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Prospective study on MALDI-TOF technology for the identification and characterisation of phytopathogenic fungi

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Abstract

MALDI-TOF technology enables the identification of micro-organisms by comparative analysis of their protein spectrum to reference databases. The main objective of this study was to assess the potential of this technology for the identification of phytopathogenic fungi. Using the genus *Fusarium* as a model, we showed that MALDI-TOF analysis was a very efficient tool for identifying species, but also proved to be able to discriminate closely related *Fusarium* taxa, including species that cannot be distinguished morphologically or that only differ in their pathogenicity. However, the initial step of fungal protein extraction was shown to be of paramount importance, and the results suggest that ad hoc optimisation of this step is needed prior to the study of any fungal genus.

Keywords

- *Forma specialis*
- *Fusarium*
- Maldi-Tof
- Plant health
- Race
- Taxonomy

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Introduction

Fungal organisms (or fungi sensu lato) are uni- or multicellular eukaryotes within two kingdoms of the tree of life: Fungi (sensu stricto) and Protista. They encompass a very large variety of taxa, whose common feature is heterotrophy, and nutrition by absorption. Most of the hundred thousand species described are saprobes, playing the essential role of organic matter decomposer. On the other hand, a minority of the species act as symbiotic organisms (mycorrhizae), with mutual benefits between fungi and hosts, while more than ten thousand other species are parasites, causing diseases on plants [Agrios, 1997].

Plant diseases have a major impact both on the agricultural and forestry sectors and on the environment. It is estimated that 85% of these diseases are caused by fungi or fungal-like organisms, of which 30% are currently causing emerging diseases [Anderson *et al.*, 2004]. Control of these plant diseases requires correct and precise identification of the causal agent, in order to implement appropriate and relevant management options to suppress or at least mitigate the impact of the disease, or to adopt preventive strategies or prophylactic measures. Indeed, for a fungal genus, only a few species are true pathogens, while within a fungal species, sometimes only certain particular *formae speciales* are able to cause disease.

The identification of a fungal taxon relies on the combination of two complementary approaches: (i) observation of several morphological features of the fungus in pure culture, and (ii) analysis of the sequence of one or several regions of the genome that are considered as phylogenetic markers or 'barcodes'. Unfortunately, this dual approach does not always enable reliable assignation to a taxon name, when morphological features are not discriminant enough, or when the DNA polymorphism observed for the genetic barcode(s) is too weak between closely related taxa, such as for several species of the genus *Fusarium* [Summerell *et al.*, 2003]. MALDI-TOF (Matrix-assisted laser desorption/ionisation-time of-flight) is a technology based on mass spectrometry, analysing the protein and polypeptide composition of microorganisms to serve as a landmark for acute identification. With this technique, proteins or fractions thereof are first co-crystallised in a matrix then ionised by a laser beam (MALDI). The released ions are accelerated and separated in a vacuum tube under the action of an electric field based on their mass/charge ratio, and detected individually over time by a particle detector (TOF). The entire set of molecules will therefore be displayed as a series of peaks of different areas and heights, forming a spectrum.

The spectrum of ions is characteristic of the organism spotted on the analysis plate, and may be analysed for instance by comparison to a database of reference spectra, thus enabling identification. The proteins that are ionised and therefore used for analysis are generally ribosomal, but many other types may be released and considered for analysis (*e.g.* cell-wall proteins). MALDI-TOF technology is already widely used for the identification of pathogenic or toxigenic bacteria [Croxatto *et al.*, 2012], and serves as a routine typing tool for the ANSES Laboratory of Hydrology in Nancy. However, there have been limited reports of MALDI-TOF for the identification of fungi or fungal-like organisms to date, and most studies concern fungi of industrial or medical interest [Chalupova *et al.*, 2014]. Unlike the classical approach that combines morphological features and DNA barcode analysis and that requires several days of turnaround time, MALDI-TOF is able to generate a result within a few minutes, starting from a pure culture of the isolate to identify.

The main goal of this prospective study was to assess the potential of this promising technology for the identification of phytopathogenic fungi. First, the project focused on the optimisation of the fungal protein extraction and crystallisation steps. Second, we assessed the ability of MALDI-TOF to discriminate related taxa, from species to lower taxonomical ranks such as cryptic species, *forma specialis* or even race, in a reliable and reproducible way. Different fungal strains of the genus *Fusarium* were used as models in this study. The *Fusarium* genus comprises numerous plant pathogenic species, including cryptic species complexes

(morphologically similar and only distinguishable using a multilocus DNA sequence analysis), but also *formae speciales* or races that can only be assigned and identified based on their pathogenicity.

Materials and methods

■ Fungal cultures

All the *Fusarium* strains used in the course of this study originated from the reference culture collection of the ANSES Plant Health Laboratory. A total of 23 isolates representing 20 different species, including taxa belonging to the *Fusarium graminearum* species complex (FGSC), as well as three *F. oxysporum formae speciales* were included in the tests (Table 1). In order to assess the effect of the culture method, a panel of different *Fusarium* species was selected, and included species representing different taxonomic levels. In this respect, this panel comprised species that were highly related as well as species that were more distant from a phylogenetic point of view. The isolates of the panel were cultivated both in Petri dishes, on solid Potato Dextrose Agar (PDA, Difco), and in 5 mL of Potato Dextrose Broth (PDB, Difco) liquid medium, with constant shaking. The cultures were incubated at 22°C for 3 to 5 days, until sampling for protein extraction and MALDI-TOF analysis. Potato dextrose was the only medium tested in this study as it is widely used in mycology laboratories. For the rest of the experiments, the fungal strains were cultured according to the protocol optimised in this work, in order to provide the reference spectra after MALDI-TOF analysis.

■ Sampling of mycelium

In order to provide discriminant and reproducible MALDI-TOF spectra, the fungal protein extracts had to be as homogenous and reproducible as possible. In this respect, the effect of the technique used to collect the mixture of mycelium + asexual conidia (micro and macro-) from the fungal culture on solid agar was assessed by comparing 3 different protocols. The first strategy for sampling was adapted from Brun *et al.* [2013] and Mancini *et al.* [2013] and consisted in harvesting a 3-mm mycelial pellet by scrapping the surface of the solid culture using a sterile scalpel blade. A second technique consisted in collecting a 3-mm mycelial pellet at the centre of the culture, using a sterile needle, and carefully avoiding any scraping of agar medium (adapted from Normand *et al.* [2013]). The final method of sampling made use of a sterilised 5-mm core punch extractor. A 5-mm culture diameter disc was punched out of the fungal culture, and the agar layer underneath was removed. The mycelium disc was then cut into four equal portions, and a single one was retained for protein extraction. In addition, the effect of the age of the mycelium/conidia mixture was also investigated by sampling at different concentric circles in the culture: 0 days (active growing margin of the culture that can be sampled anytime), 3 and 5 days (based on reference circles traced every 24 h at the margin of the culture).

Each mycelium sample was individually transferred into a sterile 2-mL microtube containing 900 µL of absolute ethanol mixed with 300 µL of molecular grade water.

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TABLE 1 / List of the *Fusarium* isolates used for this study.

Species	Forma speciale	Code	Host / substrate
	-	652*#	*Triticum* sp.
F. graminearum	-	830*	*Triticum* sp.
	-	808*	*Triticum* sp.
	-	823*#	*Zea mays*
F. cortaderiae	-	824*	*Zea mays*
	-	839*	*Triticum* sp.
	-	708*	*Zea mays*
F. poae	-	861*	*Triticum* sp.
	-	878*	*Zea mays*
F. gerlachii#	-	831#	*Triticum* sp.
F. asiaticum#	-	832#	*Triticum* sp.
F. vorosii#	-	833#	*Triticum* sp.
F. acaciae-mearnsii	-	834#	*Triticum* sp.
F. aethiopicum	-	835#	*Triticum* sp.
F. mesoamericanum	-	837#	*Triticum* sp.
F. austroamericanum	-	838#	*Triticum* sp.
F. brasilicum	-	840#	*Triticum* sp.
F. meridionale	-	841#	*Triticum* sp.
F. louisianense	-	842#	*Triticum* sp.
F. ussurianum	-	843#	*Triticum* sp.
F. nepalense	-	844#	*Triticum* sp.
	-	1013	Market gardening soil
	-	1026	Banana plantation soil
F. oxysporum	*radicis cucumerinum*	1010	*Cucumis sativus*
	spinaciae	1014	*Spinacia oleracea*
	melonis	1015	*Cucumis melo*

*Isolate that was included in the test panel for protein extraction optimisation
#Cryptic species belonging to Fusarium graminearum sensu lato

■ Mycelium preprocessing

The mycelium samples were homogenised following different alternatives aimed at enhancing the release of fungal proteins, while preserving their integrity, and in order to provide reproducible data: (i) direct deposit of the mycelium with no preliminary processing [Abreu *et al.*, 2014; Marinach-Patrice *et al.*, 2009], (ii) 10 min sonication of the sample (Ultrasonic bath, Bioblock Scientific 88154), grinding for 30 s with either, (iii) 500 µL of 2-mm steel beads, (iv) two 3-mm stainless steel beads, or (v) a mixture of Grenat sand and a ceramic sphere (Lysing Matrix A, MP Biomedicals) in a multidimensional shaker set at 6.5 units (FastPrep24, MP Biomedicals) (adapted from Brun *et al.* [2013]).

■ Fungal protein extraction

After preprocessing, the microtube containing the mycelium was centrifuged for 10 min at 11 000 g. The supernatant was then carefully removed with a pipette, and the microtube was centrifuged again for 2 min at 11 000 g. The supernatant was discarded without disturbing the mycelium pellet and the microtube was kept open under a sterile flow hood until total evaporation of the liquid. The pellet was resuspended in 10 µL of 99% formic acid (Biosolve

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0614143), and the mixture was manually homogenised using a sterile pipette tip. After 5 min of incubation at room temperature, 10 µL of acetonitrile (Biosolve 01204101) were added and the sample was again homogenised with a sterile pipette tip. The mixture was mixed by 10 s vortex and centrifuged for 2 min at 11 000 g. The supernatant was then directly used as a template for MALDI-TOF and spotted on the analysis plate.

■ Spotting of fungal proteins

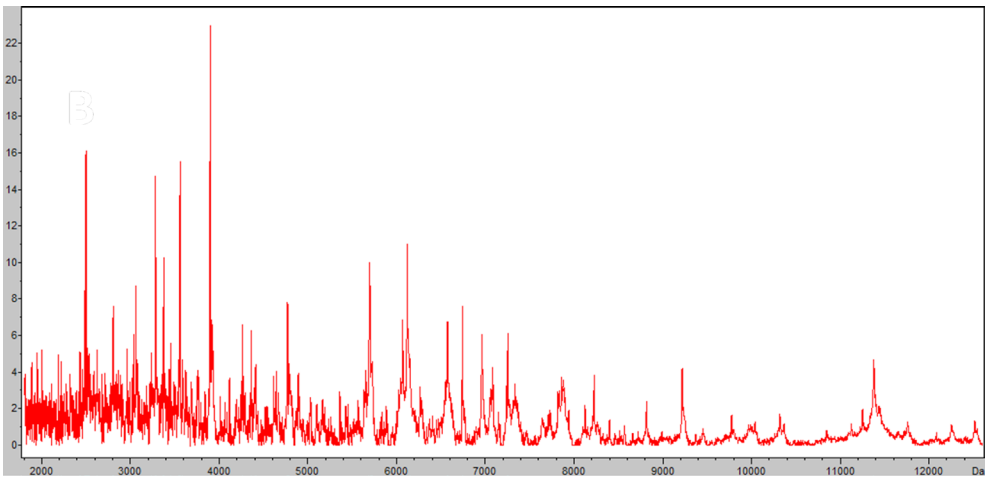
After formic acid /acetonitrile extraction, one microlitre of the supernatant was deposited onto a 96-spot MALDI-TOF plate (MSP 96 target polished steel BC, Brucker). The sample was left to dry on the bench at room temperature for 5 min, then one microlitre of matrix was added directly onto the sample, in order to activate crystallisation of the proteins. Two different types of co-crystallisation matrix were assessed and prepared according to the supplier's recommendations: alpha-cyano-4-hydroxycinnamic acid (HCCA, Brucker 8290200) and sinapinic acid (SA, Sigma-Aldrich 85429). HCCA and SA are the matrixes most frequently reported in the literature for environmental or medical fungal samples [Chalupova *et al.*, 2014].

■ Generation of MALDI-TOF reference spectra

Analyses were carried out using a Microflex MALDI-TOF spectrometer (Brucker), with default standard settings. For each fungal strain, a reference spectrum was obtained based on the mean of 24 spectra generated by 6 repetitions (*i.e.* 6 spots on the plate) for 4 independent fungal protein extractions. Each spectrum was analysed using two different software programmes. On the one hand, MALDI-Biotyper 3 (Brucker) enabled the calculation of the reference spectrum (Figure 1), and used the 70 major peaks of the spectrum for identification of the taxon by comparison with a spectrum reference database. On the other hand, two to twelve different reference spectra could be simultaneously compared, peak-by-peak by ClinProTools software (Brucker). Differences between spectra were identified based on the presence of discriminant or 'specific' peaks (present in certain strains, absent in others) and were statistically compared using principal components analysis (PCA) implemented in ClinProTools.

Results

FIGURE 1 / MALDI-TOF reference spectrum generated with *Fusarium graminearum sensu stricto* (strain LSV 652) following the preparation of fungal proteins optimised in this work.



The X-axis corresponds to the mass-to-charge ratio in Dalton, and the Y-axis to the signal intensity of the ions.

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■ Optimisation of the fungal protein extraction step

The optimal fungal protein extraction protocol was finally selected based on its ability to generate reproducible and discriminant spectra, across all the fungal strains tested. In other words, the spectra should be identical between replicate analyses and display numerous peaks, thus increasing the probability of generating discriminant peaks between different fungal taxa.

Table 2 lists the options that were finally retained for each parameter, and used for the generation of the reference spectra for all the collection fungal strains studied in this work.

TABLE 2 / Evaluation and selection of the different options during the fungal protein extraction step, prior to MALDI-TOF analysis.

Parameter	Condition
Matrix	**HCCA**
	SA
Growing condition	**Culture on solid agar**
	Liquid culture
Age of the fungal culture	**0 days**
	3 days
	5 days
Type of grinding	**None**
	Glass beads
	Stainless steel beads
	Lysing Matrix A
	Sonication
Technique for mycelium collection	**Scraping with needle**
	Scraping with scalpel blade
	Core punch extractor

The options finally retained for the preparation of reference spectra are indicated in bold.

Cultivation on solid medium was preferred based on its ability to yield more reproducible spectra and because this medium was less prone to bacterial contamination. In terms of number of peaks, no significant difference was observed between the different incubation periods before sampling. As a consequence, sampling at Day 0, or in other words, sampling mycelium samples at the growing edge of the culture, was retained, as it enabled sampling at any time during incubation of a culture. Sampling mycelium material using a sterile needle was finally chosen amongst the other harvesting procedures, despite the lower quantity of mycelium collected. Although the protocols using scalpel blade or core punch extractor enabled collection of mycelium in a standardised way, the unavoidable recovery of agar medium made them less appropriate since this caused interference with MALDI-TOF analysis and significantly reduced the number of peaks per spectrum. Amongst the different options for grinding the mycelium samples, the use of glass beads was the most efficient since it was the only way to obtain spectra with a high level of well-resolved peaks. In fact, high degradation of the fungal proteins was observed for grinding with steel beads or Lysing Matrix A, or an insufficient quantity of proteins was obtained using sonication. Nevertheless, the fungal proteins extracted without any grinding after the collection of the mycelium using a sterile needle also yielded highly reproducible spectra with numerous peaks, and this option was finally retained due to its simplicity.

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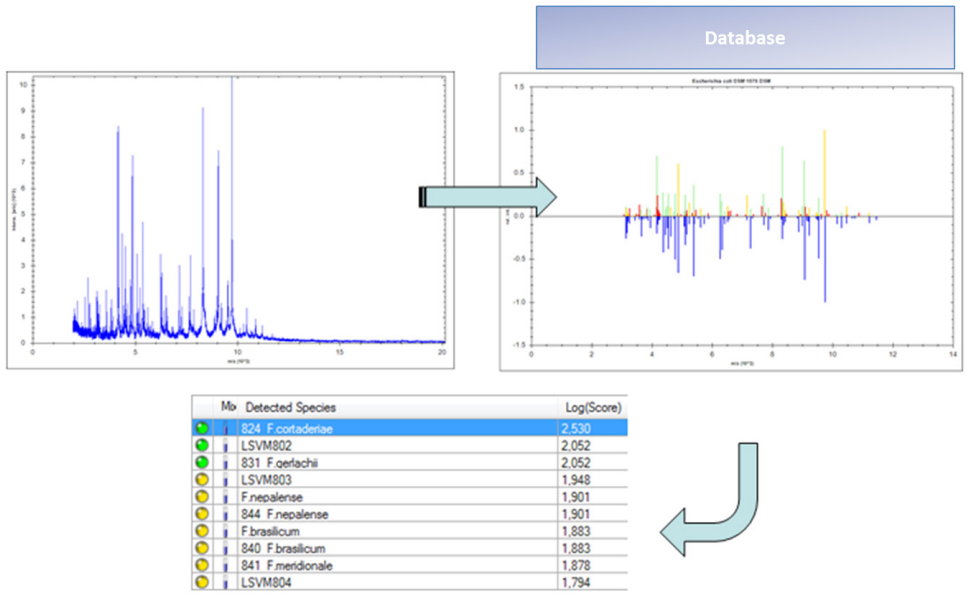
FIGURE 2 / Basic principle used for taxon identification using MALDI BIOTYPER 3 software.



The average spectrum is compared to the reference spectra database, and a homology search is conducted.

■ Identification of *Fusarium* at the species level

Overall, the majority of the ions detected in the *Fusarium* protein extracts ranged from 2 000 to 10 000 Da. Optimisation of the fungal protein extraction protocol yielded reference spectra with sufficient peak variety to discriminate the 16 different *Fusarium* species used in this study. Using MALDI-Biotyper 3 software for the comparison of the spectra to the in-house reference database produced in the framework of this study, it was always possible to assign the sample to a species, with a final log-score > 2.4 (Figure 2).

Importantly, a log-score of 1.7 is considered to be the reliability cut-off value recommended by Bruker Daltonics. In other words, the MALDI-TOF analysis of a taxon already present in the in-house database could assign a species name with a high level of certainty.

■ Identification of cryptic species of the *F. graminearum* species complex

To date, *Fusarium graminearum* sensu lato comprises 15 cryptic species, which cannot be distinguished by morphology in culture. Using the optimised protocol for sampling and extracting the fungal protein, a reference spectrum could be produced for 14 out of the 15 cryptic species. Unfortunately, in spite of repeated attempts, it was not possible to include *Fusarium boothii* in the study, since no spectrum could be generated for this species. The 14 reference spectra obtained could be analysed and compared using ClinProTools software. However, due to software limitations, it was necessary to compare them by groups of maximum seven spectra. The presence and absence of peak per spectrum for each sample were automatically assessed and the dataset was analysed by PCA. Clusters of peaks were observed on the graphic representation following principal component analyses, and were actually representative of “private” peaks observed in individual cryptic species (Figure 3). As each dot on the graph represented a peak, with associated coordinates, it was possible in return to select the discriminant peaks directly on the spectrum using ClinProTools.

Direct comparison of the different spectra confirmed that the private peaks were indeed present for some of the cryptic species, whereas they were absent in others, thus enabling discri-

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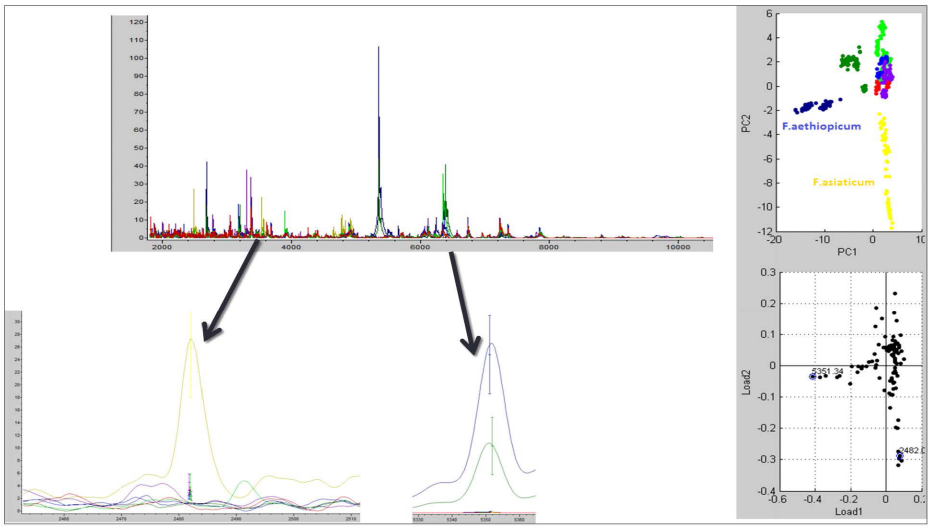
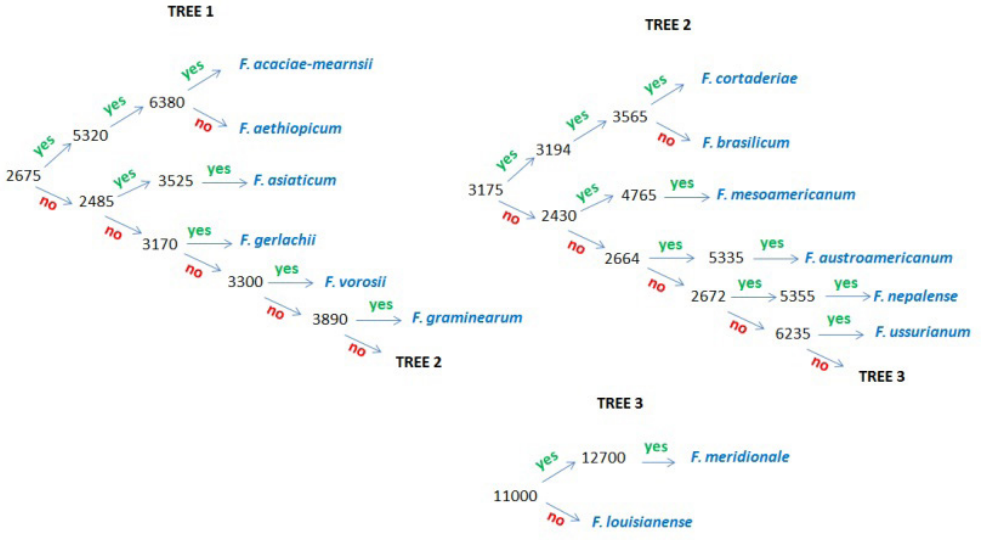
FIGURE 3 / Comparative analysis of MALDI-TOF spectra generated with several cryptic species of the *Fusarium graminearum* species complex, and associated principal components analysis enabling identification of potentially species-specific peaks.



mination of cryptic species. By combining the presence/absence of the different discriminant peaks that were identified, it was possible to define a pattern for each of the 14 cryptic species analysed, following a flow diagram, and to distinguish all species based on their spectrum (Figure 4).

■ Identification of *Fusarium oxysporum formae speciales*

FIGURE 4 / Flow diagram for the identification of *Fusarium graminearum sensu lato* cryptic species, based on the unique combinations of presence/absence of discriminant peaks in the MALDI-TOF spectra. The unique peaks were picked out by parallel analysis of the spectra using ClinProTools.



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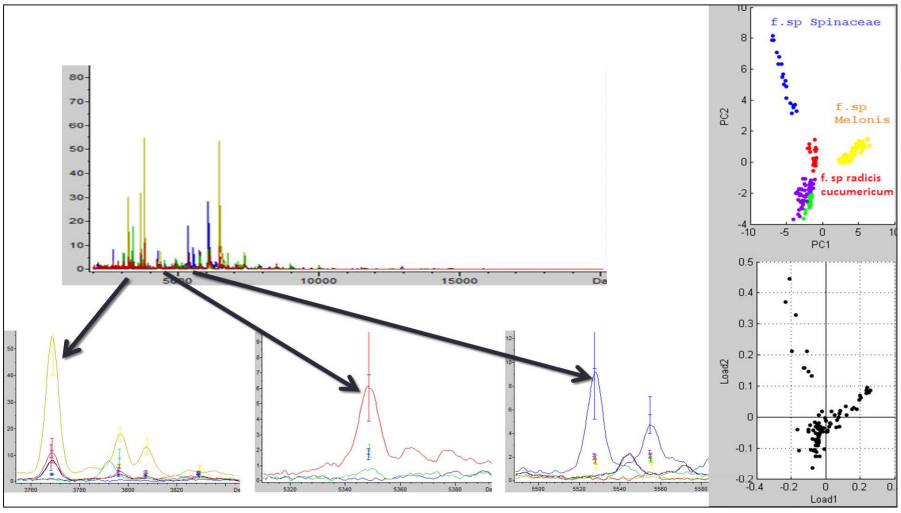
In the framework of this preliminary study, we limited the panel of *Fusarium oxysporum formae speciales* to three taxa (f.sp. *melonis*, f.sp. *radicis cucumerinum*, and f.sp *spinaciae*, pathogenic on melon, cucumber and spinach, respectively) and following the same approach described for cryptic species discrimination, it was possible to differentiate the *formae speciales*. We identified peak combinations whose presence or absence enabled us to distinguish the taxa (Figure 5).

FIGURE 5. Comparative analysis of MALDI-TOF spectra generated with three *Fusarium oxysporum formae speciales*, and associated principal components analysis enabling identification of discriminant peaks for f. *sp. melonis*, *radicis-cucumerinum* and *spinaciae*, respectively.



Discussion

This prospective study showed that after a preliminary optimisation step, MALDI-TOF was a powerful technique to identify and distinguish *Fusarium* species, and also *Fusarium* taxa at lower taxonomic levels such as cryptic species and *formae speciales*. MALDI-TOF mass spectrometry is a technology that is more and more widely used in bacteriology, and has proved to be particularly useful and accurate for identification and typing of strains [Bizzini and Greub, 2010]. The reliability and the robustness of the results depend entirely on the exhaustiveness of the database of reference spectra which is used by the associated analysis software. Several databases are available and can be purchased directly from the manufacturer. However, several suppliers of MALDI-TOF equipment are present on the market, and unfortunately, the databases are not transferable from one brand to another. Another issue is that the databases currently available are somewhat specific to medical or environmental applications. To date, Bruker Daltonics proposes two different sets of data to be used with the Micro Flex equipment, one for bacterial identification (Bruker Taxonomy/Bacteria) and another for eukaryotic organisms, including several genera of fungi (Bruker Taxonomy/Eukaryota). Unfortunately, the second one only contains a very limited set of phytopathogenic fungi, which can be easily explained by the fact that up to now the use of MALDI-TOF technology for plant pathology was scarcely reported in the scientific literature. This meant that in our case, the reference database had to be produced in-house. Only a few protocols are described in the

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literature regarding fungal protein extraction for MALDI-TOF analysis, and even fewer report efforts for standardisation.

The preliminary results of this prospective study focused on plant pathogenic fungi first confirmed that the availability of a comprehensive dataset was mandatory for reliable identification. In order to discriminate the *Fusarium* species that were used as a model in this study, it was necessary to implement an in-house database containing reference spectra. As a direct consequence, it was first mandatory to work on optimisation and standardisation of the fungal protein extraction step, and to produce robust reference spectra with repeated measures. Preliminary attempts to use the protocol suggested by the manufacturer, *i.e.* using liquid culture cultivation and a simple ethanol extraction method, were unsuccessful in producing good quality and repeatable spectra with our set of fungi. The disruption of mycelium and the rough extraction of proteins appeared to be more difficult than with prokaryotes, probably because the cell walls are in general far more resistant. We also observed that numerous parameters might greatly influence the rate of recovery and the quality of the fungal proteins before ionisation. Some of the options that were tested in our context, such as the use of SA as a matrix or the use of an unsuitable grinding technique, did not enable us to recover any ionisable proteins at all for some strains tested, which may be explained by either a poor level of protein release in the solution, their destruction or fragmentation, or their recovery with inappropriate polarity.

Moreover, given the extraordinary richness of the taxa within the Fungi kingdom, it was necessary to set up a protocol that combined high reproducibility and the capacity to discriminate by generating spectra containing a massive quantity of information, to improve the odds of identifying discriminating peak combinations.

The protocol that was optimised here for the efficient ionisation of fungal proteins in the genus *Fusarium* was also evaluated with several other genera of fungi (Monilinia spp., Colletotrichum spp.) or oomycetes (Phytophthora spp.), which include numerous aggressive plant pathogens. Unfortunately, the spectra that were obtained were poorly reproducible (data not shown), which suggested that the recovery rate for fungal proteins varied considerably across genera, probably depending on the chemical composition of the fungal cell walls. Our results confirmed the hypothesis that for each type of microorganism, and even for each genus of fungi, preliminary work had to be done to optimise the steps leading to the extraction and purification of the set of proteins before ionisation and MALDI-TOF analysis [Chalupova *et al.*, 2014]. As a result, it is unfortunately unlikely that a common and standardised protocol could be developed and used across the fungal kingdom.

However, provided that an appropriate extraction protocol is available, our preliminary results with *Fusarium* showed that MALDI-TOF analysis was quite promising for the identification of taxa, even at very low taxonomical levels.

The initial results of Marinach-Patrice *et al.* [2009] have already demonstrated that MALDI-TOF analysis enabled the identification of several *Fusarium* species involved in human dermatitis, but these were genetically distant. The results obtained here highlight the potential of MALDI-TOF to discriminate even closely related taxa, such as cryptic species for the genus *Fusarium* and are in line with the recent conclusions of Al-Hatmi *et al.* [2016] regarding the *F. fujikuroi* species complex. These cryptic species can only be identified and distinguished based on sequence analysis of several DNA markers, which requires several days of turnaround time. Likewise, through statistical comparison of the spectra, specific combinations of presence/absence of exclusive peaks made it possible to distinguish among several *formae speciales* within *F. oxysporum*. *Forma specialis* and race represent two particular taxonomic levels only observed in certain fungal species such as *F. oxysporum*, which are directly related to the pathogenicity towards specific host plants, or a particular set of varieties of the same host plant, respectively. The identification of the *forma specialis* or race typically requires several days or weeks of pathogenicity testing on plants, and MALDI-TOF analysis would

provide a quicker and easier means of obtaining a result. Following our work, preliminary attempts to discriminate races within a *forma specialis* yielded very promising results with *F. oxysporum* f. sp. *cubense* (Foc), causing the destructive Panama disease on banana trees (data not shown). Several races of Foc are described based on their pathogenicity towards certain banana varieties. One of them, *i.e.* Tropical Race 4 is currently threatening the Cavendish variety, which is the most cultivated banana cultivar throughout the world, and therefore accurate identification of this race is of paramount importance for the enforcement of disease management strategies. However, several strains of the same race, with different geographical origins, should be collected and assessed in order to confirm the potential of MALDI-TOF analysis.

Testing a larger set of *Fusarium formae speciales* and races would of course be necessary for a comprehensive assessment of the reliability of MALDI-TOF technology. However, preliminary results are definitely promising and we may be optimistic about the usefulness of MALDI-TOF for acute identification, at very low taxonomic levels. It may also be imagined that on some occasions and after a thorough comparison, identification of *formae speciales* and races would no longer require pathogenicity testing, thus saving time and money. Of course, by using software that enables a comparison of spectra using a peak–by-peak approach, such as ClinProTools, peaks representing proteins or portions thereof might be connected to pathogenicity features.

As a conclusion, this preliminary study supports the potential of MALDI-TOF for the identification and the acute characterisation of phytopathogenic fungi, taking the *Fusarium* genus as a model. The results also suggest that prior optimisation of the protein extraction step is a prerequisite for MALDI-TOF analysis of different fungal genera, which will make it difficult to build a universal fungal reference database. Once the initial investment has been made for the purchase of the equipment, routine analyses using MALDI-TOF are very affordable in terms of reagents and consumables, and enable simultaneous processing of more than 90 samples within less than 2 hours, which is undoubtedly an advantage over the currently available identification techniques.

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