profiles of fungi following the exposure to antifungal compounds could be similarly used to monitor fungicide resistance in agriculturally relevant species.

Sample processing can broaden the use of MALDI biotyping

As for the highly sensitive species identification, discussed above, sample processing (e.g., tryptic digestion, acidic/organic extraction, nano-liquid chromatography) can greatly increase the sensitivity and specificity of a MALDI-TOF MS method and thus enable subspecies identification [66, 75]. A further possibility is the functional modification of the MALDI target plates themselves, which was for example performed with antibodies for direct immunoaffinity MALDI-TOF MS of haptoglobin or by dioxide coating in order to enrich phosphopeptides [76, 77]. In order to increase the number of mass peaks that may be used as biomarkers or the sensitivity of a MALDI-TOF MS analysis, samples have been successfully treated with detergent, sonication, corona plasma discharge, or heat [78–80]. In another study, target bacteria were separated and enriched without prior cultivation by using magnetic nanobeads that were functionalized with specific antibodies [20]. A rapid sample pretreatment consisting of removal of interfering blood cells and centrifugation and washing steps, which can also be performed with a commercially available kit (Sepsityper), is crucial for obtaining high identification rates of microorganisms, including yeasts, directly in positive blood cultures [81, 82]. These examples highlight that sample pretreatments are promising tools that may also benefit applications in agricultural diagnostics.

MALDI-TOF MS of polymicrobial samples and infected material

A standard MALDI biotyping experiment requires pure cultures of the microbial species to be identified. However, the identification of microorganisms from mixed or complex samples derived, for example, from patients, the environment, or infected plant tissue or food by MALDI-TOF MS is an important goal. So far, only a few reports have demonstrated the identification of bacteria and microalgae in mixtures by using biomarkers or correlation coefficients [83–85]. These studies have shown that ion suppression can affect the detection of specific masses of one or the other microorganism and thus complicate species identification in mixed samples [83, 84, 86]. On the other hand, novel mass peaks only observed in the mixed sample were discovered and may serve as biomarkers for mixtures or contaminations [83, 86]. For samples that contain more than two organisms, species identification based on biomarkers that invariably indicate the presence of a particular species performed better than correlation-based methods [84].

Fungal MALDI biotyping as a tool for agricultural diagnostics and research

MALDI biotyping of yeasts and filamentous fungi is more difficult than bacterial identifications, because the former result in less mass peaks and fewer reference spectra are available [4, 5]. In contrast to clinically relevant fungi, only a limited number of fungal plant pathogens, postharvest diseases, or food contaminants have been detected by MALDI biotyping. The non-medical applications of MALDI-TOF MS for fungal diagnostics and research that we are aware of are summarized in Table 2. These studies document that in particular the genera *Fusarium*, *Trichoderma,* and *Saccharomyces* are being used as models for the development of MALDI-TOF MS applications and for assessing the potential of this technology. In contrast to clinical studies, naturally

Table 2 Overview of MALDI-TOF MS studies of agriculturally relevant fungi

Samples/organisms	Highlights	Reference
Alternaria	Detection of the *Alternaria* mycotoxins alternariol, alternariol monomethyl ether, and tentoxin by MALDI-TOF MS	Sivagnanam et al. [67]
Alternaria	Separation of *A. dauci*, *A. porri*, *A. solani*, and *A. tomatophila* into three clusters by molecular analyses and MALDI-TOF MS	Brun et al. [102]
Alternaria	Identification of 60 isolates of 12 *Alternaria* species by intact cell MALDI-TOF MS with small mycelium samples	Chowdappa et al. [103]
Aspergillus	Optimization of protein extraction for 24 *Aspergillus* species from as few as 10,000 spores and identification of 11 proteins	Sulc et al. [104]
Aspergillus	Identification of 12 *Aspergillus* strains by preparing crude extracts by bead beating	Hettick et al. [105]
Aspergillus	Characterisation of *A. ibericus* strains by MALDI-TOF MS and comparison with related species	Kallow et al. [106]
Aspergillus	Analysis of several aflatoxigenic and non-aflatoxigenic strain belonging to four *Aspergillus* species	Li et al. [107]
Aureobasidium	Analysis of extracellular liamocins (mannitol oils) produced by *A. pullulans*	Price et al. [108]
Aureobasidium	Determination of oil structures of different *A. pullulans* strains	Manitchotpisit et al. [109]
Beer spoilage microorganisms	Detection and distinction of beer spoilage yeasts and bacteria from brewing yeasts	Turvey et al. [19]
Bremia, Oidium	Identification of ribosomal proteins and histones as markers for the biotyping of plant pathogens	Beinhauer et al. [97]
Chalara	In vitro and in vivo identification of *C. fraxinea* by secondary metabolites collected in methanol extracts	Pham et al. [110]
Clonostachys	Cluster analysis of MALDI-TOF MS data of 45 *Clonostachys* strains from different substrates	Abreu et al. [111]
Downy and powdery mildews	Identification of the obligate biotrophic mildew fungi *Bremia lactucae* and *Oidium neolycopersici*, also from infected leaves	Chalupova et al. [96]
Fusarium	Identification and characterisation of *F. verticillioides* and fumonisins by MALDI-TOF MS and MALDI-TOF MS/MS	Chang et al. [112]
Fusarium	Differentiation of *Fusarium* subspecies based on spores collected and prepared from isolates	Marchetti-Deschmann et al. [113]
Fusarium	Optimisation of MALDI biotyping of three *Fusarium* species (16 isolates) and identification of proteins following on-target tryptic digestion	Dong et al. [114]
Fusarium	Optimized sample preparation for strongly colored *Fusarium* conidia	Dong et al. [115]
Fusarium	Mixed volume spore preparation for five *Fusarium* species	Kemptner et al. [116]
Fusarium	Differentiation of *Fusarium* species with ferulic acid as the matrix and the dried-droplet technique	Kemptner et al. [117]
Gibberella	Characterisation of *G. zeae* conidia by on-target trypsin digestion	Dong et al. [118]
Metarhizium	Reference spectra for distinguishing 51 isolates of the *M. anisopliae* species complex	Lopes et al. [119]
Monilinia	Identification of *Monilinia* brown rot fungi directly from infect fruits	Freimoser et al. [18]
Monilinia	Identification distinction of four *Monilinia* species cultivated in vitro	Horka et al. [120]
Penicillium	Discrimination of 12 *Penicillium* species based on crude extracts obtained by bead beating	Hettick et al. [121]
Penicillium	Six *Penicillium* species directly detected on citrus and apple fruits	Chen et al. [122]
Puccinia	Identification of different species and pathotypes of *P. triticina* and *P. graminis* by intact spore MALDI-TOF MS	Beinhauer et al. [123]
Rhizopus, Trichoderma, Phanerochaete	Comparison of sample preparation, matrices, and double-stick tape for collection of fungal material	Valentine et al. [124]
Saccharomyces	Fingerprinting of 33 *Saccharomyces* strains commonly used for wine fermentation	Usbeck et al. [88]
Saccharomyces	Comparison of SAPD-PCR (specifically amplified polymorphic DNA) and MALDI-TOF MS for identifying related *Saccharomyces* species	Blattel et al. [72]
Saccharomyces	Identification of yeasts involved in chichi fermentation	Vallejo et al. [98]
Saccharomyces	MALDI-TOF MS characterization of protein biomarkers desorbed from *S. cerevisiae* by formic acid	Amiri-Eliasi et al. [125]
Sepedonium	Characterisation of mycoparasitic *Sepedonium* species and analysis of low-molecular weight peptides	Neuhof et al. [126]
Spoilage yeasts	Optimization of MALDI-TOF MS assay for *Saccharomyces*, *Wickerhamomyces* and *Debaryomyces* isolated from beverages	Usbeck et al. [87]
Trichoderma	Analysis of 129 *Trichoderma* strains by MALDI-TOF MS as well as ITS and tef1 sequencing	De Respinis et al. [127]

Table 2 continued

Samples/organisms	Highlights	Reference
Trichoderma	Characterisation and clustering of *Trichoderma* strains, their peptaibiotics, and hydrophobins	Degenkolb et al. [128]
Trichoderma	Detection of peptaibols in 28 *Trichoderma* species	Neuhof et al. [129]
Trichoderma	Direct identification of hydrophobins in *Trichoderma* isolates by MALDI-TOF MS	Neuhof et al. [130]
Trichoderma, Rhizoctonia	Visualization of metabolites produced during the antagonistic interaction of *T. atroviride* and *R. solani*	Holzlechner et al. [131]
Verticillium	Identification of six pathogenic *Verticillium* isolates with a protocol involving sonication	Tao et al. [132]
Wood decay fungi	Differentiation of closely related indoor wood decay fungi by MALDI-TOF MS (*Serpula lacrymans, S. himantioides, Coniophora puteana, C. marmorata,* and *Antrodia vaillantii, A. sinuosa*)	Schmidt and Kallow [133]
Yeasts and filamentous fungi	MALDI lipid phenotyping as an alternative method for characterizing and identifying fungi	Stübiger et al. [134]
Yeasts	Identification of food-borne yeasts (≥33 species, 96 isolates) by MALDI-TOF MS and conventional methods	Pavlovic et al. [99]

occurring yeasts have been rarely studied by MALDI-TOF MS, except in the context of fermentations, for example, during winemaking and brewing [19, 72, 87, 88]. In general, the large majority of (non-clinical) fungal MALDI-TOF MS studies were directed towards phytopathogens (e.g., *Aspergillus*, *Fusarium*, *Monilinia*, *Penicillium*, *Puccinia*, mildews) or potential biocontrol strains (e.g., *Trichoderma*, *Metarhizium*). MALDI biotyping tools for other important soilborne or phyllosphere plant pathogens have not been established. Examples include *Armillaria* and *Thielaviopsis*, genera of worldwide root pathogens that attack hundreds of plant species [89, 90] or important oomycete pathogens such as *Pythium*, *Plasmodiophora,* or *Phytophthora* [91]. Also in the phyllosphere, pathogens such as *Venturia inaequalis*, the causative agent of apple scab [92], the apple blotch fungus *Diplocarpon mali* (*Marssonina coronaria*) [93, 94], and many others may be identified by MALDI biotyping and thus facilitate monitoring and studying these diseases. It is also worthwhile realizing that MALDI-TOF MS methods for identifying the majority of the fungal diseases recommended by the European and Mediterranean Plant Protection Organisation (EPPO) to be regulated as quarantine pests (List A1: https://www.eppo.int/QUARANTINE/listA1.htm, List A2: https://www.eppo.int/QUARANTINE/listA2.htm) do not exist. The lack of MALDI-TOF MS protocols for such a wide range of economically important plant pathogens is surprising and highlights avenues for future research.

As in clinical diagnostics, the need to identify fungi often goes along with the goal to identify a particular organism directly in the environment, infected plant tissue, or food, which can harbor complex microbial communities and potentially host tissue. Examples of in situ identifications of microorganisms by MALDI-TOF MS include plant invasive *Rhizobia* [95], obligate biotrophic fungal pathogens [96, 97], or *Monilinia* brown rot fungi [18]. In food science, MALDI biotyping has been mainly used for the identification of food-borne yeasts, which were pre-cultivated prior to MALDI-TOF MS analysis [87, 98, 99]. In own, unpublished work, we have developed an approach for identifying food quality and safety-relevant fungi directly from contaminated apple juices by MALDI biotyping without pre-cultivation of the fungus. MALDI-TOF MS spectra of *Byssochlamys fulva* derived from both approaches—without and with pre-cultivation of the fungus in liquid medium—revealed the same protein profiles and allowed accurate species identification (Fig. 2). These studies and results document the potential of MALDI-TOF MS to identify fungal pathogens directly in infected tissue or food.

In particular for the diagnosis of plant pathogens, food contaminants, and spoilage organisms, biomarker-based identification will permit identifying key species in complex mixtures by MALDI-TOF MS. However, so far the MALDI-TOF MS-based identification of fungal mixtures has not yet been reported.

Conclusions

Although numerous fungi have been identified by MALDI-TOF MS already, this technology is far from being a standard tool for the identification of fungi and their functions. Overall, the examples highlighted here demonstrate that the potential of MALDI-TOF MS for clinical diagnostics, and diagnostic and research applications in general, is far from being exhausted. None of the recent methodological improvements (e.g., direct smear after short cultivation times, sample concentration, immunoaffinity enrichment, subspecies identification, functionalization of MALDI target plates, biomarker-based identification in polymicrobial samples) is inherently limited to clinical samples or only bacteria. Translating these improvements and developments to other areas of diagnostics (e.g., of plant pathogens or

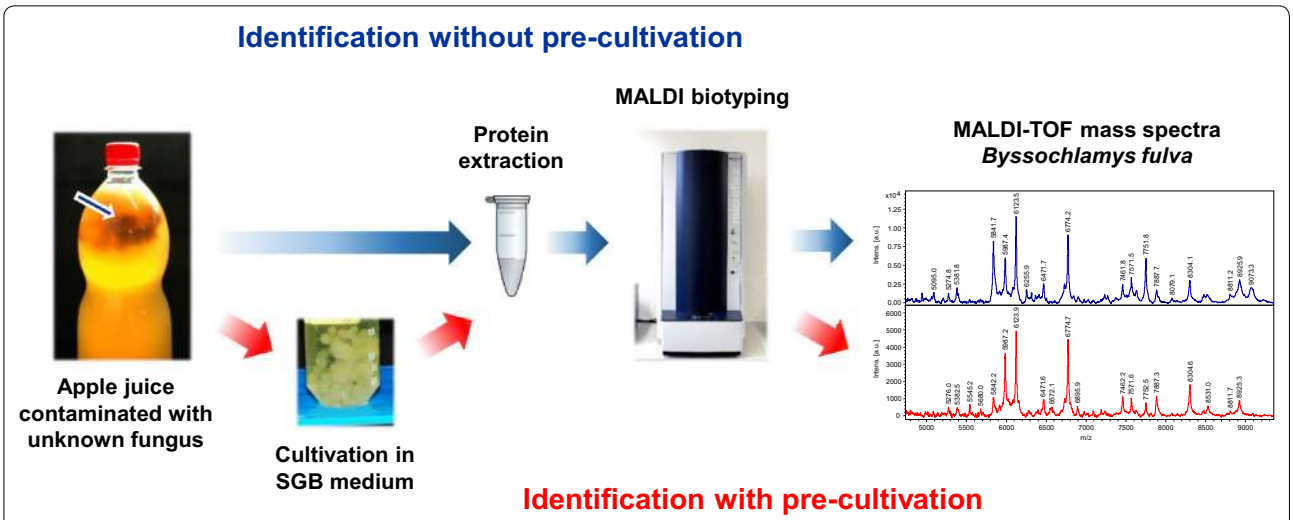

Fig. 2 Direct identification of *Byssochlamys fulva* in contaminated apple juice by MALDI-TOF MS

food spoilages) and to other microorganisms, such as filamentous fungi and yeasts, will thus enable experimental research in bacterial and fungal diagnostics, ecology, physiology, or pathology.

Authors' contributions

Both authors planned and wrote the manuscript together. Both authors read and approved the final manuscript.

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Competing interests

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