REVIEW ARTICLE

Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

C. Santos[1,2], R.R.M. Paterson[2,3], A. Venâncio[2] and N. Lima[2,4]

1 Fundamental Chemistry Department, Federal University of Pernambuco, Recife, PE, Brazil
2 IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal
3 Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia
4 Biochemistry Department, Federal University of Pernambuco, Recife, PE, Brazil

Summary

Matrix-assisted laser desorption/ionization time-of-flight intact cell mass spectrometry (MALDI-TOF ICMS) is coming of age for the identification and characterization of fungi. The procedure has been used extensively with bacteria. UV-absorbing matrices function as energy mediators that transfer the absorbed photoenergy from an irradiation source to the surrounding sample molecules, resulting in minimum fragmentation. A surprisingly high number of fungal groups have been studied: (i) the terverticillate penicillia, (ii) aflatoxigenic, black and other aspergilli, (iii) *Fusarium*, (iv) *Trichoderma*, (iv) wood rotting fungi (e.g. *Serpula lacrymans*) and (v) dermatophytes. The technique has been suggested for optimizing quality control of fungal Chinese medicines (e.g. *Cordyceps*). MALDI-TOF ICMS offers advantages over PCR. The method is now used in taxonomic assessments (e.g. *Trichoderma*) as distinct from only strain characterization. Low and high molecular mass natural products (e.g. peptaibols) can be analysed. The procedure is rapid and requires minimal pretreatment. However, issues of reproducibility need to be addressed further in terms of strains of species tested and between run variability. More studies into the capabilities of MALDI-TOF ICMS to identify fungi are required.

Introduction

Fungi are very important organisms. They are employed to produce pharmaceuticals, enzymes, organic acids and foods. Many are used as Chinese herbal medicines in significant markets worldwide (Paterson 2006a, 2008a). The number of fungal diseases of humans is growing and particularly in those people with compromised immune systems. Mycotoxin production is of primary concern to the food industry, and fungi cause devastating diseases of crops. The primary role of fungi in nature is in the recycling of organic plant material.

However, it has been argued that an understanding of speciation, population biology, phylogeny and evolution of fungi are not as developed as in other organisms (Burnett 2003). While recognizing the wealth of excellent research that addresses fungal phylogeny and systematics, evolutionary studies have focussed more on plants, animals and bacteria, because of, amongst other things, more researchers being employed in these disciplines and the existence of extensive fossil records in plants and animals. Fungi are inherently more difficult to study. Plant pathology, fungal ecology, animal/human mycosis and commercial exploitation of fungi require an improved comprehension of the structure and behaviour of fungal populations. Fungi as a whole remain difficult to identify because of the biological complexity: classification undergoes almost continual change, particularly since the recent introduction of nucleic acid-based methodology (Hibbett *et al.* 2007) where 'cryptic species' are revealed. To illustrate the points made, some important penicillia (Paterson *et al.* 2004), *Cordyceps* (Paterson

2008a) and *Ganoderma* (Paterson 2006a, 2007b; Paterson *et al.* 2009) remain problematic despite decades of study. Additional complexity arises as systematic mycologists must work within the botanical code of nomenclature that emphasizes the naming of sexual states (telomorphs) over asexual vegetative growth forms (anamorphs). For example, the recent discovery published by O'Gorman *et al.* (2009) of a sexual stage of the pathogen fungus *Aspergillus fumigatus* introduces a new scientific name with priority for it, *Neosartorya fumigata*. In this case, *A. fumigatus* must remain from pragmatic and clinical point of view (supplemental information in O'Gorman *et al.* 2009). This would be in line with the practice in *Aspergillus nidulans* because *Emericella nidulans* is largely ignored (Hawksworth 2009a).

Furthermore, internationally based culture collections are obliged to guarantee the authenticity of the fungi that they hold (Santos and Lima 2001). There are estimated to be 1.5×10^6 species of fungi of which only 5% have been identified formally (Hawksworth 2006a). Identifications are time-consuming, and decisions regarding what represents a species tend to be subjective. The standard method for identifying and classifying filamentous fungi remains morphology because, in general, filamentous fungi have more distinctive morphologies than, for example, single-celled bacteria and yeast. However, the literature provides extensive examples of problems. Unreliable morphological minutia to describe new species and variability within the morphological characters of accepted species are constant issues. The use of physiological characters has also been attempted (e.g. colony colour, growth rates) although these also are variable in many cases. There has been the creation of subspecies taxa (e.g. special forms in plant pathogenic fusaria, or varieties within penicillia), which have practical advantages in some cases, although they tend to confuse taxonomies. The situation in the terverticillate penicillia is particularly problematic in these respects and has been discussed thoroughly by the present authors (Paterson *et al.* 2004) and others (e.g. Samson and Frisvad 2004). Nevertheless, these are a necessary base from which to commence other work. The development of PCR has led to phylogenetic assessments of fungi (Hibbett *et al.* 2007), which in turn led to the discovery of cryptic species within accepted morphological species (Hawksworth 2006b) adding to the limitations associated with shape and physiological-based characters. Also, 'bar coding' to identify fungi is being considered actively (Seifert *et al.* 2007), in part because of problems with existing characters. Some of the above constraints have led to increasing interest in matrix assisted laser desorption/ionization time-of-flight intact cell mass spectrometry (MALDI-TOF ICMS) for rapid and reliable identifications (Chen and Chen 2005). The remarkable reproducibility of the methodology is based on the measurement of constantly expressed and highly abundant proteins, such as ribosomal molecules. The observable molecular mass range is between 2000 and 20 000 Da, where very few metabolites appear (Ryzhov and Fenselau 2001), which is an advantage because they can be easily used as biomarkers.

A review of the analysis of micro-organisms by MALDI-TOF is available (Fenselau and Demirev 2001) although the section on filamentous fungi is too brief to be of relevance. This present review will focus on the applications of MALDI mass spectrometry (MS) to obtain biomarker profiles directly from unfractionated filamentous fungal hyphal cells, and/or conidia.

MALDI-TOF MS/ICMS

MS has been available for *c*. 50 years for the analyses of the molecular masses of compounds. However, the application to large biomolecules has been limited because of inherent low volatility and thermal instability. Such problems have been overcome by the development of the soft ionization technology of MALDI and other mass techniques, such as Electrospray Ionization Mass Spectroscopy and Fast-Atom Bombardment. These emerged in the late 1980s as techniques for the ionization of large proteins (Tanaka *et al.* 1988). In addition, they can be applied to compounds with a wide range of molecular masses. For example, MALDI-TOF ICMS was applied to identify toxic and nontoxic cyanobacteria by analysing low molecular mass secondary metabolites (Erhard *et al.* 1997). The 2002 Nobel Prize for chemistry was awarded to Koichi Tanaka for the use of MALDI with biological macromolecules.

MALDI-TOF MS involves subjecting a sample covered with an UV-absorbing matrix that functions as an energy mediator, to a pulsed nitrogen laser. The matrix transfers the absorbed photoenergy from the irradiation source to the surrounding sample molecules, resulting in minimum fragmentation (Su and Tseng 2007). The function of the matrices containing aromatic moieties is to dilute the sample and to absorb the laser energy thus providing a 'soft' ionization of the sample (Marvin *et al.* 2003). Several matrices are available, and choosing the appropriate one for micro-organism identification is crucial. The final composition of the matrices is constituted by benzoic or cinnamic acid derivatives dissolved in an organic solvent and water. Alternatively, trifluoroacetic acid can be employed. The use of an inappropriate matrix will decrease the soft ionization leading to an excessively strong fragmentation of the analysed proteins (Hillenkamp and Peter-Katalinić 2007). The goal is to obtain an optimal signal/noise ratio with the narrowest peaks and little signal suppression by the use of the appropriate

matrix. In addition, online programs are available to allow detection of matrix cluster masses, which might interfere with those of the samples (Keller *et al.* 2008). A discussion of the protocol involved in the analysis is presented in the following paragraph.

Samples are (i) spotted onto a sample plate and (ii) covered with the matrix solution into which are embedded the target biomolecules or whole cells. These are subjected to vacuum and pulsed nitrogen laser energy in the wavelength region of 337 nm. Energy is absorbed upon the application of a laser and the matrix and analyte molecules are transformed from the solid to the gas phase hence forming a plume. The ion yield of the matrix (i.e., the ratio of ions to neutrals) is in the range of 10^{-5}–10^{-3}. The ion yield of the analyte can be much higher, in the order of 0·1–1% for typical and above 10% for exceptional cases (Hillenkamp and Peter-Katalinić 2007). The charged matrix molecules and/or clusters transfer protons onto the sample molecules (e.g. peptide or proteins) in the expanding plume. The generated ions are accelerated into the TOF analyzer, in which ions are separated according to their 'time-of-flight' which is a function of molecular mass to charge (Keys *et al.* 2004). The TOF analyser determines the molecular mass to charge (*m/z*) ratio of ions by measuring velocities from accelerating ions to defined kinetic energies after calibration of the instrument with molecules of known molecular mass. The linear mode covers the molecular masses ranging from <100 Da to more than 1000 kDa; however, the reflectron facility offers higher resolution for smaller molecules between 0 and 100 Da, although it can resolve up to 5000 Da. The detection of positive and negative ions can be achieved, although the former is optimal for the identification of micro-organisms. Obviously, the measurement of the flight time of the ions is the basis of TOF, which is based on the 'start', which is the initial laser pulse signal, and the 'stop', which is the arrival of the ions at the detector. The data generated in the detector are processed and reported by digitization of the analogue signal or by single pulse counting. The final spectrum is governed by a basic eqn (1) which results from the equality of kinetic and electrostatic energy:

$$mv^2/2 = zeU \qquad (1)$$

where, *m*, molecular mass; *z*, charge state; *e*, elementary charge; *v*, ion velocity; *U*, acceleration voltage.

However, MALDI-TOF ICMS for the identification and classification of micro-organisms needs dedicated software [e.g. BioTyper™ (Bruker Daltonics Inc., Bremen, Germany) or saramis® (AnagnosTec GmbH, Potsdam-Golm, Germany)] to enable comparisons of the unknown protein with reference molecular masses. Ribosomal proteins are used normally as reference molecular masses as they are

the most abundant in the cells. The seminal paper by Ryzhov and Fenselau (2001) characterized the *Escherichia coli* K-12 proteins that involved the establishment of a protein database. At the hearts of BioTyper™ or saramis® are growing databases of mass spectral data from a broad variety of microbial isolates. In addition, maldi software uses (i) a variety of specific and confidential advanced algorithms and (ii) clustering and phylogenetic dendrogram constructions to enable the identification of micro-organisms. Keys *et al.* (2004) developed a compilation of a MALDI-TOF mass spectral database for the rapid screening, and characterization of bacteria, and data processing was performed with MicrobeLynx™ (Waters Corp., Milford, MA). Individual spectral profiles were molecular mass corrected by a factor derived from alignment of the specific and known protein peaks obtained empirically, with specific and exact molecular masses. A composite spectrum results from a combination of valid replicate spectra. We are now in a position to assess the use of MALDI TOF with filamentous fungi.

Fungal characterization

General considerations

Fungal cells are larger than, for example, bacterial cells and have rigid walls. Generally, the walls are 80–90% polysaccharide and include the presence of chitin adding rigidity and structural support. Proteins, lipids and polyphosphates together with inorganic ions make up the cell wall cementing matrix (Welham *et al.* 2000). Proteins provide the most characteristic biomarkers available for the analysis of intact organisms and do not require extraction, separation or amplification. MALDI-TOF ICMS has been used to desorb protein biomarkers from intact fungi (Valentine *et al.* 2002; Schmidt and Kallow 2005).

A lysis step (e.g. exposure of the fungus to a strong organic acid on the sample holder) was required to obtain a spectrum in the first MALDI ICMS analyses, although is avoided in some recent works. Also, the choice of matrices is extremely important. For example, Sulc *et al.* (2008) investigated four MALDI matrices to assess utility for the analyses of *A. fumigatus* conidia. All matrices provided rich ion profiles excluding dihydroxybenzoic acid (DHB). The optimal signal/noise ratio and the narrowest peaks, with little signal suppression, were obtained with the α-cyano-4-hydroxy-cinnamic acid matrix. In contrast, other authors used DHB [2-(4-hydroxyphenylazo)] benzoic acid or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid) matrices for typing *Penicillium* spp., *Scytalidium dimidiatum* and *Trychophyton rubrum* conidia in the 2000–13 000 Da molecular mass range (Welham *et al.* 2000). Finally, many of the

peaks in the MALDI spectrum of *Saccharomyces cerevisiae* correlated with the molecular masses of the proteins predicted from its genome (Amiri-Eliasi and Fenselau 2001) indicating the potential of the technique to proteomic analysis of fungi.

Early studies on fungi

All fungi analysed by Welham *et al.* (2000) demonstrated distinctive spectra that provided profiles of the cellular material. However, the work is preliminary in nature when one considers the requirements for repeated analyses of (i) numerous strains of the same taxon and (ii) similar species within groups as undertaken in more complete taxonomic studies (see Paterson 1998; Samson and Frisvad 2004). The high percentage of carbohydrate in the fungal cell wall indicates that the ions observed in the mass spectrometric experiments may be of carbohydrate origin (Welham *et al.* 2000) rather than protein. Discrete peaks were observed over the molecular mass range 2000–13 000 Da, and the spectra obtained were reproducible. Nine matrices were examined in a comparative evaluation within the *Penicillium* spp. Simple and consistent spectra were obtained over the molecular mass range 2000–8000 Da. Furthermore, *S. dimidiatum* and *T. rubrum* were compared with the spectra obtained from the *Penicillium* spp. Equally simple and distinctive spectra were obtained in the molecular mass range 2000–8000 Da. All of the spectra exhibited a group of peaks in the range 3000–3500 Da, although *Penicillium* spp. and *S. dimidiatum* revealed additional groups at m/z 5156 and 7882 Da, respectively. *Penicillium* spp. gave an additional small group of peaks at m/z 12 236 Da. The simplicity of the spectra will facilitate a more rapid identification of the cell wall components giving rise to the observed ions. However, an alternative approach employing negative ion MALDI-MS was also being investigated given the relatively high polysaccharide composition within the cell wall. Finally, the authors stated that comparisons with previous analyses of micro-organisms were being made for the purposes of biomarker selection and pattern recognition.

Predominately terverticillate penicillia

The conidia of *Penicillium expansum*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Penicillium digitatum*, *Penicillium italicum* and *Penicillium pinophilum* were analysed using MALDI-TOF ICMS by Chen and Chen (2005). Different strains within some of the species were also compared to determine variability. The first five species of the above list are from the terverticillate penicillia, and the fungi are isolated frequently from grain and fruit. In

addition, *P. expansum* and *P. pinophilum* are common in apples, and *P. expansum* is associated with production of the patulin, a mycotoxin detected in fruits and vegetables (Paterson 2004). The identification of all these fungi by conventional methods is notoriously difficult (see Paterson *et al.* 2004; Samson and Frisvad 2004). The mass spectra were from intact conidia mixed with the MALDI matrix. Characteristic ions representing the different species were obtained with sufficiently high reproducibility that they could be employed to identify the different fungal species, as the authors claimed. The effect of the age of the conidia was determined, and the ions at m/z 2662, 2880, 3267, 4823, 5676 and 7242 Da were consistent. Furthermore, several strains of *P. chrysogenum* were analysed. The ion peaks at m/z 3140 and 5173 Da appeared in each mass spectrum with good reproducibility, which indicates that these two ions can be used as biomarker ions for *P. chrysogenum*. Furthermore, the mass spectral profiles were similar for different strains within the same species, while the biomarker ions for different *Penicillium* species were different, one from another. The variation observed may have arisen from the different compositions of the cell walls. The majority of the biomarker ions are below 5000 Da, and the approach provided a rapid method to characterize the fungal species (Chen and Chen 2005). However, there were considerable differences between strains when the spectra are examined carefully. No doubt computer analysis and numerical analysis will assist in determining the value of the spectra more objectively, because all valid raw data are taken into consideration to perform the analysis. It is noted that perhaps the most important species, *P. expansum* was represented by only one strain although it was tested under a variety of conditions. *Penicillium pinophilum* had different spectra compared to the others, which is reassuring as this fungus is not terverticillate. Furthermore, the spectrum resembling most closely the spectrum for the *Penicillium* sp. discussed in Welham *et al.* (2000) is *P. chrysogenum* from data in Chen and Chen (2005) based on the peaks at *c.* 3140 and 5173 Da, although more work is required to establish this definitively. It will be necessary to test the other terverticillate penicillia which now consist of over 50 species, some of which are very similar (Samson and Frisvad 2004). These results suggest that conidia removed directly from the surface of rotted fruit could be rapidly identified to species. However, this appears premature given how similar some of the species are within these fungi and the need for sufficient repeat analysis of strains of the same species. Contamination may also represent a problem in this type of application (Keller *et al.* 2008).

MALDI-TOF MS was used to generate highly reproducible mass spectra for *Penicillium aurantiogriseum*, *Penicillium brevicompactum*, *P. citrinum*, *P. chrysogenum*,

P. expansum, *Penicillium fellutanum*, *Penicillium jensenii*, *Penicillium melinii*, *Penicillium purpurogenum*, *Penicillium roqueforti*, *Penicillium simplicissimum* and *Penicillium variabile* (Hettick *et al.* 2008a). Eight replicate cultures of each were given three 1-min bead beating cycles in solvent (Hettick *et al.* 2008a) and so were applied with pretreatment which is a disadvantage. The mass spectra contained abundant peaks in the range *m/z* 5000–20 000 Da and allowed unambiguous discrimination between species. A biomarker common to all *Penicillium* mass spectra was observed at *m/z* 13 900 Da. It is interesting to compare these peaks with those of Chen and Chen (2005) where peaks were distributed evenly between *m/z* 2600–7378 Da, and pretreatment was not employed, although adequate discrimination was obtained. In Hettick *et al.* (2008a), 100% correct identifications were observed, indicating that MALDI-TOF MS data are a useful diagnostic tool for the objective identification of *Penicillium* species. However, it should be noted that variations in morphological and biochemical characteristics within *Penicillium* species and the lack of consensus on *Penicillium* taxonomy may limit accuracy. It was noticeable that ex-type strains were not included in the study, which are generally required for control purposes (Hettick *et al.* 2008a). In addition, Hettick *et al.* (2008a) mentioned that the Chen and Chen (2005) MALDI-TOF mass spectra of *P. citrinum*, *P. chrysogenum* and *P. expansum* differed significantly from their own. The fingerprint mass spectra of Chen and Chen (2005) are dominated by peaks at *c. m/z* 3000 Da, with ions of significantly reduced intensity in the range *m/z* 5000–8000 Da. Hettick *et al.*'s (2008a) spectra are characterized by a larger number of peaks in the range *m/z* 10 000–20 000 Da. The differences in the appearance of the fingerprint mass spectra between the two laboratories are attributable to several factors (e.g. instrumentation, culture conditions, sample preparation and MALDI matrix). These differences underscore the importance of standardized methodology for MALDI-TOF MS and MALDI-TOF ICMS. Indeed, given the wide variety of commercially available mass spectrometers for use in laboratories worldwide, a standard reference library of biological mass spectra needs to be implemented based on inter-laboratories tests.

Aspergillus

Chen and Chen (2005) claimed that the MALDI-TOF ICMS profiles for aflatoxigenic and non-aflatoxigenic *Aspergillus* conidia were different, as reported by Li *et al.* (2000). However, Li *et al.* (2000) reported certain discrepancies. This group of fungi is extremely important from the toxicological perspectives and for food manufacture (Rodrigues *et al.* 2007, 2009). It is necessary to separate

toxigenic strains from those used to produce soya sauce. Intact conidia from several strains of *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus parasiticus* and *Aspergillus sojae*, were analysed. The spectrum of *A. oryzae* ATCC 22788 was different from those of other three *A. oryzae* as it produced a single peak at 6106 Da which was not apparent from the others. Strain ATCC 11491 was also different. Three of, what were referred to as, non-aflatoxigenic strains of *A. flavus* were analysed, and they also appeared different from each other. Also, the spectra for aflatoxigenic strains did in fact appear similar to each other, although they also were similar to the non-aflatoxigenic strain ATCC 10124. The authors state that the peak at *m/z* 7292 Da is present in each strain of *Aspergillus* and could provide the unique distinguishing peak of *Aspergillus* species. However, strain ATCC 13697 did not have this peak, and only one of the spectra from *A. parasiticus* ATCC 26865 contained the peak in the supernatant wash samples. Obviously, it is important to interpret carefully the spectra if MALDI-TOF ICMS is to become accepted as a useful technique. Computer analysis may help to resolve some of these misinterpretations, and a numerical analysis approach would be advantageous, as described previously. Nevertheless, the authors concluded that the MALDI results demonstrated aflatoxigenic strains and non-aflatoxigenic strains have different mass peak profiles. Furthermore, the MALDI results of non-aflatoxigenic *A. flavus* and *A. parasiticus* conidia were stated to resemble those of the closely related *A. oryzae* and *A. sojae* spores, respectively (Li *et al.* 2000).

Highly reproducible mass spectral fingerprints for 12 species of *Aspergillus* and 5 strains of *A. flavus* were obtained by Hettick *et al.* (2008b). However, it was pointed out that the important species *Aspergillus niger* was not resolved from *Aspergillus chevalieri*. Prior to MALDI-TOF ICMS analysis, the fungi were subjected to three 1-min bead beating cycles in a solvent and so were pretreated. The mass spectra contain abundant peaks in the range 5000–20 000 Da. These data indicate that MALDI-TOF ICMS data may be used for unambiguous identification of members of the genus *Aspergillus* at the species and strain levels. In the case of large datasets, a subset of the 'most significant' peaks allows 100% identification accuracy; however, all of the peaks in the dataset must be included to identify highly similar fingerprint mass spectra such as those from different strains of the same species. The authors conclude that to identify correctly unknown species and strains, a comprehensive database of the 180 species of *Aspergillus* will be required (Hettick *et al.* 2008b).

Mass spectra of intact fungal conidia provided signals predominantly below 20 000 Da (Sulc *et al.* 2008). This approach was used successfully for distinguishing

A. flavus, *A. parasiticus*, *A. oryzae* and *A. fumigatus* species from each other. The set of identified protein molecules present on fungal conidia can be used potentially in human/veterinary medical mycology for early diagnostic purposes, although a general and specific marker from all *Aspergillus* isolates of a clinical origin was not found. Furthermore, there was considerable variation in the spectra for strains of the same species in some cases. The reasons for the consistency in Hettick *et al.* (2008b) and yet variation in Sulc *et al.* (2008) require careful consideration. Sulc *et al.* (2008) tested several protein extraction protocols to determine the limit of conidia detection in MALDI-TOF ICMS analysis. *Aspergillus fumigatus* conidia (0·1 mg) were used, and MALDI-TOF ICMS was much more sensitive than 1D PAGE.

Black aspergilli

Species of *Aspergillus* section *Nigri* have been used extensively for biotechnological purposes such as citric acid production. Recent studies using RFLP after an ITS rDNA PCR amplification demonstrated that some *A. niger* and *Aspergillus tubingensis* could be distinguished readily, although these are difficult employing conventional methods. The ITS rDNA region gave 519- and 76 -bp fragments for *A. niger* (pattern N) after digestion with *Rsa*I and a single fragment of 595 bp for *A. tubingensis* (pattern T) (Accensi *et al.* 1999). Kallow *et al.* (2006a) used MALDI-TOF ICMS to study these two taxa. A dendrogram computed for mass spectra in the range from 5000 to 20 000 Da showed three distinct clusters for the strains studied. *Aspergillus flavus* formed a clear out group as expected because it is related loosely. The *A. niger* pattern T strain clustered with *A. tubingensis*. In contrast, *A. niger* pattern N strain was clearly related and aggregated with the *A. niger* type strain. Finally, MALDI-TOF ICMS analysis discriminated between these two taxa, which was feasible otherwise only by the nucleic acid techniques. An example of the 'problem' of cryptic species is the differentiation between *A. niger* and *A. tubingensis*, which only can be undertaken using molecular biology (i.e. RFLP) (Accensi *et al.* 1999) and/or MALDI-TOF ICMS techniques (Kallow *et al.* 2006a,b; Santos *et al.* 2008).

Fusarium

MALDI-TOF demonstrated the value of the technique when it was compared to ITS rDNA sequencing, in a situation where an initial diagnosis of aspergillosis was changed to a rare case of *Fusarium* infection (Seyfarth *et al.* 2008). The mass spectral technique produced corroboratory evidence that *Fusarium proliferatum* caused the disease, the identification of which was based on the

fundamental procedures of isolation and morphological identification. The authors stressed that the worth of MALDI is dependant on adequate spectral libraries from other relevant organisms. Recently, Marinach-Patrice *et al.* (2009) submitted 62 clinical *Fusarium* isolates to both MALDI-TOF analysis and partial *TEF*1 gene sequencing for species identification. In cases of disagreement between morphological and molecular identification, these authors were able to, in five of six cases, find an identical identification between molecular identification and MS. Another important achievement of this study is that fusaria produce more distinguishable peaks when grown on malt agar and PDA medium than on SCG medium. However, with the exception of *Fusarium verticilloides*, the observed differences did not impede identification. Finally, the authors pointed out that in 1 h, including the extraction step, they were able to identify the strains using MALDI-TOF in contrast to at least 1 week to subculture the strains on PDA medium for morphological analysis and 2 days for molecular identification.

Trichoderma (Hypocreales, Ascomycota)

Peptaibols

Peptaibols are linear, α-aminoisobutyrate-containing peptides produced by certain ascomycetes, including the genus *Hypocrea/Trichoderma*. Neuhof *et al.* (2007b) investigated whether phylogenetic relationships within *Trichoderma* permitted a prediction of peptaibol production by particular taxa. Representative strains from a third (i.e. 28) of the known species of *Trichoderma* were investigated using MALDI-TOF ICMS. Peptaibols were detected in all strains, and some were found to produce five peptide families of different sizes. Comparison of the data with phylogenies derived from ITS rDNA and RNA polymerase subunit B (*rpb2*) gene sequences did not show a strict correlation with the types and sequences of the peptaibols produced. However, the production of some groups of peptaibols appeared to be found only in some clades or sections of the genus. The phylogenies obtained were not concordant with those of their producers *Hypocrea virens*, *Hypocrea jecorina* and *Hypocrea atroviridis* and suggest a complex history of peptaibol diversity. Finally, Degenkolb *et al.* (2008) described the *Trichoderma brevicompactum* clade in *Trichoderma/Hypocrea* by employing a polyphasic study that demonstrates the value of MALDI TOF in taxonomy *per se*, rather than only as a procedure to distinguish strains or investigate fungal biology. The clade includes *T. brevicompactum* and the new species *Trichoderma arundinaceum*, *Trichoderma turrialbense*, *Trichoderma protrudens* and *Hypocrea rodmanii*. Fungi in the clade produced harzianum A or trichodermin with the exception of *H. rodmanii*, although this may be a

feature of detection limits. All produced peptaibiotics, including alamethicins. Importantly, trichothecene-producing strains reported as *Trichoderma harzianum*, *Trichoderma viride* or *Hypocrea* sp. were (re)identified as *T. arundinaceum*.

Hydrophobins

Hydrophobins are small proteins that are common in filamentous fungi and are usually present on the outer surfaces of cell walls. They mediate interactions between the fungus and the environment, e.g. surface recognition during pathogenic interactions with plants, insects or other fungi and in symbiosis. The size of hydrophobins ranges from 75 to 400 amino acid residues. *Trichoderma* contains cosmopolitan soil-borne fungi with economic importance as biocontrol agents and producers of beneficial metabolites and enzymes. In addition, *Trichoderma* spp. have been reported to occur as endophytes, eliciting positive plant responses against potential pathogens. Hydrophobins are likely to play a role in this process. However, hydrophobins may also be involved in the mechanism of mycoparasitism and the colonization of decaying wood. Little is known about the occurrence and processing of the individual hydrophobins on the fungal surface. MALDI-TOF MS demonstrated that class II hydrophobins account for the main characteristic peaks of *Trichoderma*. The intact cell extraction procedure employing a solvent mixture of acetonitrile and methanol is suitable for dissolving these cell wall constituents (Neuhof *et al.* 2007c).

Wood rotting fungi

Certain fungi cause wood decay in buildings, and identification of such species is problematic. *Serpula lacrymans* is the notorious dry rot fungus. However, Schmidt and Kallow (2005) demonstrated that MALDI-TOF MS differentiated each of the following closely related indoor wood decay fungi: *S. lacrymans*, *Serpula himantioides*, *Coniophora puteana*, *Coniophora marmorata*, *Antrodia vaillantii* and *Antrodia sinuosa*. Incidentally, some authors referred to the *Ganoderma* rot of oil palm as a dry rot or dry stem rot, which is erroneous as it is a white rot fungus (Paterson 2007b), and a clear distinction requires to be maintained. The evaluation by Schmidt and Kallow (2005) provided a convincing dendrogram with respect to species separation, which may have utility for identifying unknown samples by comparison. However, the molecular mass data-derived dendrogram does not reflect the phylogenetic relationship among the six species. The authors claim that further experiments will attempt to identify fungi from naturally decayed samples using comparisons with reference spectra.

Sepedonium

Certain fungi of the genus *Sepedonium* are interesting as they infect fruiting bodies of Boletales. Twelve *Sepedonium* strains were analysed by MALDI-TOF ICMS using respective biomarkers with a focus on peptaibol production. The structures of two new peptaibols, chalciporin A and chalciporin B, were presented. The MALDI-TOF ICMS technique was applied for *in situ* peptaibol analysis of *Sepedonium* strains growing on Boletales, in particular *Sepedonium chrysospermum* infecting *Xerocomus* cf. *badius*. Chrysospermins were detected at the surface and within basidiomycete tissue, and in the cultivated parasite (Neuhof *et al.* 2007a). Finally, the biomarker patterns of the fingerprint spectra allowed discrimination between the species.

Dermatophytes

Recently, the most important clinical fungal dermatophyte species, *T. rubrum*, *Trychophyton interdigitale*, *Trychophyton tonsurans* and *Arthroderma benhamiae*, were identified by MALDI-TOF ICMS using the saramis® database and software package (Erhard *et al.* 2008). A high level of confidence (99·9%) was achieved for the studied fungi with only one *T. rubrum* strain yielding 80% confidence. Fungi originating from skin and nails were characterized morphologically, and by sequencing ITS rDNA regions of the rDNA clusters. In addition, samples were analysed by MALDI-TOF ICMS for 'blind' comparison. The mass spectra varied with the growth conditions; nevertheless, a sufficient number of biomarkers signals were observed, and a 'superspectrum' could be determined for each species. A superspectrum is a final spectrum containing only those molecular mass signals common to all strains analysed for each species. MALDI-TOF ICMS with appropriate databases can be used to identify dermatophyte species independently of culture conditions if reference strains are also employed. However, contamination by keratins from the patient could be an issue in this case (Keller *et al.* 2008).

The yeast⁄filamentous dichotomy

In an interesting paper, Qian *et al.* (2008) investigate MALDI-TOF mass signatures for differentiation of yeast species and the monitoring of morphogenesis markers for the single cells to hyphal transition in *Candida*. The process is useful for marking changes during that transition. However, there was some confusion concerning the filamentous nature of *Aspergillus* in the paper, and Yang Cai (Personal communications) stated that, 'the *Aspergillus* species (in our study) were not clearly referred as

filamentous fungi'. In addition, the author reiterated that, 'The important point of this paper was that alcohol fixation improves the quality of fungal mass signatures'.

Discussion

MALDI-TOF ICMS represents a growing technology although at an early stage of development with respect to its use with fungi. Previous studies on fungal identifications and characterization using MALDI-TOF ICMS have shown the potential of this new approach with good discrimination in most cases. Indeed, the technique has even been suggested for optimizing quality control of Chinese fungal medicines (e.g. *Cordyceps*) (Zhao *et al.* 2007). The development of algorithms to generate dendrograms is indispensable to differentiate the spectra and to undertake meta-analysis of the data. However, the reproducibility of spectra of complex biochemical mixtures such as occur with fungi is known to be problematic.

Validation of MALDI-TOF can be achieved using reference fungal strains to obtain corresponding spectra for databases. Furthermore, the data can be included with those from morphology, biochemistry and molecular biology (i.e. a polyphasic approach). For example, the ITS1-5.8S-ITS2 rDNA sequence regions have been used in phylogenetic studies and fungal identifications. These and similar data are required to be compared to and validate with MALDI-TOF ICMS; levels of confidence must be determined.

When nucleic acid methodology is employed with, *inter alia*, fungi, it is 'essential that users are aware of possible caveats that need to be considered when using them' (Hawksworth 2009b). The current authors are surprised that predictable potential problems with growth media have not been anticipated in protocols devised for growing fungi to produce representative DNA/RNA, especially when the other sophisticated resources required for phylogenetics and diagnostic investigations are considered. Internal amplification controls (IAC) must be used to account for, amongst other things, PCR inhibition (Paterson 2007a, 2008b). Gratifyingly, the use of IACs is beginning to increase (Paterson *et al.* 2008). 'Self'-produced mutagens in culture may change target DNA and compromising diagnostic and phylogenetic data. Similarly, the stabilization of DNA could be inhibited by secondary metabolites in culture leading to increased mutations. In general, the conclusion is that diagnostic and phylogenic protocols which employ nucleic acids must avoid mutagen and inhibitor production in culture (Paterson *et al.* 2008; Hawksworth 2009b; Paterson and Lima 2009).

A corollary to the issue of secondary metabolites affecting nucleic acid analysis is that media that support the production of mutagens and inhibitors may also affect

phenotypes such as morphology, proteins and indeed MALDI profiles; hence, the need to avoid such compounds is universal in fungal/microbial taxonomy, including when using MALDI-TOF analysis. Contamination requires to be avoided in PCR to stop false results, which is not of such significance in the other physical/chemical methodologies (Paterson 2006b, 2007a). Finally, the running costs and time for analyses can be lower for MALDI-TOF compared to PCR (e.g. Erhard *et al.* 2008).

MALDI-TOF ICMS has important advantages over PCR methods. The technology is rapid and straightforward to use, often requiring a simple scraping of an agar plate yielding a small amount of material to apply a sample. Care is required to avoid or take into account contaminants and interferences (Keller *et al.* 2008). In addition, the spectral signals of intact cells may be influenced by the phenotype of the fungus. These may include basidiospore, monokaryon, dikaryon, fruit body, surface mycelium, strands and substrate mycelium, etc. Vegetative mycelium grown on agar presents different zones that correspond to different ages or developmental stages. For examples, differences may be observed from (i) an apical growth zone with an extending hyphal tip and many organelles, (ii) an absorption zone involved in nutrient uptake, (iii) a storage zone with reserve materials, and (iv) a senescence zone with pigmentation and lysis. Thus, variation of the biomolecules in intact cells may influence the spectral reproducibility in repeated measurements of the same isolate. To overcome these putative problems, the present authors have employed active mycelia after 3-day growth in nutritive broth. Notwithstanding this, it may be advisable to study different fungal zones within a culture to determine these profiles. The current authors have discussed the issues of mutagen and inhibitor production earlier.

Finally, a novel bioinformatics-based approach has been suggested recently, which characterizes micro-organisms based on matching protein molecular masses in the spectrum with protein molecular masses predicted from sequenced genomes (Fenselau and Demirev 2001). This method utilizes the fact that the majority of observed biomarkers above m/z 4000 Da in MALDI spectra of intact organisms are proteins. However, its application is limited to those micro-organisms where the genomes have been sequenced. A similar approach by Amiri-Eliasi and Fenselau (2001) was mentioned previously.

Conclusions

MALDI-TOF ICMS requires to be investigated further for filamentous fungi. Much more is required to be known about reproducibility between strains of the same species.

Growth of fungi on media that permit secondary metabolite production needs to be avoided, so that mutagens and inhibitors are not produced; consequently, analysis immediately after isolation is recommended. A recommendation for testing repeatability in chemotaxonomy is provided in Paterson (1998) and could be applied to MALDI-TOF ICMS. The procedure benefits from numerical analysis of the data to avoid subjectivity and misinterpretations. More information is required on the structure of compounds being detected (e.g. are the spectra of fungi derived from proteins only?). A comprehensive study on the terverticillate penicillia would be beneficial to build on those already published using other techniques. Authentication of fungal herbal medicines is of increasing concern.

Of course, the equipment is expensive although not excessively so. Moreover, MALDI-TOF ICMS is (i) rapid (Dickinson *et al.* 2004), (ii) inexpensive in terms of labour and consumables and (iii) reliable when compared with other biological techniques. Recently, a breakthrough has been witnessed in that entire cells were subjected to MALDI-TOF ICMS analysis, resulting in patterns that were generated within minutes. These large samples might be an additional advantage in terms of reducing the significance of interferences and contaminants observed in small sample sizes, although adequate care remains relevant (see Keller *et al.* 2008). Finally, the potential of MALDI-TOF for the characterization of filamentous fungi is ready to be explored fully.

Acknowledgements

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