ISSN 1330-9862
(FTB-3058)

scientific note

Using Spores for *Fusarium* spp. Classification by MALDI-Based Intact Cell/Spore Mass Spectrometry

Martina Marchetti-Deschmann[1], Wolfgang Winkler[1], Hongjuan Dong[1], Hans Lohninger[1], Christian P. Kubicek[2] and Günter Allmaier[1]*

[1]Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164, AT-1060 Vienna, Austria

[2]Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166, AT-1060 Vienna, Austria

Received: March 31, 2012
Accepted: July 26, 2012

Summary

Fusarium is a widespread genus of filamentous fungi and a member of the soil microbial community. Certain subspecies are health threatening because of their mycotoxin production that affects the human and animal food chain. Thus, for early and effective pest control, species identification is of particular interest; however, differentiation on the subspecies level is challenging and time-consuming for this fungus. In the present study, we show the possibilities of intact cell mass spectrometry for spore analysis of 22 different *Fusarium* strains belonging to six *Fusarium* subspecies. We found that species differentiation is possible if mass spectrometric analyses are performed under well-defined conditions with fixed parameters. A critical point for analysis is a proper sample preparation of spores, which increases the quality of mass spectra with respect to signal intensity and *m/z* value variations. It was concluded that data acquistion has to be performed automatically; otherwise, user-specific variations are introduced generating data which cannot fit the existing datasets. Data that show clearly that matrix-assisted laser desorption ionization-based intact cell/intact spore mass spectrometry (IC/ISMS) can be applied to differentiate closely related *Fusarium* spp. are presented. Results show a potential to build a database on *Fusarium* species for accurate species identification, for fast response in the case of infections in the cornfield. We furthermore demonstrate the high precision of our approach in classification of intact *Fusarium* species according to the location of their collection.

Key words: intact cell/spore mass spectrometry, fungi, *Fusarium*, species classification

Introduction

Fungi are essential microorganisms in our modern society. Besides their use as treating agents in wastewater management, biofuel production, pulp and paper industry, fungi have also gained importance in antibiotic, protein, organic acid and food production (*1*). However, fungal diseases in humans are growing – especially in those with compromised immune systems (*2*,*3*). Also, a dramatic economic impact on farming has to be mentioned. If ripening cereals, seeds or food stocks are infected, crops have to be disposed of because of diseases like *Fusarium* head blight (FHB), which is responsible for high mycotoxin levels in affected plants. This *Fusarium*-based disease plays a crucial role especially in the animal and human food chain (*4*). Eckard *et al.* (*5*) showed on the morphological and mycotoxin level that silage maize is frequently infected by *Fusarium* species in Switzerland. It was shown that 12 different species contributed to maize infections in a concerted manner. A pre-

*Corresponding author; Phone: ++43 1 58801 15162; Fax: ++43 1 58801 915162; E-mail: martina.marchetti-deschmann@tuwien.ac.at

vious study in European countries (*6*) revealed that in wheat mainly 3 species, namely *F. graminearum*, *F. avenaceum* and *F. culmorum*, are responsible for FHB with less frequent observation of *F. poae*, *F. cerealis*, *F. equiseti*, *F. sporotrichioides*, *F. tricinctum* and *F. acuminatum*, making the former three the most important ones to be identified at an early stage. Additionally, a difference in host preference was suggested for *F. graminearum* after studying 560 members of the species complex collected from different infected hosts like wheat, maize and barley in South Africa (*7*). To fight FHB in the cornfield, biocontrol agents for European cereals are of interest and the latest studies suggest mycoparasitic effects of certain fungal species like *Trichoderma* against *Fusarium* (*8*,*9*). *Trichoderma* was positively tested against *Fusarium oxysporum* f. sp. *lentis* (*10*) and it has been shown that *Trichoderma gamsii* 6085 is able to antagonize *F. culmorum* and *F. graminearum* on rice but is severely inhibited on wheat haulms (*11*).

Taking all these findings together, it seems obvious that there is a trend to develop biocontrol agents showing good growth on certain agricultural products, targeting furthermore the correct plant pathogen, *e.g.* a specific member of the *Fusarium* species complex. Thus, a quick identification of *Fusarium* sp. on the species level is highly desirable to bring the perfect biocontrol agent into action. Unfortunately, the taxonomy of *Fusarium* has been a subject of controversy for many years (*12*,*13*), making species identification difficult. Identification of fungi can be straightforward like in the case of *Candida albicans*, *Aspergillus fumigatus* or *Candida glabrata*, but in some cases it is very challenging, as it has been shown for *Fusarium* (*14*), because the morphological species concept does not reflect the phylogenetic tree of the *Fusarium* genus (*12*). Morphological and biochemical traits often fail to identify these fungal pathogens. Nevertheless, early detection of *Fusarium* crop infections like FHB is important; however, it is often based on questionable genetic markers or methods detecting a long list of mycotoxins (*4*) when the exact composition of fungal subspecies is not known. Therefore, both approaches can identify a plant pathogen as *Fusarium*, but subspecies differentiation, important for targeted antagonistic treatment, is very often not possible.

Furthermore, these analytical approaches are very time consuming. For fast and accurate analysis, intact cell mass spectrometry (ICMS) has been shown to be a powerful tool. For ICMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is used to desorb and ionize analytes from the surface of intact or partially lysed cells. For this, samples are directly embedded into a MALDI matrix without laborious sample clean-up or enrichment steps and subsequently surface-associated analytes are desorbed and ionized by a UV laser and the resulting ions are mass analyzed. This technique has already helped to identify bacteria (*15–17*), to diagnose *Fusarium* proliferation during infection (*18*), and to identify *Bacillus pumilus* (*19*). Detailed discussions of ICMS used for the analysis of microorganisms (*20*), and for fungal identification in particular (*21*), are available. Robust protocols for bacterial classifications have been developed (*22*) taking mixed cultures (*23–25*) and experimental factors (*26–28*) into account. Well developed software algorithms (*29–32*) sometimes allow better species identification than classical biological approaches. Only recently, a study on clinically relevant *Fusarium* species (*33*) has been published showing that species differentiation is possible after acid extraction of fungal colonies cultivated for 72 h. Another approach differentiates for the first time *Fusarium* sp., *Aspergillus* sp. and *Mucorales* sp. directly from the fungal culture surface (*34*). The latest trends have started to focus on fungal spores (*35–37*), where no cell lysis is taking place and only surface-associated analytes are used for species differentiation. However, no study on fungal spore classification for closely related *Fusarium* subtypes is available so far, addressing the final demand for building a robust database for rapid and direct species identification.

In this paper, we present for the first time that differentiation at the lower taxonomic level is possible directly from intact spores using MALDI-based ICMS, as demonstrated for 22 *Fusarium* species. We call this specific approach also intact spore mass spectrometry (ISMS). A simple and fast sample preparation of fungal spores, applicable to coloured and non-coloured spores, allows for the first time to differentiate closely related *Fusarium* spp. solely based on mass spectrometric peptide/protein fingerprinting (by IC/ISMS) and statistical analysis of surface-associated analytes. The presented results are a first approach towards the development of a database.

Materials and Methods

Chemicals

Ferulic acid (FA) was obtained from Fluka (Buchs, Switzerland). Acetonitrile and glycerol were of analytical grade and purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Riedel-de-Haën (Seelze, Germany). Water was purified with a Simplicity® water purification system (Millipore, Billerica, MA, USA). The protein MALDI-MS calibrants cytochrome c and the ACTH 7-38 fragment were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Fusarium spores

Austrian *Fusarium* isolates, collected from different hosts, were prepared as previously described (*38*). In short, *Fusarium* spp. were cultivated on nutrient plates and spore productions were performed in shake flasks. After sterile filtration, the collected spores were mixed with 20 % glycerol in water and stored at –20 °C. Spore concentrations were determined by spore counting using a light microscope (Nikon Instruments, Tokyo, Japan). Before IC/ISMS, 50- to 100-μL spore solutions were purified using a Nanosep® centrifugal device (Pall, Ann Arbor, MI, USA) with a molecular mass cut-off of 10 kDa and the final retentate was suspended in water to obtain a concentration of 3.5×10^6 spores per μL. *Fusarium* spp. used in this work and strain details are listed in Table 1.

IC/ISMS

For reproducibility studies, multiple replicates were prepared for each sample, and then the selected samples were chosen to evaluate MALDI sample preparation techniques (for details see Table 1).

Table 1. List of *Fusarium* strains used during this study. For each species the host and location of collection together with the MS sample preparation technique and number of mass spectrometric preparation replicates is listed

Fusarium	Strain (CPK number)	Host of isolate	Location of collection in Austria	Number of IC/ISMS replicates	
				Sample preparation 1	Sample preparation 2
graminearum	1122	unknown	unknown	13	13
	2761	maize	Wieselsdorf	12	12
	2763	maize	Wieselsdorf	16	13
	2764	maize	Wieselsdorf	14	14
	2765	barley	Probstdorf	14	14
	2766	wheat	unknown	15	15
poae	2774	maize	IFA-Tulln	–	11
	2775	barley	Probstdorf	–	11
	2781	barley	Probstdorf	–	11
	2793	barley	Lambach	–	11
	2794	barley	Lambach	–	10
cerealis	2739	maize	Wieselsdorf	–	10
	2740	maize	Wieselsdorf	–	10
	2741	maize	Mogersdorf	–	10
	2743	barley	Lambach	–	10
culmorum	2745	wheat	unknown	–	10
	2752	wheat	unknown	–	10
sporotrichioides	2796	maize	Wieselsdorf	–	10
	2797	maize	Wieselsdorf	–	10
equiseti	2755	wheat	unknown	–	10
	2756	wheat	unknown	–	10
	2760	walnut	unknown	–	10

Sample preparation 1 (SP1): Samples for analysis were prepared by the mixed volume method (*39*). Briefly, MALDI matrix solution (10 mg of FA in 1 mL of aqueous 0.1 % TFA containing 70 % acetonitrile) was mixed with the same volume of freshly prepared spore suspension in a sample tube using a table-top vortexer. A volume of 1 μL of this mixture was applied on a stainless steel MALDI-MS target and dried at room temperature.

Sample preparation 2 (SP2): An enhanced sample preparation especially suitable for coloured *Fusarium* samples was used (*40*). The preparation procedure is the same as SP1, but after drying at room temperature another 5 μL of MALDI matrix solution were deposited on top and again allowed to dry at ambient temperature.

Mass spectrometry

Positive ion MALDI mass spectra were acquired on an AXIMA CFR+ (Kratos Analytical, Shimadzu Biotech Ltd, Manchester, UK), equipped with a nitrogen laser. The instrument was operated in a fully automated manner by predefining the following parameters: linear ion mode, delayed extraction set to *m/z*=5000 for optimal resolution, accumulation of 2500 unselected and consecutive laser pulses for one mass spectrum per sample omitting the automated removal of mass spectra of low mass spectrometric quality (*e.g.* low absolute signal intensity or signal-to-noise (*S/N*) ratios), mass spectra acquired from the whole sample spot according to a predefined raster covering the whole sample preparation, ion gate set to *m/z*= 1000 to remove unspecific fragment and cluster ions, and the *m/z* range from 2500 to 15 000 used for data analysis. Mass spectra were calibrated externally using singly and doubly charged cytochrome c ions (*m/z*=12361.2 and 6181.1, respectively) and the singly charged ACTH 7-38 fragment (*m/z*=3657.9).

Data analysis

Basic statistical evaluation

The detailed analysis of sample preparation techniques was performed on *m/z* aligned data. For this purpose mass spectra were smoothed using the Savitzky-Golay algorithm (number of channels: 20) and baseline subtracted (filter width 200), both by using the vendor-provided acquisition software (Launchpad v. 2.8.4.2008 1127, Kratos Analytical, Shimadzu Biotech Ltd). After that, data were exported as mzXML files (*41*) and reimported into mMass v. 2.4 (*42*) for signal assignment. Mass spectra were normalized with respect to the highest signal and an alignment was automatically generated by an in-house script considering peaks from different mass spectra to be identical if their *m/z* differences were less than [1+(*m/z*)/3000], giving a variable threshold of 1.8–6 Da for signals between *m/z*=2500 and 15 000. Signal intensity for *m/z* values that did not appear in all replicates was set as 0.

Feature extraction

After smoothing and baseline subtraction using again the above-mentioned Launchpad, mass spectra were also exported as text files to another in-house software called MS Feature Extractor (v. 1.01). All peaks with an *S/N* ratio exceeding a threshold of 3 were marked between *m/z*= 2500 and 15 000 and extracted as features for further analysis.

Partial least square discriminant analysis (PLS-DA) was carried out using DataLab v. 3.5 (*43*). Classifier metrics were calculated as follows:

$$\text{recall}=[(\text{true positive})/(\text{true positive}+\text{false negative})\cdot 100] \quad /1/$$

$$\text{precision}=[(\text{true positive})/(\text{true positive}+\text{false positive})\cdot 100] \quad /2/$$

$$\text{accuracy}=[(\text{true positive}+\text{true negative})/213\cdot 100)] \quad /3/$$

Results and Discussion

Fusarium strains relevant for Austrian agriculture were classified on the species level by IC/ISMS. Here we present that mass spectrometry is capable of distinguishing species at low levels of the *Fusarium* lineage tree by investigating solely their spores in a quick and time-saving manner.

Reproducibility of sample preparation

Up to now, only little attention has been paid to changes in spectrum quality of replicate measurements, and predominantly descriptive surveys of mass spectra after changes in sample preparation are available. Recently, the first statistical approach to data evaluation after ICMS analysis has been published for clinically relevant pathogens (*44*). However, no information on reproducibility and precision of an experiment is available for fungal spore analysis where cell lysis was particularly avoided (mild pH conditions); therefore, no species-characteristic intracellular material is released during sample preparation. One can assume that intact spores are a prerequisite for better reproducibility. As we have reported previously, mass spectra generated from *Fusarium* spores of different subtypes exhibit differences with respect to the observed *m/z* values and signal intensities using a certain sample preparation, namely SP1 (*38*). We also reported that for coloured *Fusarium* species an enhanced sample preparation, SP2, can help to give better mass spectra with respect to useful *S/N* ratios (*40*).

In the present study, we thoroughly evaluated our first observations by a rigorous statistical analysis. First, the same set of mass spectra was provided to two different users and their peak picking process was investigated. We observed a user-dependent number of assigned peaks, although both users were experienced in the field of MALDI-TOF-MS (both have more than 6 years of experience) and both were given the same criteria for peak picking: signals had to be picked from all sample replicates in a mass range from *m/z*=2500 to 15 000 with the *S/N* ratio above 3. All mass spectra related to *F. graminearum* (CPK 1122, 2761, 2763, 2764, 2765, 2766) and *F. poae* (CPK 2774, 2775, 2781, 2793, 2794) samples were chosen and in total peaks of 135 mass spectra were picked by two MS users. Fig. 1 represents the most intriguing data from samples CPK 2774, 2775 and 2781. The total number of peaks picked by the users from 10 IC/ISMS preparation replicates of the same spore preparation varied by 100 % for CPK 2774 (108 and 212 signals assigned) and up to 400 % for CPK 2775 (65 and 265, respectively).

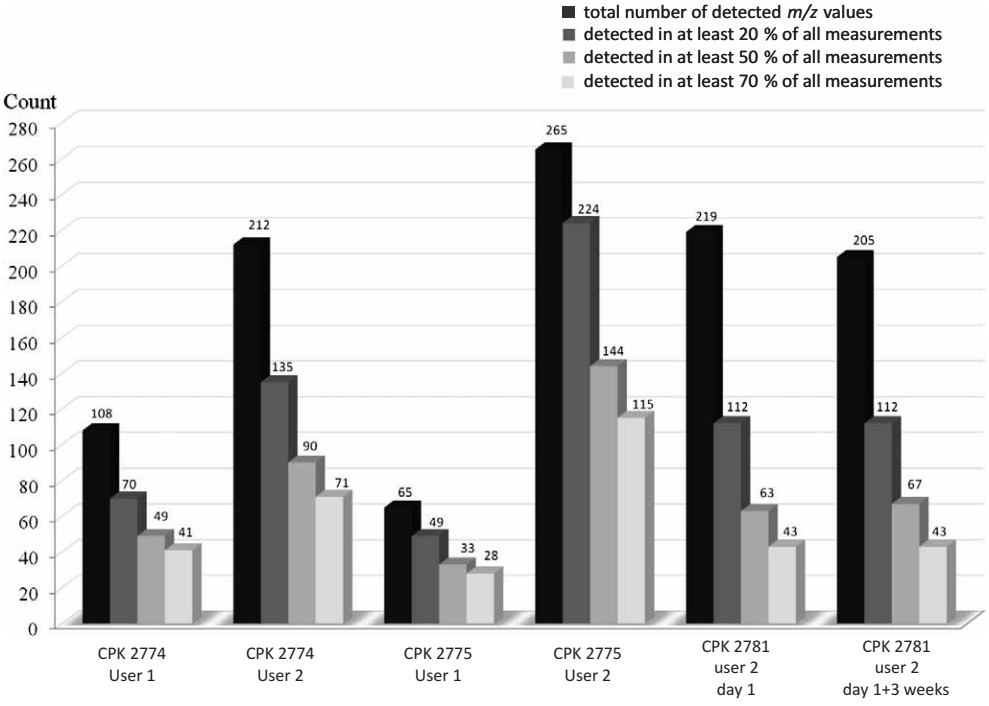

Fig. 1. Peak counts obtained by manual peak assignment for three different *Fusarium graminearum* species evaluated by two different users given the same parameters for peak assignment: *S/N*>3 and peaks have to be picked in the mass range from *m/z*=2500 to 15 000

We evaluated the manual peak picking approach as experienced users, especially those who have to interpret non-isotopically resolved mass spectra, tend to trust their own peak picking process more than automated procedures which very often miss the correct peak maximum of badly resolved signals. Laboratory practice showed that automatically retrieved peak lists are often manually revised, which we wanted to prove to be an incorrect approach even for badly resolved mass spectra recorded in the linear ion mode.

After taking a closer look at the data and assigned signals, it was found that user 1 intuitionally did not assign alkali adduct ions as $[M+Na]^+$ or $[M+K]^+$ species and user 2 tried to avoid exactly this, therefore assigning more signals than usual. This shows that even for experienced users manual peak assignment is not consistent, although peak picking defaults had been agreed upon. It has to be pointed out here that at the moment an automated peak picking algorithm is not capable of distinguishing alkali adduct ions from $[M+H]^+$ ions in the case of IC/ISMS data due to broad peak shapes. Nevertheless, statistical analysis gives better results if only features of high significance are processed, but Na^+ or K^+ adduct ions provide only redundant information. Also, the intuitive removal of supposed adduct ions can lead to missed signals from less abundant species showing the same *m/z* value.

Fascinatingly, it was observed that the percentage of significant signals decreased to the same extent for both users when looking at the number of peaks assigned in at least 20, 50 or even 70 % of all 10 replicates. In CPK 2774 for user 1 62 % (62), and for user 2 66 % (141) of the signals were found in at most 3 out of 10 replicates. These similar rates prove the consistency of peak assignment for both users. Furthermore, it also shows that a stringent peak picking process is not better suited for omitting multiple analyte assignments (*i.e.* $[M+H]^+$, $[M+Na]^+$, $[M+2Na-H]^+$ and $[M+K]^+$). If this was a fact, the percentage of reoccurring signals would have to be higher. The reproducibility of data handling was also documented by the fact that the number of assigned peaks for sample CPK 2781 handled by user 2 on two days within three weeks remained roughly the same.

Although it was ascertained that the number of peaks found in at least 2, 5 or 7 out of 10 mass spectra decreased to the same percentage for two different users within one sample, the extent cannot be generalized. For CPK 2774 38 and 34 % of the signals were found in at least 70 % of the replicates. Although for CPK 2775 up to 43 % (for both users) were highly reproducible analyte signals, for CPK 2781 only 19 and 21 % were redundant. Therefore, for manual peak picking it was concluded that peak assignment is reproducible for one user if clear definitions are available. However, two users working within the same project may encounter difficulties in the process of species classification since a different number of features (peaks) may be extracted from the same species, leading to various principal components (details discussed later). In conclusion, an automated peak picking algorithm allowing only little influence by the user is proposed.

We also studied spectral information, reproducibility and precision of detected *m/z* values and respective intensities by comparing SP1 with the improved procedure of SP2 for all available *F. graminearum* species (in total 6 samples and 165 mass spectra). For this, mass spectra were assigned only by one user, namely user 2, applying the same criteria as for the first stage of evaluation (smoothed and baseline subtracted mass spectra, *m/z*=2500–15 000, *S/N*>3). As an example of the overall observed results only, *F. graminearum* CPK 1122 is discussed in detail. Results of the application of SP1 and SP2 are presented in Figs. 2a and b, respectively. The average mass spectra of *F. graminearum* CPK 1122 are displayed as their centroid spectra showing that for SP2 the number of overall detected peaks is significantly reduced (SP1: 112, SP2: 47). But considerably more important is the fact that for SP2 most of the signals were detected in more than 50 % of the mass spectra, reflecting the high reproducibility of this sample preparation. For SP1 only 44 out of 112 signals (39 %) were detected in more than 50 % of the mass spectra.

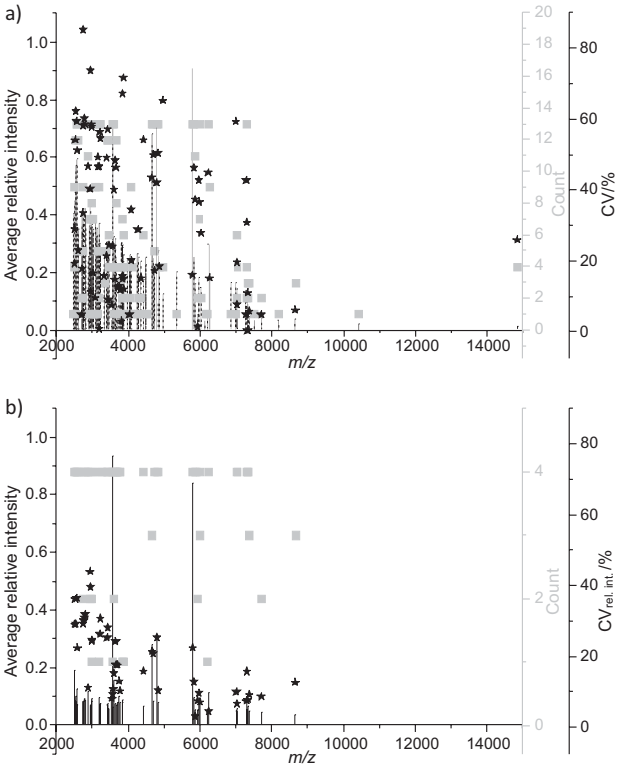

Fig. 2. Average, centroid mass spectra calculated from *Fusarium graminearum* CPK 1122: a) 13 replicates of sample preparation 1, and b) 4 replicates of sample preparation 2 were evaluated according to the number (■) of counts for every detected *m/z* value and the CVs (★) of the respective relative intensities

Especially the high background signal in the lower *m/z* region (*m/z*<5000) was decreased for SP2, improving the relevance of the information for the developed technique. For better illustration of this finding, Fig. 2 exemplifies the variation of the relative intensity of all signals. It was observed that the coefficients of variation (CVs) of the relative signal intensities were between 5 and 45 % in SP2 but varied between 5 and 85 % in SP1.

Besides this, the reduced chemical noise resulted in better *S/N* ratios, which can also be observed if Figs. 2a and b are compared. In Fig. 3a the precision for the determined *m/z* values giving CVs of 0.01–0.07 % even for very low abundance peaks (<0.1 % relative intensity) is presented as a model for *F. graminearum* CPK 1122 prepared using SP2. For SP1 unspecific adduct ion formation contributed adversely to mass precision, giving finally an average mass deviation of 0.11 ppm for SP1 in comparison with 0.06 ppm for SP2. Higher reproducibility of relative signal intensities and more precise *m/z* value determination were not specific for *F. graminearum* CPK 1122 alone, but were observed for all *F. graminearum* species. This fact is seen in Fig. 3b where SP1 is compared to SP2. The latter exhibits significantly better CV values for all *m/z* values independent from the respective signal intensity. Taking all evaluation steps together, it can be stated that for future applications an automated, unambiguous detection and classification of *Fusarium* species by IC/ISMS SP2 is the method of choice for sample preparation.

Fusarium species classification

Twenty-two agriculturally relevant *Fusarium* species were selected and at least ten replicates were measured by IC/ISMS with SP2 method. All mass spectra were exported as a text file to the MS Feature Extractor. Spectral characteristics, so-called features, for 245 mass spectra had to be evaluated according to their suitability for *Fusarium* species classification. In the MS Feature Extractor, *m/z* signals relevant for each mass spectrum were marked according to their peak widths and the resulting features were extracted with respect to their signal intensity maxima, their average signal intensities and their *S/N* ratios. In total 164 different features were assigned for the 22 species. The same procedure was used for smoothed and baseline-subtracted mass spectra.

The gained feature list was exported for classification analysis. In a first approach, we had already successfully tested supervised hierarchical cluster analysis for its capability to differentiate *F. poae* and *F. gramine-*

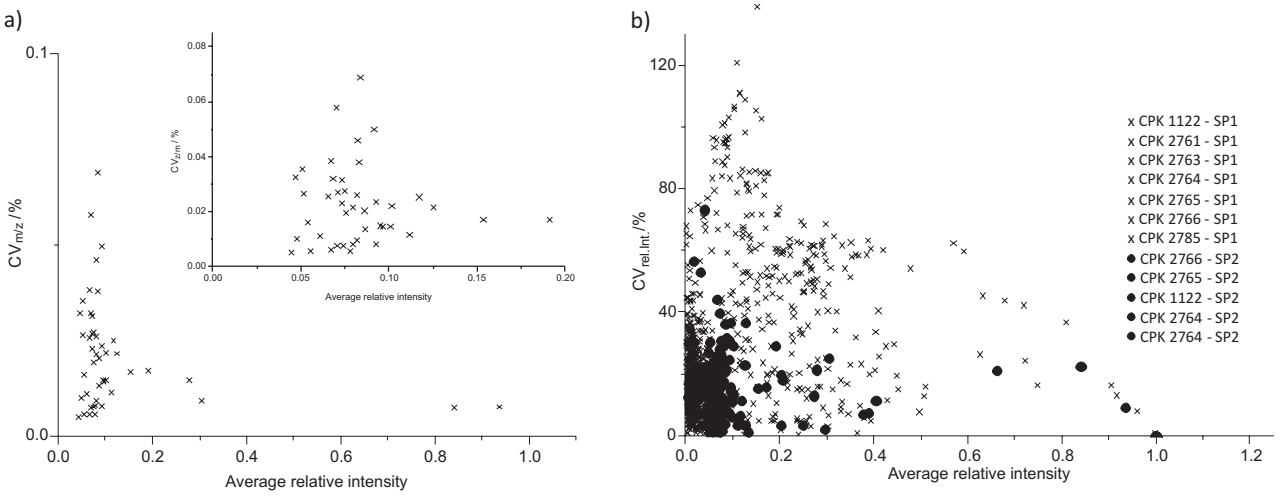

Fig. 3. Determination of: a) the precision of the detected *m/z* values of *Fusarium graminearum* CPK 1122 evaluated according to the respective relative intensities; b) reproducibility of the relative intensities of all detected *m/z* signals in sample preparations 1 (×) and 2 (●)

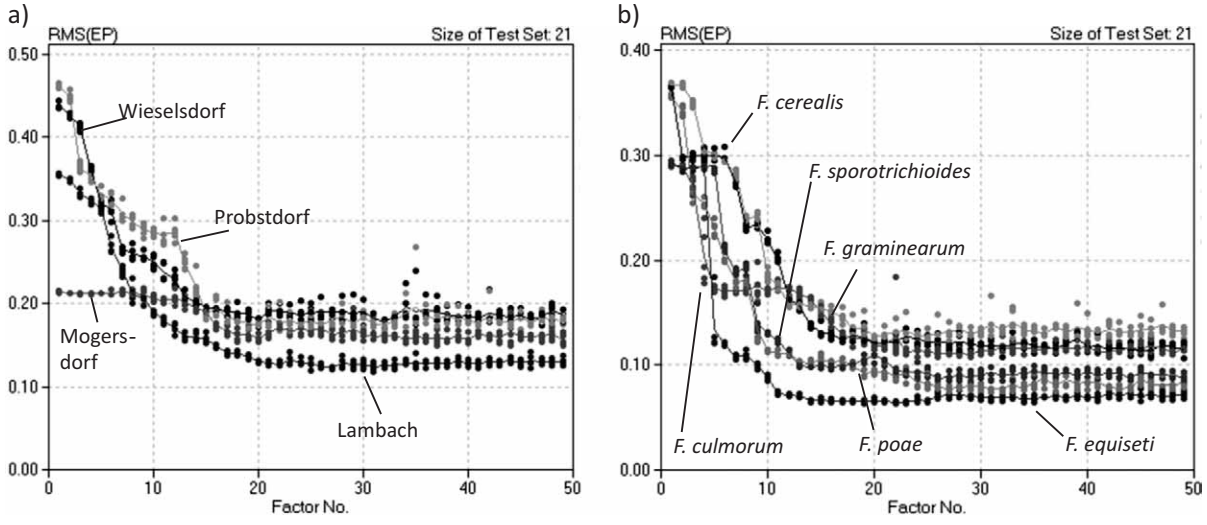

Fig. 4. Tenfold cross-validation: a) classifiers of the locations, b) classifiers of the species. The cross-validation was repeated five times to show the stability of the model. The average values of the root mean square errors of prediction are indicated by lines. The unusual behaviour of the data for the location Mogersdorf may be due to the small number of samples from this location

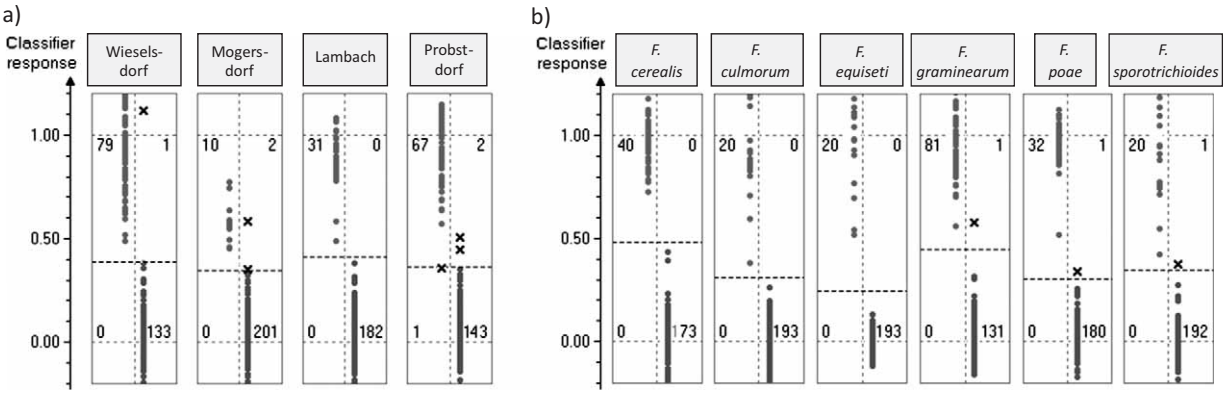

Fig. 5. Application of the classifiers to the test sets using a leave-a-quarter-out approach: a) results of the locations, and b) the species; wrong classification results are indicated by crosses, true positives are in the upper left, true negatives in the lower right corner

Table 2. Common classifier metrics showing the performance of the classifiers

Location	Recall/%	Precision/%	Accuracy/%
Wieselsdorf	100.0	98.8	99.5
Mogersdorf	100.0	83.3	99.1
Lambach	100.0	100.0	100.0
Probstdorf	98.5	97.1	98.6
Species			
F. cerealis	100.0	100.0	100.0
F. culmorum	100.0	100.0	100.0
F. equiseti	100.0	100.0	100.0
F. graminearum	100.0	98.8	99.5
F. poae	100.0	97.0	99.5
F. sporotrichioides	100.0	95.2	99.5

arum (*45*). Nevertheless, increasing the number of species makes supervised analysis complicated and time consuming, which makes this approach less attractive. Furthermore, hierarchical cluster analysis was no longer capable of clearly distinguishing the species.

We therefore developed two models based on partial least square discriminant analysis (PLS-DA) to classify all involved species. For the development of the models we used a dataset containing 213 observations of six species at four locations. The information on the species was encoded by using binary indicator variables which formed the Y-matrix (target values) for the PLS--DA classifiers. The independent X-matrix was based on 49 spectral features derived by the MS Feature Extractor. The dataset was split into test and training data using the leave-a-quarter-out approach. In order to determine the optimum number of PLS factors, we applied 10-fold cross-validation (Figs. 4a and b, respectively), which was repeated five times to get an impression of the stability of the models. About 20 factors proved to be sufficient for both models to get excellent classification results. The validity of the classifiers was checked using the leave--a-quarter-out approach. The results of the validation step are shown in Figs. 5a and b. Both the classifier of locations and the classifier of species performed well. In the case of locations (Fig 5a), five out of 213 samples classified as false positive and one classified as false negative. The classifier of species (Fig. 5b) performed even better, only three out of 213 samples were classified as false positive, and none as false negative.

The overall performance of the classifiers is shown in Table 2. Except for the classification of the location Mogersdorf, which shows a precision of only 83 %, all other classifier metrics are close to 100 %. The low precision of Mogersdorf may be due to the small sample size from this location. Further investigations based on more sampes should be carried out to check whether this deviation is only a singular event.

Conclusions

Up to this study, it has not been shown in detail that the differentiation of *Fusarium* species can be facilitated from fungal spores by the applied classification method. We were able to show clearly that a differentiation of related subspecies (according to Watanabe *et al.* (*12*)) can be achieved using a proper sample preparation technique and classification method.

At this point, it has to be stated that we are aware of the fact that the presented results were generated from *Fusarium* isolates. We are also aware of the fact that a plant infection like FHB is usually not caused by only one *Fusarium* species and we want to point out that we understand that there can be a regional change in the distribution of *Fusarium* species (*46*).

Nevertheless, the presented data clearly show that a proper MS sample preparation and a consistent way of peak picking following predefined parameters allows the classification of intact *Fusarium* spores by IC/ISMS according to the species and location of harvesting. This study is considered a good basis for further investigations towards an IC/ISMS-based *Fusarium* spore database. Expanding the number of studied species, including different hosts and regions, can provide more general results for a fast and reliable species identification, maybe even of spore mixtures – allowing a targeted treatment of crops with the respective antagonist.

Acknowledgements

This study was partly financed by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (grant no. 100053). Spores were kindly prepared by Marietta Pauls.

References

1. *Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine*, J.S. Tkascz, L. Lange (Eds.), Kluwer Academic/Plenum Publishers, New York, NY, USA (2004).
2. T.N. Helm, D.L. Longworth, G.S. Hall, B.J. Bolwell, B. Fernandez, K.J. Tomecki, Case report and review of resolved fusariosis, *J. Am. Acad. Dermatol. 23* (1990) 393–398.
3. R.C. Summerbell, S.E. Richardson, J. Kane, *Fusarium proliferatum* as an agent of disseminated infection in an immunosuppressed patient, *J. Clin. Microbiol. 26* (1988) 82–87.
4. N. Magan, Mycotoxin contamination of food in Europe: Early detection and prevention strategies, *Mycopathologia, 162* (2006) 245–253.
5. S. Eckard, F.E. Wettstein, H.R. Forrer, S. Vogelgsang, Incidence of *Fusarium* species and mycotoxins in silage maize, *Toxins, 3* (2011) 949–967.
6. A. Bottalico, G. Perrone, Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe, *Eur. J. Plant Pathol. 108* (2002) 611–624.
7. A.L. Boutigny, T.J. Ward, G.J. Van Coller, B. Flett, S.C. Lamprecht, K. O'Donnell, A. Viljoen, Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference, *Fungal Genet. Biol. 48* (2011) 914–920.
8. R.R. Bélanger, N. Dufour, J. Caron, N. Benhamou, Chronological events associated with the antagonistic properties of *Trichoderma harzianum* against *Botrytis cinerea*: Indirect evidence for sequential role of antibiosis and parasitism, *Biocontrol Sci. Tech. 5* (1995) 41–54.
9. A. Pellegrini, P.E. Corneo, F. Camin, L. Ziller, S. Tosi, I. Pertot, Studying trophic interactions between a plant pathogen and two different antagonistic microorganisms using a 13C-labeled compound and isotope ratio mass spectrometry, *Rapid Commun. Mass Spectrom. 26* (2012) 510–516.
10. H. Kari Dolatabadi, E. Mohammadi Goltapeh, N. Mohammadi, M. Rabiey, N. Rohani, A. Varma, Biocontrol potential of root endophytic fungi and *Trichoderma* species against *Fusarium* wilt of lentil under *in vitro* and greenhouse conditions, *J. Agr. Sci. Tech. 14* (2012) 407–420.
11. F. Matarese, S. Sarrocco, S. Gruber, V. Seidl-Seiboth, G. Vannacci, Biocontrol of *Fusarium* head blight: Interactions between *Trichoderma* and mycotoxigenic *Fusarium*, *Microbiology, 158* (2012) 98–106.
12. M. Watanabe, T. Yonezawa, K.I. Lee, S. Kumagai, Y. Sugita-Konishi, K. Goto, Y. Hara-Kudo, Molecular phylogeny of the higher and lower taxonomy of the *Fusarium* genus and differences in the evolutionary histories of multiple genes, *BMC Evol. Biol. 11* (2011) Article No. 322.
13. P.E. Nelson, M.C. Dignani, E.J. Anaissie, Taxonomy, biology, and clinical aspects of *Fusarium* species, *Clin. Microbiol. Rev. 7* (1994) 479–504.
14. B. Lievens, M. Rep, B.P. Thomma, Recent developments in the molecular discrimination of *formae speciales* of *Fusarium oxysporum*, *Pest Manag. Sci. 64* (2008) 781–788.
15. R.D. Holland, J.G. Wilkes, F. Rafii, J.B. Sutherland, C.C. Persons, K.J. Voorhees, J.O. Lay Jr., 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. 10* (1996) 1227–1232.
16. T. Krishnamurthy, P.L. Ross, Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells, *Rapid Commun. Mass Spectrom. 10* (1996) 1992–1996.
17. T. Krishnamurthy, P.L. Ross, U. Rajamani, Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom. 10* (1996) 883–888.
18. F. Seyfarth, M. Ziemer, H.G. Sayer, A. Burmester, M. Erhard, M. Welker *et al.*, The use of ITS DNA sequence analysis and MALDI-TOF mass spectrometry in diagnosing an infection with *Fusarium proliferatum*, *Exp. Dermatol. 17* (2008) 965–971.
19. D.N. Dickinson, M.T. La Duc, M. Satomi, J.D. Winefordner, D.H. Powell, K. Venkateswaran, MALDI-TOFMS compared with other polyphasic taxonomy approaches for the identification and classification of *Bacillus pumilus* spores, *J. Microbiol. Methods, 58* (2004) 1–12.
20. C. Fenselau, P.A. Demirev, Characterization of intact microorganisms by MALDI mass spectrometry, *Mass Spectrom. Rev. 20* (2001) 157–171.
21. C. Santos, R.R.M. Paterson, A. Venâncio, N. Lima, Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *J. Appl. Microbiol. 108* (2010) 375–385.
22. A. Freiwald, S. Sauer, Phylogenetic classification and identification of bacteria by mass spectrometry, *Nat. Protoc. 4* (2009) 732–742.
23. D.J. Evason, M.A. Claydon, D.B. Gordon, Exploring the limits of bacterial identification by intact cell-mass spectrometry, *J. Am. Soc. Mass Spectrom. 12* (2001) 49–54.
24. A.J. Saenz, C.E. Petersen, N.B. Valentine, S.L. Gantt, K.H. Jarman, M.T. Kingsley, K.L. Wahl, Reproducibility of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for replicate bacterial culture analysis, *Rapid Commun. Mass Spectrom. 13* (1999) 1580–1585.
25. J. Walker, A.J. Fox, V. Edwards-Jones, D.B. Gordon, Intact cell mass spectrometry (ICMS) used to type methicillin-resistant *Staphylococcus aureus*: Media effects and inter-laboratory reproducibility, *J. Microbiol. Methods, 48* (2002) 117–126.
26. T.L. Williams, D. Andrzejewski, J.O. Lay Jr., S.M. Musser, Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells, *J. Am. Soc. Mass Spectrom. 14* (2003) 342–351.
27. S.L. Gantt, N.B. Valentine, A.J. Saenz, M.T. Kingsley, K.L. Wahl, Use of an internal control for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of bacteria, *J. Am. Soc. Mass Spectrom. 10* (1999) 1131–1137.
28. J.H. van Adrichem, K.O. Börnsen, H. Conzelmann, M.A. Gass, H. Eppenberger, G.M. Kresbach *et al.*, Investigation of protein patterns in mammalian cells and culture supernatants by matrix-assisted laser desorption/ionization mass spectrometry, *Anal. Chem. 70* (1998) 923–930.
29. J.J. Bright, M.A. Claydon, M. Soufian, D.B. Gordon, Rapid typing of bacteria using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and pattern recognition software, *J. Microbiol. Methods, 48* (2002) 127–138.
30. K.H. Jarman, S.T. Cebula, A.J. Saenz, C.E. Petersen, N.B. Valentine, M.T. Kingsley, K.L. Wahl, An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry, *Anal. Chem. 72* (2000) 1217–1223.
31. K.H. Jarman, D.S. Daly, C.E. Petersen, A.J. Saenz, N.B. Valentine, K.L. Wahl, Extracting and visualizing matrix-assisted laser desorption/ionization time-of-flight mass spectral fingerprints, *Rapid Commun. Mass Spectrom. 13* (1999) 1586–1594.
32. Z. Wang, L. Russon, L. Li, D.C. Roser, S.R. Long, Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom. 12* (1998) 456–464.

33. C. Marinach-Patrice, A. Lethuillier, A. Marly, J.Y. Brossas, J. Gené, F. Symoens *et al.*, Use of mass spectrometry to identify clinical *Fusarium* isolates, *Clin. Microbiol. Infect. 15* (2009) 634–642.
34. E. De Carolis, B. Posteraro, C. Lass-Flörl, A. Vella, A.R. Florio, R. Torelli, 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. 18* (2012) 475–484.
35. T.Y. Li, B.H. Liu, Y.C. Chen, Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom. 14* (2000) 2393–2400.
36. N.B. Valentine, J.H. Wahl, M.T. Kingsley, K.L. Wahl, Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry, *Rapid Commun. Mass Spectrom. 16* (2002) 1352–1357.
37. K.J. Welham, M.A. Domin, K. Johnson, L. Jones, D.S. Ashton, Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom. 14* (2000) 307–310.
38. J. Kemptner, M. Marchetti-Deschmann, R. Mach, I.S. Druzhinina, C.P. Kubicek, G. Allmaier, 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. 23* (2009) 877–884.
39. J. Kemptner, M. Marchetti-Deschmann, C.P. Kubicek, G. Allmaier, Mixed volume sample preparation method for intact cell mass spectrometry of *Fusarium* spores, *J. Mass Spectrom. 44* (2009) 1622–1624.
40. H. Dong, J. Kemptner, M. Marchetti-Deschmann, C.P. Kubicek, G. Allmaier, 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. 395* (2009) 1373–1383.
41. P.G. Pedrioli, J.K. Eng, R. Hubley, M. Vogelzang, E.W. Deutsch, B. Raught *et al.*, A common open representation of mass spectrometry data and its application to proteomics research, *Nat. Biotechnol. 22* (2004) 1459–1466.
42. M. Strohalm, M. Hassman, B. Košata, M. Kodíček, *mMass* data miner: An open source alternative for mass spectrometric data analysis, *Rapid Commun. Mass Spectrom. 22* (2008) 905–908.
43. J. Lohninger, DataLab, Epina Software Labs, Pressbaum, Austria (2011) (*http://www.lohninger.com/datalab/*).
44. S. Schumaker, C.M. Borror, T.R. Sandrin, Automating data acquisition affects mass spectrum quality and reproducibility during bacterial profiling using an intact cell sample preparation method with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom. 26* (2012) 243–253.
45. H. Dong, M. Marchetti-Deschmann, W. Winkler, H. Lohninger, G. Allmaier: Intact Cell/Spore Mass Spectrometry of *Fusarium* Macro Conidia for Fast Isolate and Species Differentiation. In: *Detection of Biological Agents for the Prevention of Bioterrorism*, *NATO Science for Peace and Security, Series A: Chemistry and Biology*, J. Banoub (Ed.), Springer Netherlands, Dordrecht, The Netherlands (2011) pp. 47–63.
46. T. Aoki, A taxonomic and ecological study of the genus *Fusarium*, *Nippon Kingakukai Kaiho, 41* (2000) 19–32.