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Research review paper

Identification of fungal microorganisms by MALDI-TOF mass spectrometry

Jana Chalupová [a,1], Martin Raus [a,1], Michaela Sedlářová [b], Marek Šebela [a,*]

[a] Department of Protein Biochemistry and Proteomics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic
[b] Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a reliable tool for fast identification and classification of microorganisms. In this regard, it represents a strong challenge to microscopic and molecular biology methods. Nowadays, commercial MALDI systems are accessible for biological research work as well as for diagnostic applications in clinical medicine, biotechnology and industry. They are employed namely in bacterial biotyping but numerous experimental strategies have also been developed for the analysis of fungi, which is the topic of the present review. Members of many fungal genera such as *Aspergillus*, *Fusarium*, *Penicillium* or *Trichoderma* and also various yeasts from clinical samples (e.g. *Candida albicans*) have been successfully identified by MALDI-TOF MS. However, there is no versatile method for fungi currently available even though the use of only a limited number of matrix compounds has been reported. Either intact cell/spore MALDI-TOF MS is chosen or an extraction of surface proteins is performed and then the resulting extract is measured. Biotrophic fungal phytopathogens can be identified via a direct acquisition of MALDI-TOF mass spectra e.g. from infected plant organs contaminated by fungal spores. Mass spectrometric peptide/protein profiles of fungi display peaks in the *m/z* region of 1000–20 000, where a unique set of biomarker ions may appear facilitating a differentiation of samples at the level of genus, species or strain. This is done with the help of a processing software and spectral database of reference strains, which should preferably be constructed under the same standardized experimental conditions.

Contents

Abbreviations: AFST, antifungal susceptibility testing; CA, caffeic acid; CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; FA, ferulic acid; HABA, [2-(4-hydroxyphenylazo)]benzoic acid; IC/IS, intact cell or intact spore; IGS, intergenic spacer; ITS, internally transcribed spacer; LSU, large subunit; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; SA, sinapinic acid; SSU, small subunit; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +420 585634927; fax: +420 585634933.
E-mail address: marek.sebela@upol.cz (M. Šebela).

[1] Both authors contributed equally.

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1. Introduction

Microfungi are described as a group of eukaryotic organisms such as molds, rusts and yeasts plus fungi-like microorganisms (belonging to the taxonomic ranks Protista, Chromista and Myxomycota), which are no longer classified in the kingdom Fungi. A broad spectrum of these microorganisms with miscellaneous ecology, physiology and morphology covers important producers of enzymes, organic acids, pharmaceuticals, alcohols or antibiotics. Many of them also synthesize harmful toxins causing human and animal diseases. As natural recyclers of organic plant material, fungal plant pathogens have a negative impact on agriculture (Bennett, 1998; Cannon and Sutton, 2004; Santos et al., 2010). Studies on fungal organisms are complicated because of an inadequate comprehension of the whole fungal speciation connected with population biology, ecology, evolution and phylogeny. As regards to the detection of human/animal and plant mycoses and identification of the causal agents, standard biological methods become insufficient in many cases. They are often time consuming and tend to fail.

Since the advent of mass spectrometry (MS), numerous identification methods for microorganisms based on profiling of cell surface proteins have been described. They include namely the intact cell or intact spore mass spectrometry (IC/IS MS), but also rely on an initial extraction of proteins by acidified solvents (Welham et al., 2000) or with the help of a bead beating prior to the MS analysis (Hettick et al., 2008a,2008b). MS measurements with bacterial cells evolved hand in hand with the development of MS itself (Meuzelaar and Kistemaker, 1973). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as one of the most reliable tools for fast and easy identification, differentiation and classification of microorganisms. IC/IS MALDI-TOF MS operates with unique mass spectrometric profiles (fingerprints) acquired by the desorption of specific peptide/protein biomarkers from the cell/spore surface of a particular pathogen (Fenselau and Demirev, 2001). Based on results and experience gained in bacterial identification, IC/IS MALDI-TOF MS has also been introduced for a differentiation of microscopic fungi. Contrary to bacteria, fungal cells are larger in size and their cell wall is more rigid. It is usually based on glucans and chitin, rarely on glucans and cellulose (in the distinct phylogenetic lineage of fungi-like Oomycota). Mannoproteins are also major cell wall components, especially in yeasts (Carlile et al., 2001). Taking this into consideration, modified approaches had to be developed as regards to the procedure of sample preparation, selection of a proper matrix compound, sample deposition techniques etc.

A review article describing characterization of filamentous fungi by MALDI-TOF MS appeared in 2010 (Santos et al., 2010) but the text naturally does not cover yeast analysis. More recently, Havlicek et al. (2013) summarized current trends in MS-based microbial diagnostics with a special focus on the instrumentation (fungi were included but only marginally). In 2013, two comprehensive reviews appeared (Clark et al., 2013; Posteraro et al., 2013), which emphasized the use of MALDI-TOF MS for the analysis of fungi in clinical microbiology laboratories. However, other applications than those related to medicine were not included. The present review deals with MALDI-based identification of fungi in various branches of science and diagnostics. Standard biological methods of determining fungal species are briefly discussed together with their limitations, which provide a space to be filled up with mass spectrometric strategies. There are first attempts of fungal identification mentioned together with a further progress illustrated on specific examples. A special attention has been paid to potential applications in biotechnology, medicine and phytopathology.

2. Biological methods of fungal strains identification

The main goal in all fields of diagnostics is to identify the origin of a human, animal or plant disease in such a way, which is fast, reliable and effective. Basic methods for the detection of fungal pathogens are based on host specificity, disease symptoms and microscopic characters. Although the host specificity and disease symptoms can preliminary help to estimate a causal agent, signs usually change during the disease progression and thus they can give unclear information (Carlile et al., 2001; Doohan, 2005). Because of considerable variability in fungal morphology, microscopy still remains an indispensable tool for identifying individual species. Microscopic techniques, which are commonly used for this purpose, include observation and evaluation of different infectious structures and reproductive organs (sexual and asexual spores) as regards to the color, shape and surface. Together with light microscopy, scanning electron microscopy can be used to reduce the rate of misdiagnose. Evaluation of different shapes of spores (e.g. spherical, oval, ovate, with or without papillae) or various branching of spore-carrying structures (monopodial, sympodial, dichotomous) requires experts and specialists with a practice in identification of fungal agents (Sedlář et al., 2009). In certain cases, fungi are isolated and grown in culture media, which brings the possibility of evaluating physiological characteristics such as colony color or growth rate (Santos et al., 2010). For example, yeasts show a limited morphology, which complicates their identification. On the other hand, yeast cells produce many metabolites, which can be used for biochemical tests and metabolomic profiling. Immunological aspects are taken into account in many cases when no morphological characters are visible. Pathogens can be identified and quantified using species-specific antibodies coupled with a fluorescent dye or enzyme. Enzyme-linked immunosorbent assay was applied for the detection of mycelium of a rice spoilage agent *Humicola lanuginosa*. Similarly, *Botrytis cinerea* was found in grape juice (Carlile et al., 2001). However, immunological methods work reliably only at the genus level and sometimes it may be difficult and expensive to generate the required antibodies.

Molecular biology methods are highly specific. They benefit from a variability in DNA sequences, which allows determining and differentiating closely related species or strains and detect pathogens at early stages of host infection with no visible signs. Specific genetic features such as host resistance can also be recognized (McCartney et al., 2003). In contrast to the morphology-based examinations, these methods are independent of operator's experience. The existence of conserved genes on one side and different DNA sequence regions, which are unique for individuals, on the other side makes this approach suitable for analyzing both common and different phylogenetic features. Data derived from ribosomal DNA (rDNA) sequences are often used for classification and identification of fungi. From this point of view, 18S ("small subunit", SSU), 5.8S and 28S ("large subunit", LSU) gene sequences coding for ribosomal RNA are interesting (Martin and Rygiewicz, 2005). Their significance for fungal identification can be summarized as follows: 1) the 18S gene has varied enough during evolution and can help to determine the taxonomic kingdom and reveal the phylogenetic aspects and relationships of fungal classes; 2) the 28S gene is more variable and it has been used in classification at levels from genus to phylum; 3) the 5.8S gene does not contain much information but is still useful, e.g. for the identification of ascomycetes, basidiomycetes and zygomycetes. The regions between the 18S, 5.8S and 28S genes on rDNA are not highly conserved and constitute internally transcribed spacers 1 and 2 (ITS1 and 2), respectively, while regions beyond the rDNA genes are known as externally transcribed spacer and intergenic spacer (IGS), the latter separating rDNA copies. By amplification of the ITS regions, different fungal species can be recognized. IGS has been used at the subspecific level to recognize races and populations (Carlile et al., 2001; Mitchell and Zuccaro, 2006). Interestingly, it has been shown that the ITS2 region is highly conserved between *Aspergillus* and *Penicillium* and thus namely the ITS1 region is promising for the development of specific probes to distinguish between the genera (Gaskell et al., 1997). Mitochondrial DNA genes can also be utilized for fungal identification. The *cox2* locus coding for subunit 2 of cytochrome *c* oxidase was employed in a phylogenetic analysis of Peronosporomycetes (Hudspeth et al., 2000). A gene coding for cytochrome *b* allowed discrimination of *Aspergillus*

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species (Wang et al., 1998). Other genes are also becoming used as targets for diagnostic purposes and pathogen characterization: e.g. β-tubulin, calmodulin, actin, hydrophobins and ascomycete mating-type genes (Balajee et al., 2009; Geiser et al., 1998; Hong et al., 2005; McCartney et al., 2003).

Although the identification procedures involving molecular tools are shortened by avoiding a culture cultivation, the whole process of molecular analysis of target genes remains time consuming and costly. Thus, looking for a more rapid and reliable way for determining causal agents is still the main challenge in the diagnostics of fungi.

3. Mass spectrometry of peptides and proteins

Detailed analyses of all proteins from an organism (=proteome) fall into the scope of proteomics. The discipline is built on high-resolution separation methods for proteins plus MS with electrospray ionization or MALDI (Aebersold, 2003). There are three common strategies for protein identification: 1) "classical" bottom-up strategy; it relies on separation of protein mixtures, proteolytic digestion of individual separated proteins and mass spectrometric analysis of the resulting peptides allowing the determination of a peptide mass fingerprint (PMF) and/or of sequence tags; 2) shotgun proteomics; this approach involves proteolytic digestion of unseparated complex protein mixtures, which is followed by capillary chromatographic or electrophoretic separation of peptides from the digests and tandem MS (MS/MS) allowing the determination of sequence tags, and 3) top-down proteomics; here isolated proteins are obtained by complex purification procedures as intact molecules and they are subjected to fragmentation in the mass spectrometer (Domon and Aebersold, 2006). In both latter cases, sequencing by tandem or multistage MS allows identifying the protein unambiguously. Mass spectrometry of peptides for protein identification is generally performed using two approaches: 1) PMF and peptide sequencing with database search and 2) de novo peptide sequencing plus sequence-similarity search for proteins which sequences are not yet present in protein databases (Shevchenko et al., 1997).

Besides, MS of intact proteins has been frequently used in experiments performed for diagnostic purposes and protein characterization. A comparison of protein species and levels among individual organisms with and without a disease can be used for diagnosis and prognosis based on biomarker discovery (Minerva et al., 2012). The related technique MALDI imaging has been introduced for the laser ablation of proteins or other biomolecules (e.g. lipids) of interest directly from cryo-sliced tissue samples with the subsequent MS or even MS/MS analysis (Cazares et al., 2011; McDonnell and Heeren, 2007). MALDI-TOF MS was shown to be a reliable approach for solving some clinical as well as food microbiology problems such as rapid and accurate identification and strain differentiation of microorganisms, overcoming the existing limitations of classical cultivation methods (Šedo et al., 2011). The most important advantages of the MALDI mass spectrometric approach are: 1) only small amount of biological material is required (i.e. less than 100 ng); 2) both measurement and data interpretation processes are very fast and relatively easy. MALDI-TOF MS of surface peptides/proteins released from microorganisms by an acidic sample treatment provides singly charged ions covering a broad range of m/z values of 1000–20 000 (Šedo et al., 2011). This means there is enough peptide/protein signals to generate a high-quality peptide/protein fingerprint mass spectrum. Database searches with a comparison of typical and unknown mass spectra then allow identification and taxonomical classification of microorganisms (Šedo et al., 2011; Seng et al., 2009). MALDI MS offers relatively high tolerance against sample impurities (salts and detergents), as well as fast and accurate molecular mass determination and the possibility of automation, which makes it a powerful alternative to classical biological methods.

4. The dawn of MALDI-TOF MS of intact fungal microorganisms

The development of intact cell/intact spore matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (IC/IS MALDI-TOF MS) of fungal microorganisms began around the year 2000 by first experiments with *Penicillium* spp., *Scytalidium dimidiatum*, *Trichophyton rubrum* and *Aspergillus niger* (Li et al., 2000; Welham et al., 2000). The experimental approaches used at that time were adopted from initial protocols for MALDI-TOF MS measurements with whole bacterial cells (Holland et al., 1996; Jarman et al., 1999; Welham et al., 1998). Fungal cells are larger in size than bacterial cells and possess a rigid cell wall, which is similar by its arrangement (but not by composition) to that of plant cells. The cell walls of fungi are mainly composed of different polysaccharides according to taxonomic group (either chitin, glucans, mannoproteins, chitosan, chitin, polyglucuronic acid or cellulose), together with smaller quantities of proteins, lipids and polyphosphates. The vegetative part of a fungus, the mycelium, consists of branching hyphae. Spores (Fig. 1) are produced on the tips of hyphae (Valentine et al., 2002).

During measurements on a MALDI instrument, mass spectra are obtained by the laser ablation of cell wall structures of spores because of their erosion due to the acidity of the matrix solution (Li et al., 2000). *Penicillium* spp. spores were washed with 0.1% trifluoroacetic acid (TFA) and, after collecting by a centrifugation, resuspended in methanol. Prior application to the target plate, the suspension was mixed in a volume ratio of 1:1 with matrix in the form of a saturated solution (in a suitable solvent). Welham et al. (2000) examined in their pioneering work 9 different matrix compounds including for example α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), [2-(4-hydroxyphenylazo)]benzoic acid (HABA), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), 2-amino-4-methyl-5-nitropyridine and *trans*-3-indoleacrylic acid. From those, the latter two were shown to provide peaks of similar intensities up to a molecular mass limit of 8 kDa, which was extended up to 12 kDa using DHB, HABA and SA. At the experimental conditions used, the mass spectra were found relatively simple. In a similar study from that time (Li et al., 2000), intact spores of several strains of four *Aspergillus* species (*Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus parasiticus* and *Aspergillus sojae*) were analyzed by MALDI-TOF MS. The aim was to investigate whether the mass spectra of aflatoxigenic and non-aflatoxigenic spores are similar to each other as well as to evaluate the relationships between individual species. The spores were obtained by washing out from cultivation agar plates with 0.1% Tween 20. After centrifugation, aliquots of spore pellets were mixed with matrix solutions (30 mg ml^{-1} in 0.1% TFA/acetonitrile, 1:2, v/v) on the sample plate. The best results were obtained with CHCA, SA and 3,4-dihydroxycinnamic acid (caffeic acid, CA) as matrices (chemical formulas are given in Fig. 2). The mass spectra were simple again: only a few characteristic peaks were observed in the mass range of m/z 3800–8000. A peak with m/z 7292 appeared typical for all studied *Aspergillus* strains. It has been shown that aflatoxigenic and non-aflatoxigenic strains have different peak profiles. Furthermore, the analyzed spores of non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* displayed mass spectra similar to closely related non-aflatoxigenic species *A. oryzae* and *A. sojae*, respectively. Both studies from the year 2000 indicated a potential of MALDI-TOF MS to be a rapid screening tool for monitoring spore-like contaminations.

There are three major groups of fungi: yeasts, molds and mushrooms (Welham et al., 2000). For the first time, the yeasts *Candida albicans* and *Saccharomyces cerevisiae* were studied by IC/IS MALDI-TOF together with the ascomycete *Epidermophyton floccosum* (Amiri-Eliasi and Fenselau, 2001). Several lysis methods were examined for on-target disruption of *S. cerevisiae* cells from which 25% formic acid was found the best as regards to the number of peaks and signal-to-noise-ratio observed in the resulting mass spectra. SA was the matrix of choice in this case. Many of the peaks in the MALDI spectrum of *S. cerevisiae* correlated with molecular masses predicted from its genome, including the

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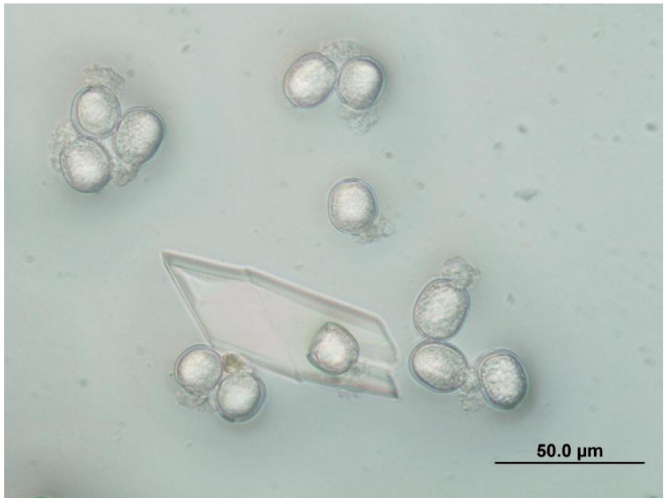

Fig. 1. Optical microscopy of *Bremia lactucae* spores. Panel A) a suspension containing 1×10^9 spores in 1 ml of water was mixed in a volume ratio of 1:1 with matrix solution containing FA and SA (5:15 mg ml^{-1} in acetonitrile/0.5% v/v TFA, 7:3, v/v) on a cover slide. This was done to simulate the conditions on a MALDI target, which occur when the spores are deposited by the dried droplet technique. A matrix crystal is also clearly visible. Panel B) a conidiophore of *B. lactucae*, which is a specialized tree-like hypha bearing fresh conidia. Asexual fructification of lettuce downy mildew occurs 1–2 weeks after conidia germination. In both panels, magnification is shown by a bar in the right bottom corner.

eukaryotic marker ubiquitin, ribosomal and mitochondrial proteins (Amiri-Eliasi and Fenselau, 2001).

5. MALDI-TOF MS analysis of *Aspergillus* species

Concerning Aspergilli molds, they belong to the most common and widely distributed fungi (Gugnani, 2003). Certain representatives of this group are important in brewing and food industry as well as in the biotechnological production of industrial enzymes (*A. niger*, *A. oryzae*). Other species are known to produce toxic aflatoxins in infected crops. Many species have been found as causative agents of opportunistic infections in humans — *Aspergillus fumigatus*, *A. flavus*, *A. niger* (Gugnani, 2003). Thus it is not surprising that various species of the genus were subjected to IC/IS-MALDI-TOF MS in order to generate fingerprint mass spectra for a rapid differentiation of isolates.

A double-stick tape was used by Valentine et al. (2002) to collect hyphae and mycelia of *A. niger*, *Rhizopus oryzae*, *Trichoderma reesei* and *Phanerochaete chrysosporium* and transfer them to the target plate. In parallel, ammonium chloride was used for a standard wash of the spores

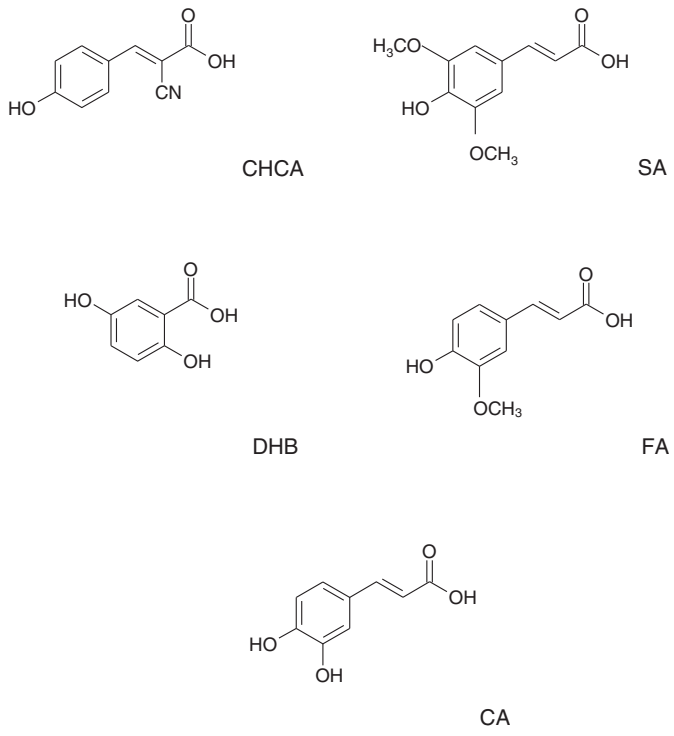

Fig. 2. Chemical formulas of matrix compounds that are commonly used in MALDI-TOF MS analyses of fungi.

and/or hyphae. Ferulic acid (FA) was found to be the matrix of choice in this work but SA was also efficient (chemical formulas are given in Fig. 2). The analysis of *A. niger* was challenging as the authors met difficulties in the detection of biomarkers for this species. Kalow et al. (2006) transferred freeze-dried mycelia of Aspergilli grown in liquid medium onto a MALDI target in the form of a thin layer, where they were mixed with a DHB matrix for crystallization. A newly recognized species *Aspergillus ibericus* was clearly distinguished from the others based on mass spectra clustering and shown to be related to *Aspergillus carbonarius*.

Hettick et al. (2008b) described MALDI-TOF measurements with 12 species and 5 strains of *A. flavus*. After cultivation, conidia and hyphae were collected and pretreated by beating with zirconium beads to extract surface molecules. When CHCA was used as matrix, highly reproducible fingerprints were acquired with abundant peaks in the *m/z* region of 1 to 20 000. As an exception, two of the analyzed species, *Aspergillus chevalieri* and *A. niger*, provided only a few low-abundance peaks and they were not fully resolved in a cluster analysis of MS data. A more recent MS-based work by Sulc et al. (2009) investigated numerous strains of *A. fumigatus*, *A. flavus*, *A. niger* and some other Aspergilli to find biomarkers for discrimination analysis. The experimental protocol was optimized with several variable parameters such as amount of spores, extraction solvent, conditions of a sonication treatment and matrix selection for the subsequent preparation of MALDI probes (CHCA was finally chosen). Despite well pronounced peak characteristic for individual species, no general and specific marker was registered. The difficulties in recording mass spectra of intact *A. niger* have been rationalized in terms of the presence of low-molecular-weight black pigments, which possibly interfere with the ionization of biomolecules (Hettick et al., 2008b; Sulc et al. 2009).

In a recent study by Cassagne et al. (2011) aiming at the development of a standardized procedure for identification of molds in clinical isolates, a database was built using MALDI-TOF MS and reference strains belonging not only to the genus *Aspergillus* but also to the genera *Penicillium*, *Rhizopus*, *Trichoderma* and others were represented. Sample preparation was optimized by involving extraction of the fungi with

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formic acid and acetonitrile. The extract was then measured using CHCA as a matrix. With the help of the reference database, almost 200 isolates were analyzed from which 87% were correctly identified at the species level. It has been shown that invasive aspergillosis may be caused by previously unrecognized Aspergilli. To consider this fact in routine species identification by MALDI-TOF MS, a database including reference spectra of 23 unusual species was engineered (Alanio et al., 2011). Both young and mature colonies were analyzed to cover possible differences. The database was validated with numerous clinical and environmental *Aspergillus* isolates and 98.6% of them were correctly identified.

Lau et al. (2013) developed a comprehensive database for the identification of molds by MALDI-TOF MS (294 isolates were used comprising 58 reference strains from several well-maintained collections and 236 clinical strains). When published, the NIH mold database included 76 genera and a total of 152 species. Aspergilli represented a majority of database entries. For protein extraction, a mold sample was picked up from the culture plate and placed into absolute ethanol mixed with zirconium-silica beads. The sample was emulsified by repeated squeezing with a sterile wooden stick against the tube wall, vortexed and centrifuged. The remaining pellet was resuspended in 70% formic acid and processed by a repeated centrifugation with the addition of acetonitrile in the middle step. Then the supernatant was either directly measured or stored frozen. When challenged against 421 isolates, the database allowed obtaining an accurate species identification rate of around 90%.

6. MALDI-TOF MS analysis of *Penicillium*, *Trichoderma* and wood decaying fungi

Penicillium is a large group of ubiquitous fungi with representatives that are important for various biotechnological and health applications (Paterson et al., 2004). Among others, *Penicillium expansum* is well known for the production of patulin, a mycotoxin which spoils for example apple juices (Andersen et al., 2004). *Penicillium citrinum*, *Penicillium italicum* and *Penicillium digitatum* (contaminating frequently citrus fruits) together with *P. expansum* and *Penicillium pinophilum* (contaminating apples) were subjected to IC/IS MALDI-TOF MS analysis in order to differentiate them and to compare various strains within each individual species (Chen and Chen, 2005). Fungal spores were scratched from agar media plates by a pipette tip and washed off by a matrix solution based on DHB (30 mg ml^{-1}). The mixture containing spores and matrix was then deposited on a MALDI target and left to dry. In the observed mass spectrometric profiles, characteristic (= biomarker) ions for each of the six species were found to appear in the *m/z* region of 2500–7500. The method was successfully evaluated when measuring with rotten fruits obtained in a local supermarket, where *P. citrinum* and *P. digitatum* were identified (Chen and Chen, 2005). In another study, twelve *Penicillium* species were analyzed by MALDI-TOF MS. After collecting from culture media, conidia and hyphae were subjected to three 1-min-long bead-beating cycles (zirconium beads) in acetonitrile/4% TFA, 1:1, v/v. The resulting extract was premixed with a CHCA matrix solution and spotted on the target. Following disruption, fungal isolates provided spectra exhibiting significantly higher number of peaks than untreated samples. Characteristic peaks appeared in the *m/z* region of 5000–20 000, where a signal with *m/z* 13 900 was found in all spectra as a potential biomarker. Both discriminant and cluster analyses confirmed high reproducibility of the acquired data (Hettick et al., 2008a).

The dual modality of fungal propagation, i.e., sexual (teleomorph) and asexual (anamorph) has been reflected in a dual nomenclature. The anamorph and teleomorph generally develop at different times and on different substrates (Guarro et al., 1999). For example *Hypocrea* and *Trichoderma* are the names for the teleo- and anamorph forms, respectively, of the same taxon from the phylum Ascomycota. IC/IS MALDI-TOF MS analysis of *Hypocrea* and *Trichoderma* strains comprising 29 different species was performed to investigate their diversity as regards to metabolite production and low-mass proteomics. Around 10^6 cells were used per spot on the target, DHB served as a matrix. Characteristic peaks appeared in the *m/z* range of 5000–10 000. They were assigned to hydrophobins, small proteins from the outer surfaces of cell walls of hyphae and conidia, whereas ribosomal proteins (typical for a MALDI profiling of bacteria) were not identified (Neuhof et al., 2007b). Hydrophobin patterns were found diverse due to post-translational N- and C-terminal processing. It has been shown that sporulating and nonsporulating mycelia of several species differ in their hydrophobin composition. In a similar study, 129 morphologically and genetically well characterized strains of *Hypocrea* and *Trichoderma* belonging to 25 species were analyzed by IC/IS MALDI-TOF MS using growing mycelia and DHB as a matrix (De Respinis et al., 2010). In a taxonomic tree analysis, the results accurately reflected previous phylogenetic classifications derived from DNA sequence analysis of ITS regions and specific genes such as *tef1* (translation elongation factor 1 gene). Hydrophobins were also detected by IC/IS MALDI-TOF MS of *Trichoderma* species from the *Brevicompactum* clade demonstrating the value of the method in taxonomic studies (Degenkolb et al., 2008). In their enormous work, the authors have analyzed numerous *Trichoderma*/*Hypocrea* isolates using a battery of experimental methods (microscopy and morphological characterization; DNA-based phylogenetic analyses; analytical profiling of secondary metabolites — except for hydrophobins, also peptaibiotics and mycotoxins were studied) to reexamine phylogenetic lineages.

IC/IS MALDI-TOF MS of *Hypocrea*/*Trichoderma* has also been performed with a special focus on the detection of peptaibol profiles for studying phylogenetic relationships (Neuhof et al., 2007c). Peptaibols are antibiotic peptides, which contain a non-proteinogenic acid alpha-aminoisobutyrate and a hydroxylation of the C-terminus, which are produced by certain ascomycetes. Their name is derived from the chemical composition: linear **pept**ides containing α-aminoisobutyric acid (**Aib**) and ending with an alcoh**ol** (Chugh and Wallace, 2001). Spectral data were acquired with a DHB matrix in a peptide mass range up to *m/z* 2000. Peptaibols were detected in all strains. In some cases, up to five families of the compounds were found differing in size. But there was no strict correlation between the phylogeny based on peptaibol masses and phylogenies derived from rDNA spacer regions and RNA polymerase (subunit B) gene sequences (Neuhof et al., 2007c). Peptaibol production was also analyzed in *Sepedonium* strains infecting fruiting bodies of Boletales. Neuhof et al. (2007a) used IC/IS MALDI-TOF MS for monitoring of chrysospermin and chrysospermin-related peptaibols in the mycelia of *Sepedonium* strains. Peptaibol patterns alone were not sufficient for unambiguous taxonomic identification. Yet, the microorganisms could be readily assigned from fingerprint spectra by recognizing biomarker ions in the *m/z* range of 4000–8000.

Species of the genera *Serpula*, *Coniophora* and *Antrodia* represent common wood decay fungi in Europe. Wet mycelia of the basidiomycetes (three pairs of similar but different species that were undistinguishable from each other by traditional methods) were subjected to IC/IS MALDI-TOF MS analysis (e.g. *Serpula lacrymans* and *Serpula himantioides*). A rapid and clear differentiation was achieved by unique peak patterns (Schmidt and Kalow, 2005).

7. MALDI-TOF MS analysis of *Fusarium* species

Fusarium is a genus of numerous filamentous fungi, which are widely distributed in soil and in association with plants. Most species are harmless, but there are others, which produce mycotoxins. The mycotoxigenic *Fusarium* species become problematic when for example ripening cereals (wheat, maize, rice etc.) are infected by them and the mycotoxins may consequently enter the food chains of humans and animals (Bennett and Klich, 2003). Toxins of *Fusarium moniliforme* are known to be cancerogenic (Nelson et al., 1992). In rare case, *Fusarium* may cause human infections with the most severe manifestations in immunocompromised patients.

Fusarium species differentiation by IC/IS MALDI-TOF MS has been attempted for many times. Fusariosis in clinical diagnostics is confirmed

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for example by conventional methods such as positive cultures from blood, sputum and skin. Polymerase-chain reaction and nucleotide sequence analysis represent typical molecular biology methods. A high-specificity is achieved by analysis of ribosomal DNA (Abd-Elsalam et al., 2003; Ninet et al., 2005). In a comparative study (Seyfarth et al. 2008), a patient suffering from acute lymphoblastic leukemia had a mortal fungal infection. To determine the infectious species, the ITS region of rDNA from cultured cells was sequenced and the cells were also directly analyzed by MALDI-TOF MS using DHB as a matrix. All results were consistent with the presence of *Fusarium proliferatum* despite false-positive serologic indications for an *Aspergillus*. For MS-based identification, multiple mass spectra were used to compute a consensus spectrum containing mass signals that were present in more than half of the individual spectra, which was then used for software comparison with data from reference strains.

Aspects of *Fusarium* species identification by MALDI-TOF MS were explored in several recent studies. Conventional identifications of fungi based on morphological traits are hampered by phenotypic polymorphism. Marinach-Patrice et al. (2009) performed an extensive study with 62 strains belonging to nine species (isolated mostly from human infection sites) and compared the MS results with sequencing of a characteristic gene coding of the translation elongation factor 1-α (*tef1*). In this case, samples for MALDI-TOF MS were obtained by an extraction of mycelia with 80% TFA. Prior to measurements, CHCA matrix was spotted on the target over the dried sample. Spectral data were processed by commercial software when a database was constructed comprising profiles of five species that are most frequently isolated in human infections (including e.g. *Fusarium solani*, *Fusarium oxysporum* and *F. proliferatum*) and the procedure of strain identification was shown reliable. Another investigation involving fungal biotyping used strains of *Aspergillus*, *Fusarium* and Mucorales (De Carolis et al., 2012). First a reference database was built with spectra of 55 species and then 103 isolates from a microbiological laboratory were measured by IC/IS MALDI-TOF MS and their profiles searched over the database. As comparative methods, ITS region nucleotide sequencing was carried out together with sequencing of specific genes. Mycelium/spore samples (water suspensions) for MS were dried on the target plate, wetted with ethanol and, after drying on air, overlaid with a CHCA matrix. Length of incubation of the fungal cultures was shown to influence significantly spectral features. For that reason, both young and mature colonies were included into the database. MS experiments allowed achieving an identification rate of 96.8% with respect to the species level.

Researchers from the Vienna University of Technology, Austria, have been concerned in studying fundamental tasks of IC/IS MALDI-TOF MS of *Fusarium* species. Kemptner et al. (2009b) optimized sample preparation techniques for plant pathogenic Fusaria (e.g. *Fusarium graminearum*, *Fusarium poae*, *Fusarium cerealis*). The optimization involved testing of a panel of matrix compounds (CHCA; DHB; FA; SA; 2,4,6-trihydroxyacetophenone; 5-methoxysalicylic acid), which were used individually, in certain mixtures, or after adding additives (butylamine, aniline). The matrices were dissolved at various concentrations (10, 20 or 30 mg ml^{-1}). Solvent mixtures used were also variable and consisted of 0.1% TFA or water plus and organic component (acetonitrile, isopropanol, acetone or methanol). Major peaks in acquired mass spectrometric profiles were shown to be peptides or proteins by post-source decay on the MALDI instrument and the subsequent reading amino acid sequence tags. FA was found to be the most suitable matrix for a direct measurement with *Fusarium* spores as regards to information provided (number of ion species, signal intensities). Another crucial point was the content of organics in the matrix solvent: a final ratio of acetonitrile/0.1% TFA of 7:3 was found optimal. The dried droplet sample preparation technique was superior over the others bringing highly reproducible spectra. In a continuation study (Kemptner et al., 2009a), a mixed volume sample preparation technique was introduced as the most effective, where a spore suspension sample is first premixed with a FA matrix solution and then aliquots are deposited on the target plate. This procedure ensures even better reproducibility of results. IC/IS MALDI-TOF MS has not only a potential to differentiate *Fusarium* species from each other but may also serve for a classification according to the location of collecting isolates (Marchetti-Deschmann et al., 2012). Except for the importance of sample preparation protocol, the necessity of automatic data acquisition (i.e. a consistent way of peak picking) for reproducibility and precision of detected *m/z* values was recognized.

Because of the complexity of microorganisms, there is no standardized protocol available with a broad applicability. Colored Fusaria represent a challenge for IC/IS MALDI-TOF MS as the presence of pigments of slightly orange to deep brown color hinders on-target crystallization with matrix and the subsequent measurements of mass spectrometric peptide/protein profiles. Dong et al. (2009) investigated the use of washing steps prior to the sample preparation of conidia spores of *F. graminearum* and *F. poae*. Several solvent systems composed of water, acetonitrile, methanol, ethanol, isopropanol, organic acids and the detergent *n*-octylglucoside were used for that purpose (each alone or in mixtures). The presence of an acid such as formic acid or TFA in the washing solution brought about lighter co-crystals with FA matrix and then high-quality spectra could be generated. The best results were achieved after the washing with acetonitrile/0.5% formic acid (7:3, v/v) when a two-layer volume technique for sample preparation was employed, which is similar to the above mentioned mixed volume technique. The difference resides in adding a matrix solution drop onto already dried sample/matrix spot.

8. MALDI-TOF MS analysis of fungal phytopathogens

Plants are exposed to interactions with many organisms and environmental stress conditions. Phytopathogens disturb plant metabolism resulting in a disease, manifested by characteristic symptoms. The major part of economically important plant diseases are represented by mycoses, i.e. diseases caused by fungi and fungi-like organisms (Bennett and Klich, 2003). Chalupová et al. (2012) performed development and evaluation of a methodology for IC/IS MALDI TOF MS of fungal and fungal-like pathogens representing obligate biotrophic parasites of crop plants. Typical examples of the phytopathogens belong to two rather distant taxonomic groups: downy mildews are classified in the kingdom Chromista, class Oomycota, order Peronosporales, whereas powdery mildews appear in the kingdom Fungi, class Leotiomycetes, order Erysiphales. Experimental approaches for the MS analyses were elaborated with *Bremia lactucae*, cause of lettuce downy mildew, and *Oidium neolycopersici*, cause of tomato powdery mildew. This was based on determining a suitable concentration of spores in the analyzed suspension (2–5 × 10^9 per ml), selection of a proper MALDI matrix compound (CA, CHCA, DHB, FA, norharman and SA were tested), looking for the optimal solvent composition (acid concentration, additives etc.) and comparison of different sample preparation methods (dried droplet, double layer, mixed volume) as regards to the sample amount applied, using different MALDI targets (stainless steel, polymer-based) and various conditions for sample exposure to the acidic matrix system. The dried droplet method involving solvent evaporation at room temperature was found the most suitable for the deposition of spores and matrix on the target and the subsequent crystallization. The best peptide/protein profiles were obtained by combining FA and SA as a mixed matrix (FA:SA, 5:15 mg ml^{-1}, in acetonitrile/2.5% TFA, 7:3, v/v). A pretreatment of the cell wall with hydrolytic enzymes such as cellulases and chitinases prior to MS measurements was successfully introduced.

Following the described experimental procedure, mass spectrometric peptide/protein profiles of numerous reference phytopathogenic microorganisms were acquired (Chalupová J. et al., Palacký University, unpublished data) and, in consequence, a database has been constructed for the identification of new field isolates (Fig. 3). The species characterized in this way belonged to the genera *Bremia*, *Peronospora*,

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Pseudoperonospora and *Plasmopara* (all downy mildews), *Oidium*, *Erysiphe* and *Blumeria* (all powdery mildews) and *Botrytis* including the gray mold (*B. cinerea*). To perform database searches with new spectral data, home-made software Biospean (Raus M. et al., Palacký University, unpublished results) was developed. The software is a web program application and utilizes Linux as an operating system on the physical server, Apache as web server software, MySQL as a database engine and PHP as a scripting language. Biospean allows detection of peaks in a mass spectrum and is able to evaluate its similarity with other spectra when searching over a spectral database (Fig. 4). The principle of peak detection resides in a local scanning of intensity values around a particular *m/z* position taking the level of noise into consideration by adjusting signal-to-noise (S/N) ratio. The score value (expressed as a percentage) for a comparison of an inspected spectrum with another spectrum is derived from the number of identical peak positions found (assigned with an adjustable mass tolerance with the corresponding penalization) divided by the total number of detected peaks in the inspected spectrum. The two spectra may optionally be compared also in the opposite way and the score values obtained are then averaged.

A novel procedure was also introduced for direct spectra acquisition from infected plant leaves (Chalupová et al., 2012). For this purpose, a special MALDI target was made using a milling machine, which had rectangular grooves of different depths on its surface. Infected leaves were cut in a size that fitted the target grooves, then fixed there by a double-stick tape and sprayed with the FA/SA matrix solution. External calibration was made by depositing peptide/protein standards at several places of the leaf surface using the dried droplet method. After desiccation, mass spectrometric peptide/protein profiles were obtained for *O. neolycopersici* (from tomato leaves) or *B. lactucae* (from lettuce leaves), which correlated well with spectra originating from harvested spores. In an alternative way (Chalupová J. et al., Palacký University, unpublished results), spores of *O. neolycopersici* were imprinted from infected tomato leaves onto a conductive double-stick tape and transferred to a MALDI target. After matrix deposition by a pipette, reliable mass spectra were acquired.

Pathogenic fungi of the genus *Verticillium*, namely *Verticillium dahliae* and *Verticillium alboatrum*, cause wilt diseases in economically important plants such as tomato and cotton. In 2009, a method was developed for rapid identification of six *Verticillium* species using MALDI-TOF MS (Tao et al., 2009). The described workflow involved fungi growth and sampling, protein extraction, measurements of mass spectra and data analysis. Harvested mycelia were washed with distilled water, fixed in 75% ethanol and extracted with 70% formic acid/acetonitrile (1:1, v/v). The extracted proteins were deposited on a polished-steel MALDI target and, on drying a CHCA matrix solution was added.

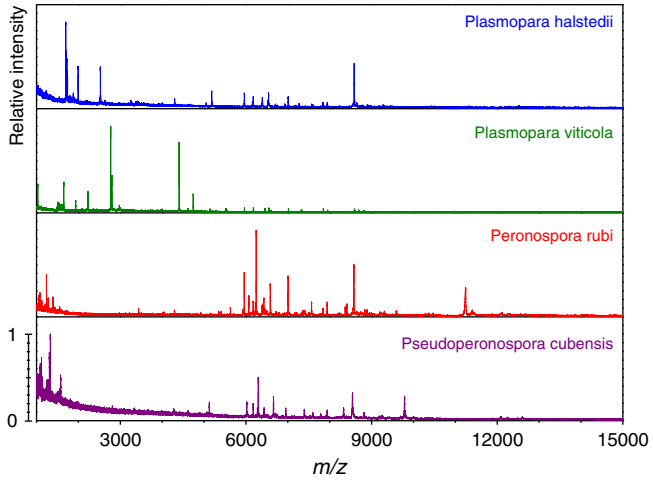

Fig. 3. Positive ion MALDI linear TOF mass spectra of intact spores of *Plasmopara halstedii*, *Plasmopara viticola*, *Peronospora rubi* and *Pseudoperonospora cubensis*. The microorganisms were used in a concentration of 2–5 × 10^9 spores per ml. All experiments were performed on a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The matrix solution used was composed of a mixture of FA and SA (5:15 mg ml^{-1} in acetonitrile/2.5% v/v TFA, 7:3, v/v).

A group of filamentous fungi from the genus *Monilinia*, which represent important fruit pathogens responsible for major losses in fruit production, have been analyzed by capillary electrophoresis, isoelectric focusing, polyacrylamide gel electrophoresis and MALDI-TOF MS to evaluate the applicability of these methods for identification of isolates collected from infected fruits (Horká et al., 2012). The high resolution and discriminative power of MALDI-TOF MS facilitated differentiation of closely related species or even different strains of the same fungal species (SA was used as a matrix). However, when applied for infected fruits, the MALDI analysis was negatively influenced by sample complexity (various strains of different fungal species seemed to be present).

MALDI-TOF MS has also been shown a powerful tool for the identification of ascomycete phytopathogenic fungi from the genus *Alternaria* (Brun et al., 2013). *Alternaria dauci*, *Alternaria porri*, *Alternaria solani* and *Alternaria tomatophila* cause blight diseases of carrots, onions, potatoes and tomatoes, respectively. Their traditional diagnostics by morphological characterization may become hampered by sterility in cultures or formation of species-complexes of similar taxa. For that reason, a secondary metabolite profiling was found more reliable (Andersen et al., 2008). When the MALDI-TOF MS results obtained with a set of 37 strains (proteins were extracted using 70% formic acid) were compared with combined DNA-based data (sequencing of ITS and two marker genes), a partial difference in clustering was found, which was attributed to different chemical profiles that are needed for the pathogenicity in different host groups (Brun et al., 2013). The major benefit of MALDI-TOF MS in this case resided in applicability for much younger cultures (3 days) than the profiling of metabolites (7–14 days), which is advantageous for an early detection.

9. Applications of MALDI-TOF MS of fungi in biotechnology and clinical diagnostics

IC/IS MALDI-TOF MS analysis was successfully employed for the characterization of *Penicillium chrysogenum* cells growing in a bioreactor for penicillin production (Posch et al., 2013). The reason was to look at the outer proteins of the cells providing a characteristic pattern in specific physiological and morphological states with the aim of determining biomarkers of highest penicillin productivity. A mixture of SA and FA of 1:4 in acetonitrile/0.1% TFA, 7:3, v/v was used as a matrix for co-crystallization with biomass samples on disposable nano-coated polymer-based targets. Statistically significant biomarker ions were found for pellet growth morphology as well as for the state of high productivity (Posch et al., 2013).

The thermophilic fungus *Malbranchea sulfurea* produces photosensitive polyketides, malbranpyrroles, as secondary metabolites. Interestingly, its ethyl acetate extract showed strong cytotoxicity against various cancer cell lines. Yang et al. (2009) demonstrated the use of intact cell/spore desorption/ionization on silicon (a matrix-free ionization technique performed on a standard MALDI-TOF instrument) for the detection of malbranpyrroles A–F in the *m/z* region of 300–400. The study showed that only brown (not white) hyphae of *M. sulfurea* can produce malbranpyrroles.

An early and accurate diagnostic is required to tackle serious and life-threatening infections in humans. Pathogenic fungi from the genera *Candida*, *Cryptococcus* or *Aspergillus* represent typical infectious agents causing diseases in immunocompromised hosts including patient with immunosuppressive therapy. IC/IS MALDI-TOF MS was shown to be useful for a rapid identification of yeast cells. Five species of the genus *Candida* (i.e. *C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida kefyr* and *Candida dubliniensis*) were analyzed together with *Cryptococcus neoformans*, *S. cerevisiae* and *Rhodotorula* sp. as well as with three *Aspergillus* mold species (*Aspergillus terreus*, *A. fumigatus* and *Aspergillus syndowii*; cultured as a yeast-like fungi) in a study, which

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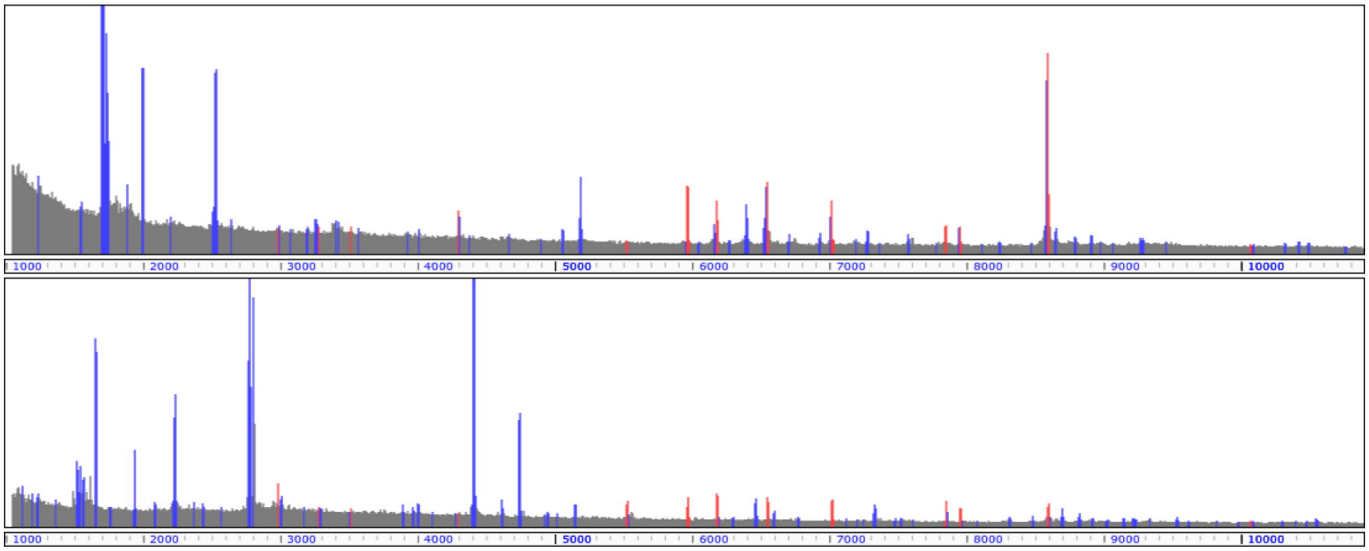

Fig. 4. A comparison of mass spectrometric peptide/protein profiles of *Plasmopara halstedii* and *Plasmopara viticola*. This is a graphical window, which appears as a result of data evaluation by the software Biospean (Martin Raus, Palacký University in Olomouc, Czech Republic). All detected peaks (ions) are highlighted in blue, whereas red color indicates the matching of peak positions between both spectra. For experimental details, see Fig. 3.

demonstrated the advantage of a fixation by 50% (v/v) methanol prior to the deposition of yeast samples on MALDI target (Qian et al., 2008). SA was found to be the most suitable matrix and was applied using the dried-droplet technique. Besides differentiation of yeast species, morphogenetic forms (germinated vs. non-germinated cells) of *C. albicans* could also be distinguished.

A large MALDI-based study with collection strains and many clinical isolates of *Candida* (n = 250), *Cryptococcus*, *Saccharomyces*, *Trichosporon*, *Geotrichum*, *Pichia* and *Blastoschizomyces* was performed by Marklein et al. (2009). Prior to measurements, yeast cells from up to five representative colonies were transferred to a tube and treated with 75% ethanol. After centrifugation, the pellet was extracted with 70% formic acid and acetonitrile, 1:1, v/v and centrifuged. An aliquot of the supernatant was applied onto a MALDI target and on drying it was overlaid with a CHCA matrix solution for crystallization. One measurement was generally completed within 10 min per isolate and within 3 h for 96 samples starting from single colonies on agar plates. With an incomplete database of reference strains, 92.5% of the clinical isolates were identified. No false-positive identifications occurred as the non-identified isolates provided low score values due to missing references in the database (Marklein et al., 2009). Another study compared results of bacteria and yeast identification (980 clinical isolates) using conventional techniques (Vitek 2 colorimetric system, API strips and biochemical tests) and MALDI-TOF MS. Correct species identification by the MALDI analysis was observed in 85.2% of yeasts (61 isolates were inspected encompassing 12 different species). As regards to yeasts and on the species level, MALDI-TOF MS had higher performance than conventional biochemical systems. Failed identifications were clearly associated with the absence of reference spectra in the database (van Veen et al., 2010). For different sets of yeast isolates of similar taxonomic composition (with various species of the genera *Candida*, *Cryptococcus*, *Trichosporon*, etc.), higher identification rates of more than 96% were obtained by other groups (Dhiman et al., 2011; Stevenson et al., 2010). To speed up the process of sample preparation, *Candida* or *Aspergillus* cells collected from culture media were transferred on a disposable MALDI target and overlaid with 25% formic acid for cell lysis. Upon drying, a CHCA matrix solution was applied to the spot for crystallization (Iriart et al., 2012). Using this approach, a high performance of correct identifications of yeasts and Aspergilli was achieved (92.3%).

Two pathogenic basidiomycetous yeasts, *C. neoformans* and *Cryptococcus gattii*, are known to cause meningoencephalitis in immunocompromised and apparently immunocompetent human hosts, respectively. The *C. neoformans*/*C. gattii* group comprises two sibling species that are divided into eight major molecular types, *C. neoformans* VNI to VNIV and *C. gattii* VGI to VGIV, which differ in host range, epidemiology, virulence, antifungal susceptibility and geographic distribution. MALDI-TOF MS of protein extracts obtained via the formic acid extraction allowed 100% identification of all *Cryptococcus* isolates. No spectra were obtained from intact yeast cells (Firacative et al., 2012). In another study, an in-house reference database of MALDI-TOF MS spectra was constructed and used for a highly reliable species identification of *C. neoformans* and *C. gattii* isolates, which had previously been characterized by DNA-based methods. Prior to spectra acquisition, proteins were extracted by suspending cells in 10% formic acid. The suspension was deposited on the target, air dried, overlaid with absolute ethanol and air dried again. Then a saturated CHCA matrix solution was added for crystallization (Posteraro et al., 2012).

MALDI-TOF MS has been frequently used in screening studies of yeast-containing positive blood cultures (Marinach-Patrice et al., 2010; Ferreira et al., 2011; Ferroni et al., 2010; Yan et al., 2011; Spanu et al., 2012). Prior to measurements on a mass spectrometer, blood culture broth samples were processed by washings and centrifugation (pelleting) followed by protein extraction with 70% formic acid and acetonitrile, 1:1, v/v. It has been shown that a complete removal of blood cells by a lysis solution was essential for obtaining a high accuracy in identification (Spanu et al., 2012). *Candida*-specific spectral differences were found namely in the *m/z* range of 5000–7400 (Marinach-Patrice et al., 2010). The whole procedure was characterized as fast (a single MS analysis is done within minutes) and economical (calculated costs of less than 1 USD per an isolate). On the other hand, the method's main weakness is its poor performance in the presence of polyfungal bloodstream infections. Fortunately, such infections occur only rarely (Spanu et al., 2012). A very recent IC/IS MALDI-TOF MS study on blood culture isolates of yeasts using commercial instrumentation showed an overall identification performance of more than 90%, which was superior to conventional procedures. Here a formic acid solution (70%) was used to treat yeast samples deposited on the target. After evaporation, CHCA was spotted for crystallization (Rosevinge et al., 2013).

Dermatophyte fungi isolates originating from skin and nail samples of patients (including *T. rubrum*, *Trichophyton interdigitale*, *Arthroderma benhamiae* and *Microsporum canis*) were first identified by morphological treats and by sequencing rDNA cluster regions including ITS1 and 2. Then they were subjected to a comparative analysis by IC/IS MALDI-TOF

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MS (Erhard et al., 2008). All isolates produced mass spectra with 60 to 120 signals in the *m/z* range of 2000–20 000 (using DHB as a matrix) and the results obtained were consistent with the ITS and 5.8S rDNA sequencing. Moreover, the method proved to be robust enough with respect to growing the same sample on different media. Dermatophytes from the genera *Trichophyton*, *Epidermophyton*, *Microsporum* and *Arthroderma* were also subjects of several recent studies employing MALDI-TOF MS (Nenoff et al., 2013; Packeu et al., 2013; Theel et al., 2011). Their identification to the species level is often needed for the accurate treatment of dermatophytoses. Common species are relatively easy to identify by standard morphological methods but others may become a diagnostic challenge. Cells from cultures were either extracted by 70% formic acid and the extracts crystallized with a CHCA matrix prior to spectra acquisition (Packeu et al., 2013; Theel et al., 2011) or transferred directly to the target plate and measured using DHB as a matrix (Nenoff et al., 2013). At the species level, MALDI-TOF MS and DNA-based sequencing strategies were largely consistent and superior to the morphological identifications (Packeu et al., 2013; Theel et al., 2011).

Infectious zygomycetes of the clinically important genera *Mucor*, *Rhizopus* and *Lichtheimia* (order Mucorales) show variable resistance to antimycotic substances. An accurate diagnosis before onset of antimycotic therapy is therefore desirable. MALDI-TOF MS was investigated for a rapid identification of *Lichtheimia* strains on the species level (Schrödl et al., 2012). A total of 53 fungal strains were available for cultivation in that study, from which 46 belonged to the genus *Lichtheimia* (distributed within five species). After washing and resuspending of post-centrifugation pellets from the cultures, mass spectra were acquired with formic acid/acetonitrile extracts using CHCA as a matrix when the dried-droplet technique was employed for sample preparation. First a database was created using peptide/protein profiles (*m/z* 2000–20 000) obtained for well-characterized strains. All five species of *Lichtheimia* could be easily distinguished in this way. Then the other isolates were measured and their mass spectrometric data searched against the database: 77% of all tested strains were identified with the highest probability (95% were at the level of a probable identification). The phylogenetic tree constructed from the MS data was almost identical with that coming from multigene genealogy results (including rDNA spacer regions).

10. Discussion and perspectives

Since the middle of the 1990s, a methodology has been continually developed for the use of MALDI-TOF MS in microbial identification and characterization. Numerous original research papers have been published on this topic so far. However, compared with bacteria, much less attention has been paid to mass spectrometric analysis of fungi (Kallow et al., 2010). Similarly to bacterial analysis, IC/IS MALDI-TOF MS of fungi provides fast and reliable experimental data. However, because of the rigidity of the fungal cell wall, sample preparation techniques including a mechanical or chemical pretreatment of harvested cells or spores have been introduced to increase the release of surface proteins for their subsequent extraction followed by MS analysis (Hettick et al., 2008a,2008b; Marklein et al., 2009; Sulc et al., 2009). The additional protein extraction step can be beneficial in terms of obtaining a higher amount of characteristic peptide or protein ions in mass spectrometric profiles, which is definitely useful for sample identification, but on the other hand, the whole sample preparation procedure is then more time consuming and less efficient than those involving direct measurements with intact cells or spores. As regards to the time scale of experiments, requirements for chemicals and consumables and demands on a skillful and experienced technical assistance, MS-based identifications of fungi provide many advantages in contrast to biological and biochemical methods such as morphological studies under microscope, rDNA-based biotyping or using colorimetric identification systems. In addition, MALDI-TOF MS shows an accuracy, which is equal or even outperforms routine diagnostic systems (Rosevinge et al., 2013). MS may fail in identifications when there is no reference spectrum available in the database; misidentifications are rare.

No versatile method for MALDI-TOF MS of fungal microorganisms has been described up to now. Thus, both the selection of a proper matrix compound and choice of an optimal sample preparation technique are still rather empiric. But there are some rules, which may be followed. DHB, FA and SA are used as typical matrices in IC/IS MALDI-TOF MS of fungi, the latter two may also be combined into a mixture and then used to facilitate sample desorption and ionization. When measuring with mycelia or spore extracts containing peptides and proteins, CHCA is the matrix of choice. The dried-droplet technique remains preferred for sample deposition. Mixed-volume techniques (Kemptner et al., 2009a,2009b) are less frequent and associated with a longer exposure of the material to solvents, which may be undesirable. Common fungi, such as members of the genera *Aspergillus*, *Fusarium*, *Penicillium* or various yeasts, can be cultured on nutrient media. When pure colony material cannot be obtained, it is possible to make a subculture (Rosevinge et al., 2013). Conversely, obligate biotrophs such as downy and powdery mildews are usually grown on host plants, which makes their analysis more difficult. However, recent experiments by Chalupová et al. (2012) suggested the possibility of a direct identification of mildews from infected plant leaves without the need of an isolation step.

From the above literature survey it becomes clear that both reference strains and studied isolates have to be analyzed by MALDI-TOF MS under the same experimental conditions (Posteraro et al., 2013). This means that not only the culture growth and sample preparation steps but also the instrumental setup for spectra acquisition should better be optimized and standardized. Nevertheless, different culture conditions including media composition (Sabouraud dextrose, *Candida* chromogenic agar or others) and temperatures of incubation may not necessarily affect obtaining good identification results (Lau et al., 2013). On the contrary, both pigment production and variability in age of colonies may cause troubles. It has repeatedly been shown that pigments interfere with the ionization of biomolecules (Dong et al., 2009; Sulc et al., 2009). Thus it is recommended here to avoid their presence especially when pigment formation is related to the composition of a selective medium e.g. for the cultivation of *B. cinerea* (Gielen et al., 2003). Possible differences between the mass spectra from young and mature colonies have to be taken into account and preferably handled by uploading two different reference spectra into the database: one acquired from young colonies and the other from mature colonies (Alanio et al., 2011). The sample preparation step is probably the most important in the whole analytical procedure. For filamentous fungi such *Aspergillus*, *Penicillium* and *Fusarium*, the "intact cell" approach is frequently used (prewashed spores are mixed on the MALDI target with a proper matrix solution) and seems to be very effective with respect to the time consumption for analysis of each sample. But yeasts have a rigid cell wall that needs to be disrupted to release more proteins for ionization. The "complete extraction" approach, typically used for *Candida* or *Cryptococcus*, consists of an ethanol fixation step for inactivation and reducing cell aggregation, which is followed by a treatment of the cells with 70% formic acid. Then acetonitrile is added to increase protein solubility and the extract is spotted on the target prior to the matrix application. To make the extraction procedure shorter, cells deposited on the target are extracted with 25–70% formic acid (Posteraro et al., 2013). To achieve reliable results, it is also necessary to use an updated commercial spectral database or a custom database engineered with the highest care. Lau et al. (2013) demonstrated the necessity of introducing less common or uncommon species into the database otherwise low identification rates can be expected for real samples such as clinical mold isolates. As regards to MALDI-TOF instruments, those that are equipped with a linear flight tube allow obtaining a sufficient resolution for constructing reference databases and routine analyses. The presence of a reflector analyzer is not needed.

The data, which came out from MALDI-TOF MS of fungi in the course of previous 20 years, clearly indicated a big potential of the method for

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1) diagnostics in human or veterinary medicine, 2) phytopathology and experimental botany, 3) monitoring of environmental contamination and industrial processes, and 4) microbial biotechnology. Most of the mentioned spheres of potential application will rely on identification i.e. distinguishing fungi at the level of species or strains. In a fundamental phylogenetic research, MS experiments followed by bioinformatics tools such as hierarchical cluster analysis are able to provide strong results, which may end up with discoveries of new species and reassignment of taxonomic affiliations based on classical biological methods (Kallow et al., 2006).

MALDI-based identification and classification of fungal microorganisms utilize peptide/protein profiles containing characteristic biomarker peaks in the *m/z* region of 1000–20 000. Their presence is important for the subsequent database searches and comparison of experimental data with those for reference strains. For that reason, a future development of MALDI-TOF MS methodology for fungal analyses will definitely involve a continuous updating of current commercial databases provided by vendors together with the instrumentation as well as building up new databases for specialized research purposes. Hand in hand with this, improved software solutions will appear for data processing and management. There is still a big gap in linking MALDI-TOF MS peptide/protein profiles with proteomic identification of individual biomarker molecules, which needs to be expanded. The outer surface proteins named hydrophobins have been recognized as the cause of predominant ions during MALDI-TOF MS profiling of *Hypocrea* and *Trichoderma* strains (Neuhof et al., 2007b). Interestingly, spores of downy and powdery mildews release ribosomal proteins when treated with acidic matrix solutions (Chalupová J. et al., Palacký University, unpublished results), which is similar to the behavior of bacteria and yeasts (Fenselau and Demirev, 2001).

Both selection and spread of drug resistant pathogens represent a serious public health problem in current medicine. In this context, recent papers have shown a big potential of MALDI-TOF MS for antifungal susceptibility testing (AFST), which represents a prerequisite for efficient antifungal therapy (Marinach et al., 2009; Vella et al., 2013). Standard susceptibility tests are based on measuring of microbial growth under different drug concentrations in order to determine the minimal inhibition concentration for a given isolate. Many different methods including colorimetric tests are used for that purpose but they may provide unsatisfactory correlations with reference data because of subjectivity in reading results (Morace et al., 2002). MALDI-based approaches offer the possibility of finding the lowest drug concentration at which a change in the fingerprint spectrum, reflecting proteome alterations, can be detected. Then a susceptibility or resistance behavior can be discriminated fast and objectively using a statistical analysis. Interesting AFST data have been published for *C. albicans* (Marinach et al., 2009; Vella et al., 2013).

Conflict of interest

There are no conflicts of interests.

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References

Abd-Elsalam KA, Aly IN, Abdel-Satar MA, Khalil MS, Verreet JA. PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. Afr J Biotechnol 2003;2:82–5.

Aebersold R. A mass spectrometric journey into protein and proteome research. J Am Soc Mass Spectrom 2003;14:685–95.

Alanio A, Beretti JL, Dauphin B, Mellado E, Quesne G, Lacroix C, et al. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Aspergillus* species. Clin Microbiol Infect 2011;17:750–5.

Amiri-Eliasi B, Fenselau C. Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. Anal Chem 2001;73:5228–31.

Andersen B, Smedsgaard J, Frisvad JC. *Penicillium expansum*: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. J Agric Food Chem 2004;52:2421–8.

Andersen B, Dongo A, Pryor BM. Secondary metabolite profiling of *Alternaria dauci*, *A. porri*, *A. solani*, and *A. tomatophila*. Mycol Res 2008;112:241–50.

Balajee SA, Borman AM, Brandt ME, Cano J, Cuenca-Estrella M, Dannaoui E, et al. Sequence-based identification of *Aspergillus*, *Fusarium*, and *Mucorales* species in the clinical mycology laboratory: where are we and where should we go from here? J Clin Microbiol 2009;47:877–84.

Bennett JW. Mycotechnology: the role of fungi in biotechnology. J Biotechnol 1998;66:101–7.

Bennett JW, Klich M. Mycotoxins. Clin Microbiol Rev 2003;16:497–516.

Brun S, Madrid H, Gerrits van den Ende B, Andersen B, Marinach-Patrice C, Mazier D, et al. Multilocus phylogeny and MALDI-TOF analysis of the plant pathogenic species *Alternaria dauci* and relatives. Fungal Biol 2013;117:32–40.

Cannon PF, Sutton BC. Microfungi on wood and plant debris. In: Mueller GM, Bills GF, Foster MS, editors. Biodiversity of fungi: inventory and monitoring methods. London: Elsevier Academic Press Inc.; 2004. p. 218–38.

Carlile MJ, Gooday GW, Watkinson SC. The fungi. 2nd ed. San Diego: Academic Press Ltd; 2001.

Cassagne C, Ranque S, Normand AC, Fourquet P, Thiebault S, Planard C, et al. Mould routine identification in the clinical laboratory by matrix-assisted laser desorption ionization-time of flight mass spectrometry. PLoS One 2011;6:e28425.

Cazares LH, Troyer DA, Wang B, Drake RR, Semmes OJ. MALDI tissue imaging: from biomarker discovery to clinical applications. Anal Bioanal Chem 2011;401:17–27.

Chalupová J, Sedlářová M, Helmel M, Řehulka P, Marchetti-Deschmann M, Allmaier G, et al. MALDI-based intact spore mass spectrometry of downy and powdery mildews. J Mass Spectrom 2012;47:978–86.

Chen HY, Chen YC. Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 2005;19:3564–8.

Chugh JK, Wallace BA. Peptaibols: models for ion channels. Biochem Soc Trans 2001;29:565–70.

Clark EA, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev 2013;26:547–603.

De Carolis E, Posteraro B, Lass-Flörl C, Vella A, Florio AR, Torelli R, et al. Species identification of *Aspergillus*, *Fusarium* and *Mucorales* with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 2012;18:475–84.

Degenkolb T, Dieckmann R, Nielsen KF, Gräfenhan T, Theis C, Zafari D, et al. The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibiotics, and mycotoxins. Mycol Prog 2008;7:177–219.

De Respinis S, Vogel G, Benagli C, Tonolla M, Petrini O, Samuels GJ. MALDI-TOF MS of *Trichoderma*: a model system for the identification of microfungi. Mycol Prog 2010;9:79–100.

Dhiman N, Hall L, Wohlfiel SL, Buckwalter SP, Wengenack NL. Performance and cost analysis of matrix-assisted laser desorption ionization-time of flight mass spectrometry for routine identification of yeast. J Clin Microbiol 2011;49:1614–6.

Domon B, Aebersold R. Mass spectrometry and protein analysis. Science 2006;312:212–7.

Dong H, Kemptner J, Marchetti-Deschmann M, Kubicek CP, Allmaier G. Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry. Anal Bioanal Chem 2009;395:1373–83.

Doohan F. Fungal pathogens of plants. In: Kavanagh K, editor. Fungi — proteomics and application. Chichester: John Wiley; 2005. p. 219–50.

Erhard M, Hipler UC, Burmester A, Brakhage AA, Wöstemeyer J. Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry. Exp Dermatol 2008;17:356–61.

Fenselau C, Demirev PA. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 2001;20:157–71.

Ferreira L, Sánchez-Juanes F, Porras-Guerra I, García-García MI, García-Sánchez JE, González-Buitrago JM, et al. Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Microbiol Infect 2011;17:546–51.

Ferroni A, Suarez S, Beretti JL, Dauphin B, Bille E, Meyer J, et al. Real-time identification of bacteria and *Candida* species in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2010;48:1542–8.

Firacative C, Trilles L. MALDI-TOF MS enables the rapid identification of the major molecular types within the *Cryptococcus neoformans/C. gattii* species. PLoS One 2012;7:e37566.

Gaskell GJ, Carter DA, Britton WJ, Tovey ER, Benyan FH, Lovborg M. Analysis of internal transcribed spacer regions of ribosomal DNA in common airborne allergenic fungi. Electrophoresis 1997;18:1567–9.

Geiser DM, Frisvad JC, Taylor JW. Evolutionary relationships in *Aspergillus* section *Fumigati* inferred from partial β-tubulin and hydrophobin DNA sequences. Mycologia 1998;90:831–45.

Gielen S, Aerts R, Seels B. Development of a selective medium for the determination of the spore concentrations of *Botrytis cinerea* in the air. Commun Agric Appl Biol Sci 2003;68:685–93.

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Guarro J, Gené J, Stchigel AM. Developments in fungal taxonomy. Clin Microbiol Rev 1999;12:454–500.

Gugnani HC. Ecology and taxonomy of pathogenic aspergilli. Front Biosci 2003;8: s346–57.

Havlicek V, Lemr K, Schug KA. Current trends in microbial diagnostics based on mass spectrometry. Anal Chem 2013;85:790–7.

Hettick JM, Green BJ, Buskirk AD, Kashon ML, Slaven JE, Janotka E, et al. Discrimination of *Penicillium* isolates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Rapid Commun Mass Spectrom 2008a;22: 2555–60.

Hettick JM, Green BJ, Buskirk AD, Kashon ML, Slaven JE, Janotka E, et al. Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Anal Biochem 2008b;380:276–81.

Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, et al. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 1996;10:1227–32.

Hong SB, Goo SJ, Shin HD, Frisvad JC, Samson RA. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia 2005;97:1316–29.

Horká M, Kubesová A, Šalplachta J, Zapletalová E, Horký J, Šlais K. Capillary and gel electromigration techniques and MALDI-TOF MS — suitable tools for identification of filamentous fungi. Anal Chim Acta 2012;716:155–62.

Hudspeth DSS, Nadler SA, Hudspeth MES. A *cox2* molecular phylogeny of the Peronosporomycetes. Mycologia 2000;92:674–84.

Iriart X, Lavergne RA, Fillaux J, Valentin A, Magnaval JF, Berry A, et al. Routine identification of medical fungi by MALDI-TOF: performance of the new VITEK MS using a new time-effective strategy. J Clin Microbiol 2012;50:2107–10.

Jarman KH, Daly DS, Petersen CE, Saenz AJ, Valentine NB, Wahl KL. Extracting and visualizing matrix-assisted laser desorption/ionization time-of-flight mass spectral fingerprints. Rapid Commun Mass Spectrom 1999;13:1586–94.

Kallow W, Erhard M, Shah HN, Raptakis E, Welker M. MALDI-TOF MS and microbial identification: years of experimental development to an established protocol. In: Shah HN, Gharbia SE, Encheva V, editors. Mass spectrometry for microbial proteomics. Chichester, UK: Wiley; 2010. p. 255–76.

Kallow W, Santos I, Erhard M, Serra R, Venâncio A, Lima N. In: Meyer W, Pearce C, editors. *Aspergillus ibericus*: a new species of section *Nigri* characterised by MALDI-TOF MS. 8th International Mycological Congress, Cairns, Australia (Proceedings Book). Pianoro (BO). Italy: MEDIMOND; 2006. p. 189–93.

Kemptner J, Marchetti-Deschmann M, Kubicek CP, Allmaier G. Mixed volume sample preparation method for intact cell mass spectrometry of *Fusarium* spores. J Mass Spectrom 2009a;44:1622–4.

Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinia IS, Kubicek CP, Allmaier G. Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2009b;23: 877–84.

Lau AF, Drake SK, Calhoun LB, Henderson CM, Zelazny AM. Development of a clinically comprehensive database and a simple procedure for identification of molds from solid media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2013;51:828–34.

Li TY, Liu BH, Chen YC. Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2000;14:2393–400.

Marchetti-Deschmann M, Winkler W, Dong H, Lohninger H, Kubicek CP, Allmaier G. Using spores for *Fusarium* spp. classification by MALDI-based intact cell/spore mass spectrometry. Food Technol Biotechnol 2012;50:334–42.

Marinach C, Alanio A, Palous M, Kwasek S, Fekkar A, Brossas JY. MALDI-TOF MS-based drug susceptibility testing of pathogens: the example of *Candida albicans* and fluconazole. Proteomics 2009;9:4627–31.

Marinach-Patrice C, Fekkar A, Atanasova R, Gomes J, Djamdjian L, Brossas JY, et al. Rapid species diagnosis for invasive candidiasis using mass spectrometry. PLoS One 2010;5: e8862.

Marinach-Patrice C, Lethuillier A, Marly A, Brossas JY, Gené J, Symoens F, et al. Use of mass spectrometry to identify clinical *Fusarium* isolates. Clin Microbiol Infect 2009;15: 634–42.

Marklein G, Josten M, Klanke U, Müller E, Horré R, Maier T, et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. Clin Microbiol 2009;47:2912–7.

Martin KJ, Rygiewicz PT. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiol 2005;5:28.

McCartney HA, Foster SJ, Fraaije BA, Ward E. Molecular diagnostics for fungal plant pathogens. Pest Manac Sci 2003;59:129–42.

McDonnell LA, Heeren RM. Imaging mass spectrometry. Mass Spectrom Rev 2007;26: 606–43.

Meuzelaar HLC, Kistemaker PG. A technique for fast and reproducible fingerprinting of bacteria by pyrolysis mass spectrometry. Anal Chem 1973;45:587–90.

Minerva L, Ceulemans A, Baggerman G, Arckens L. MALDI MS imaging as a tool for biomarker discovery: methodological challenges in a clinical setting. Proteomics Clin Appl 2012;6:581–95.

Mitchell JI, Zuccaro A. Sequences, the environment and fungi. Mycol 2006;20: 62–74.

Morace G, Amato G, Bistoni F, Fadda G, Marone P, Montagna MT, et al. Multicenter comparative evaluation of six commercial systems and the national committee for clinical laboratory standards m27-A broth microdilution method for fluconazole susceptibility testing of *Candida* species. J Clin Microbiol 2002;40:2953–8.

Nelson PE, Plattner RD, Shackelford DD, Desjardins AE. Fumonisin B1 production by *Fusarium* species other than *F. moniliforme* in section *Liseola* and by some related species. Appl Environ Microbiol 1992;58:984–9.

Nenoff P, Erhard M, Simon JC, Muylowa GK, Herrmann J, Rataj W, et al. MALDI-TOF mass spectrometry — a rapid method for the identification of dermatophyte species. Med Mycol 2013;51:17–24.

Neuhof T, Berg A, Besl H, Schwecke T, Dieckmann R, von Döhren H. Peptaibol production by Sepedonium strains parasitizing Boletales. Chem Biodivers 2007a;4:1103–15.

Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP, Nakari-Setälä T, Penttilä M, et al. Direct identification of hydrophobins and their processing in *Trichoderma* using intact-cell MALDI-TOF MS. FEBS J 2007b;274:841–52.

Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP, von Döhren H. Intact-cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus *Trichoderma/Hypocrea*: can molecular phylogeny of species predict peptaibol structures? Microbiology 2007c;153:3417–37.

Ninet B, Jan I, Bontems O, Léchenne B, Jousson O, Lew D, et al. Molecular identification of *Fusarium* species in onychomycoses. Dermatology 2005;210:21–5.

Packeu A, Hendrickx M, Beguin H, Martiny D, Vandenberg O, Detandt M. Identification of the *Trichophyton mentagrophytes* complex species using MALDI-TOF mass spectrometry. Med Mycol 2013;51:580–5.

Paterson RRM, Venâncio A, Lima N. Solutions to *Penicillium* taxonomy crucial to mycotoxin research and health. Res Microbiol 2004;155:507–13.

Posch AE, Koch C, Helmel M, Marchetti-Deschmann M, Macfelda K, Lendl B, et al. Combining light microscopy, dielectric spectroscopy, MALDI intact cell mass spectrometry, FTIR spectromicroscopy and multivariate data mining for morphological and physiological bioprocess characterization of filamentous organism. Fungal Genet Biol 2013;51:1–11.

Posteraro B, De Carolis E, Vella A, Sanguinetti M. MALDI-TOF mass spectrometry in the clinical mycology laboratory: identification of fungi and beyond. Expert Rev Proteomics 2013;10:151–64.

Posteraro B, Vella A, Cogliatti M, De Carolis E, Florio AR, Posteraro P, et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for discrimination between molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii*. J Clin Microbiol 2012;50:2472–6.

Qian J, Cutler JE, Cole RB, Cai Y. MALDI-TOF mass signatures for differentiation of yeast species, strain grouping and monitoring of morphogenesis markers. Anal Bioanal Chem 2008;392:439–49.

Rosevinge FS, Dzajic E, Knudsen E, Malig S, Andersen LB, Løvig A, et al. Performance of matrix-assisted laser desorption-time of flight mass spectrometry for identification of clinical yeast isolates. Mycoses 2013;56:229–35.

Santos C, Paterson RRM, Venâncio A, Lima N. Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Appl Microbiol 2010;108:375–85.

Sedlář J, Sedlářová M, Flusser J. Image processing methods for determination of downy mildews from light microscopy images. In: Kulpa K, Kaska W, editors. Signal Processing Symposium 2009 Proceedings. Warsaw: Warsaw University of Technology; 2009. [lecture B2_1].

Schmidt O, Kalow W. Differentiation of indoor wood decay fungi with MALDI-TOF mass spectrometry. Holzforschung 2005;59:374–7.

Schrödl W, Heydel T, Schwartze VU, Hoffmann K, Grosse-Herrenthey A, Walther G, et al. Direct analysis and identification of pathogenic *Lichtheimia* species by matrix-assisted laser desorption ionization-time of flight analyzer-mediated mass spectrometry. J Clin Microbiol 2012;50:419–27.

Šedo O, Sedláček I, Zdráhal Z. Sample preparation methods for MALDI-MS profiling of bacteria. Mass Spectrom Rev 2011;30:417–34.

Seng P, Drancourt M, Gouriet F, Scola BL, Fournier PE, Rolain JM, et al. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 2009;49(4):543–51.

Seyfarth F, Ziemer M, Sayer HG, Burmester A, Erhard M, Welker M, et al. The use of ITS DNA sequence analysis and MALDI-TOF mass spectrometry in diagnosing an infection with *Fusarium proliferatum*. Exp Dermatol 2008;17:965–71.

Shevchenko A, Wilm M, Mann M. Peptide sequencing by mass spectrometry for homology searches and cloning of genes. J Protein Chem 1997;16:481–90.

Spanu T, Posteraro B, Fiori B, D'Inzeo T, Campoli S, Ruggeri A, et al. Direct MALDI-TOF mass spectrometry assay of blood culture broths for rapid identification of *Candida* species causing bloodstream infections: an observational studying two large microbiology laboratories. J Clin Microbiol 2012;50:176–9.

Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. J Clin Microbiol 2010;48:3482–6.

Sulc M, Peslova K, Zabka M, Hajduch M, Havlicek V. Biomarkers of *Aspergillus* spores: strain typing and protein identification. Int J Mass Spectrom 2009;280:162–8.

Tao J, Zhang G, Jiang Z, Cheng Y, Feng J, Chen Z. Detection of pathogenic *Verticillium* spp. using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2009;23:3647–54.

Theel ES, Hall L, Mandrekar J, Wengenack N. Dermatophyte identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2011;49:4067–71.

Valentine NB, Wahl JH, Kingsley MT, Wahl KL. Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 2002;16:1352–7.

van Veen SQ, Claas EC, Kuijper EJ. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J Clin Microbiol 2010;48:900–7.

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Vella A, De Carolis E, Vaccaro L, Posteraro P, Perlin DS, Kostrzewa M, et al. Rapid antifungal susceptibility testing by matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. J Clin Microbiol 2013;51:2964–9.

Wang LI, Yokoyama K, Miyaji M, Nishimura K. The identification and phylogenetic relationship of pathogenic species of *Aspergillus* based on the mitochondrial cytochrome *b* gene. Med Mycol 1998;36:153–64.

Welham KJ, Domin MA, Johnson K, Jones L, Ashton DS. Characterization of fungal spores by laser desorption/ionization time of flight mass spectrometry. Rapid Commun Mass Spectrom 2000;14:307–10.

Welham KJ, Domin MA, Scanell DE, Cohen E, Ashton DS. The characterization of micro-organisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 1998;12:176–80.

Yan Y, He Y, Maier T, Quinn C, Shi G, Li H, et al. Improved identification of yeast species directly from positive blood culture media by combining Sepsityper specimen processing and Microflex analysis with the matrix-assisted laser desorption ionization biotyper system. J Clin Microbiol 2011;49:2528–32.

Yang YL, Liao WY, Liu WY, Liaw CC, Shen CN, Huang ZY, et al. Discovery of new natural products by intact-cell mass spectrometry and LC-SPE-NMR: malbranpyrroles, novel polyketides from thermophilic fungus *Malbranchea sulfurea*. Chem Eur J 2009;15:11573–80.

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