Forest Pathology WILEY-BLACKWELL

For. Path. doi: 10.1111/efp.12054

Phenotypic characterization of cryptic *Diplodia* species by MALDI-TOF MS and the bias of mycelium age

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Summary

Detection and identification of fungal cryptic species has been facilitated by DNA sequencing. However, the examination of some phenotypic traits is fundamental for the confirmation of genetic results. MALDI-TOF mass spectrometry has shown remarkable reliability in the recognition of species-specific phenotypic markers and has already been tested in several species of fungi. However, even though there is no direct evidence, the age of the mycelium appears to influence the composition of the metabolites detected. In this study, we demonstrate that MALDI-TOF is a reliable technique to identify suitable metabolites to distinguish three monophyletic species of *Diplodia* (*D. pinea*, *D. seriata* and *D. scrobiculata*), thus supporting recent DNA results. Nevertheless, different collections of samples over a period of 3 months after inoculation also revealed that the MALDI-TOF spectra are highly dependent on mycelial age. More importantly, the species-specific markers emerge only after 1 month of mycelial growth. The methodological and biological implications of these findings are discussed.

1 Introduction

During the last 20 years, the detection and identification of fungal cryptic species in the *Botryosphaeriaceae* has been highly facilitated by the sequencing of DNA markers (Pavlic et al. 2009). The use of these molecular approaches and of recently developed ones, such as high-resolution melting analysis (Luchi et al. 2011), represent reliable tools to distinguish cryptic fungal species. Despite their usefulness, DNA markers can only provide evidence for diversification amongst taxa based on a relatively small fraction of their genome and do not provide evidence for phenotypic diversification. Such information would prove invaluable for confirming identifications based on genetic markers and when trying to reconstruct the evolutionary trajectories leading to the emergence of different species.

In recent years, in addition to molecular techniques, MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry has proved useful and reliable in the identification of metabolic markers, which can univocally characterize organisms at the specific level showing small morphological and genetic variations, including viruses, bacteria and fungi (Schmidt and Kallow 2005; Vargha et al. 2006; De Respinis et al. 2010). Unlike DNA-based methods, MALDI-TOF distinguishes phenotypically functional metabolites of the target organism, including those implicated in host–pathogens interactions (Vödisch et al. 2009; Huang et al. 2010). However, unlike PCR-based methods, there is potential bias in MALDI-TOF typing due to the differential expression of such markers at different developmental stages or in different rearing conditions. For bacteria and yeasts, several studies demonstrate that sample preparation is simple and results are reproducible regardless of cultivation conditions and growth state (Sauer and Kliem 2010; van Veen et al. 2010). In contrast, filamentous fungi represent a more challenging subject of study. For example, Barros et al. (2010) showed that *Metarhizium acridum* conidia and mycelia express different sets of proteins. Moreover, Schmidt and Kallow (2005) found that different analyses of mycelia from the same fungal culture had different compositions. These authors argued that mycelia can express different substances at different ages, but did not specifically test the effect of ageing on MALDI-TOF spectra.

In this work, three *Diplodia* species representing an entire monophyletic group (Phillips et al. 2008) were studied. These species have very similar morphological and genetic characteristics (Alves et al. 2006; Crous et al. 2006; Phillips et al. 2008). In the relatively recent past, three morphotypes (A, B and C) were identified within the former *S. sapinea* species, on the basis of conidial morphology, cultural characteristics, virulence and molecular markers (Wang et al. 1985; Palmer et al. 1987; De Wet et al. 2000; Burgess et al. 2001; Zhou et al. 2001). Subsequently, morphotypes A and C were assigned to *Diplodia pinea*, whilst morphotype B was recognized as a distinct species (*Diplodia scrobiculata*; De Wet et al. 2003). The clade also includes *Diplodia seriata* (previously *Botryosphaeria obtusa* (Schwein.) Shoemaker), which has been observed in temperate areas on most continents and isolated from over 35 different hosts, including *Vitis* spp. This species is recognized as an important pathogen of several fruit trees causing cankers, leaf spots and black rot of the fruit (Urbez-Torres et al. 2008). *Diplodia pinea* and *D. scrobiculata* are parasitic fungi causing shoot tip blight in conifer crowns, especially on *Pinus* spp. (Swart and Wingfield 1991; Stanosz et al. 1996; Blodgett and Bonello 2003). *Diplodia pinea* and *D. scrobiculata* only show minor phenotypic differences: the mycelia are very similar, even though that of *D. scrobiculata* maintains a strict contact

Received: 5.6.2012; accepted: 23.4.2013; editor: S. Woodward

with the agar surface during growth in vitro, whereas that of *D. pinea* appears fluffier. Any phenotypic clue for diversification between the two species would be invaluable for confirming the specific status of these taxa.

It was demonstrated recently that *D. seriata* expresses a series of proteins that can be detected by MALDI-TOF (Cobos et al. 2010). From this perspective therefore MALDI-TOF MS is a promising technique for the recognition of phenotypic markers in these highly similar monophyletic species. The existence of such markers may confirm classifications made on the basis of DNA sequences due to functional differences in metabolism. In addition, the possible biasing effect of mycelium age on species identification was tested.

2 Materials and methods

2.1 Sample preparation

Isolates of *D. pinea*, *D. seriata* and *D. scrobiculata* used in this study were obtained from various sources (Table 1). Cultures used for MALDI-TOF analyses were grown in Petri dishes on potato dextrose agar (PDA; Difco, Detroit, MI, USA) and incubated at 25°C. An area of about 5 mm^2 was taken from the superficial mycelium of each colony 1 week after inoculation and then at intervals of 3 weeks until week 10, thus obtaining four collections (weeks 1, 4, 7 and 10). MALDI-TOF analyses were conducted on a total of 88 samples of mycelium.

2.2 Mass spectrometry analyses

Mycelial samples were placed in 1.5-ml microfuge tubes with 200 μl of methanol and sonicated in an ultrasonic bath for 5 min. The matrix for MALDI-TOF experiments was a solution of α-cyano-4-hydroxycinnamic acid (10 mg ml^{-1}) in acetonitrile and 0.1% trifluoroacetic acid (TFA) in water (1 : 1, v:v). One microlitre of the methanol solution was mixed with MALDI matrix (1 : 1, v:v) and the mixture transferred to a stainless steel target; the droplet was allowed to evaporate before the target was introduced into the mass spectrometer. The stainless steel target was analysed using an automatic procedure available on the MALDI mass spectrometer to standardize the results. Extracts from each individual colony were analysed on a MALDI Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany). The instrument was operated in positive ion reflector mode. The accelerating voltage and the ion source 2 were set to 25.0 and 21.9 kV, respectively, and the delay time was 20 ns; 800 laser shots were automatically accumulated for each spectrum. External calibration was performed using the Bruker Standard Peptide Calibration kit (Bruker Daltonics, Bremen, Germany).

Calibrated spectra were imported into the ClinProTools ™(cpt) software (Bruker Daltonics, Bremen, Germany) and processed with a procedure suggested by the software manual and similar to that used by Baracchi and Turillazzi (2010). The program extracts the areas of the most frequent peaks, which also account for most variation amongst groups. SPSS 13 ®for Windows (SSPS, Inc., Chicago, IL, USA) was used for statistical analyses.

2.3 Statistical analyses

Peak areas were transformed into percentages for the subsequent statistical analyses. A multivariate general linear model (GLM) was used in the initial exploratory analysis; both multivariate and univariate tests of significance were performed.

Table 1. Isolates of *Diplodia pinea*, *D. seriata* and *D. scrobiculata* used in the present study.

Species	Isolates	Host	Source	Origin	Collector
Diplodia seriata (=B. obtusa)	UCD244	Grapevine	Trunk	Madera County, California, USA	J.R. Úrbez-Torres
D. seriata	UCD352	Grapevine	Trunk	Monterey County, California, USA	J.R. Úrbez-Torres
D. seriata	B121	Grapevine	Trunk	Blenheim, New Zealand	M. Jaspers
D. seriata	N144	Grapevine	Trunk	Nelson, New Zealand	M. Jaspers
D. seriata	DO4	Grapevine	Trunk	Murrumbateman, NSW	S. Savocchia
D. seriata	J10	Grapevine	Trunk	Eden Valley, SA	S. Savocchia
D. seriata	V7	Grapevine	Trunk	Montepaldi, Firenze, Italy	N. Luchi
D. seriata	V9	Grapevine	Trunk	Montepaldi, Firenze, Italy	N. Luchi
D. seriata	V10	Grapevine	Trunk	Montepaldi, Firenze, Italy	N. Luchi
D. pinea (C morphotype)	S93	*Pinus patula*		Habinsaran, South Africa	M.J. Wingfield
D. pinea	411	*P. resinosa*		Clearwater Co., Minnesota, USA	M.A. Palmer
D. pinea	S91	*P. pinea*	Cone	Castel Fusano, Roma, Italy	
D. pinea	S106			Columbus, Ohio, USA	
D. pinea	128	*P. resinosa*		Grant Co., Wisconsin, USA	M.A. Palmer
D. pinea	Dip C	*P. sylvestris*	Shoot	Legnaro, Padova, Italy	C. Villari
D. pinea	Dip D	*P. sylvestris*	Shoot	Legnaro, Padova, Italy	C. Villari
D. pinea	P1	*P. nigra*	Shoot	M. Morello, Firenze, Italy	N. Luchi
D. pinea	P5	*P. nigra*	Shoot	M. Morello, Firenze, Italy	N. Luchi
D. pinea	P8	*P. nigra*	Shoot	M. Morello, Firenze, Italy	N. Luchi
D. scrobiculata	124	*P. banksiana*		Jackson Co., Wisconsin, USA	M.A. Palmer
D. scrobiculata	215	*P. resinosa*		Douglas Co., Wisconsin, USA	M.A. Palmer
D. scrobiculata	DS14	*P. menziesii*	Canker	Tocchi, Siena, Italy	N. Luchi

Table 2. Multivariate test of significance based on Wilk's lambda, *F* statistic, degrees of freedom (d.f.) for both effects (Eff.) and error (Err.).

	Wilk's λ	*F*	Eff. d.f	Err. d.f.	p
Species	0.176	4.785	22	76.00	<0.001
Sample	0.434	4.509	11	38.00	<0.001
Species × Sample	0.425	1.844	22	76.00	0.027
Strain	0.004	1.461	209	389.30	<0.001

p Values are also provided.

The percentages of each peak were entered as dependent variables, whilst species membership and mycelium age were entered as independent predictor variables; species was included as a factorial variable, whereas age was considered a covariate. To detect different trends for ages in different species, interactions between age and species were also evaluated. Finally, as repeated analyses at different ages were carried out on the same strains, strain was included as a random factor. Moreover, a discriminant analysis was applied using the identification obtained by previous molecular analyses with the *a priori*-determined grouping variable and peak percentages as predictors. Two different analyses for the early samples (1–4 weeks old) and for the late samples (7–10 weeks old) were performed to compare the discriminant power of MALDI-TOF analyses in species recognition at different mycelial ages. Wilk's lambda p values and the correct assignment of cases were used to assess the level of discrimination achieved by this model. Despite the high ratio between the number of cases and the predictor variables in our sample (approximately 6 : 1), discriminant analysis tended to provide relatively conservative results; to enhance reliability, therefore a forward stepwise entering of predictors was used and the correct attribution of cases to their original group assessed using a full cross-validation test (leave-one-out). Through this method, each specimen was blindly attributed to one of the *a priori*-determined groups (species).

3 Results

With a signal-to-noise threshold of 4.0 (on average spectrum) and a relative threshold base peak of 0.001, the software distinguished 12 peaks, with molecular weights ranging from 1032 to 3336 Da (Table 2). In 74 of the 88 processed samples, five or more peaks were identified; no peaks were extracted by the program in the remaining 14 spectra, which were excluded from the analyses. Typical spectra of *D. pinea*, *D. seriata* and *D. scrobiculata* are presented in Fig. 1. The multivariate test of significance showed that all factors and interactions had significant effects in determining the chemical composition of the three taxa (Table 2), with species membership and period of sampling giving the highest *F* values (Table 2). In the univariate results, seven peaks differed significantly amongst species and five peaks amongst the four collection periods (weeks 1, 4, 7 and 10) (Table 3, Figs 2 and 3). Two peaks also showed a significant interaction between species and period (Table 3, Fig. 3), and strain membership had a significant effect on three peaks. The peak representing the compound of 3152 m/z identified *D. pinea* without showing any effects with age, but differed between strains (Fig. 2). The peak of 1164 m/z identified *D. seriata*, and the peak at 1541 m/z identified *D. scrobiculata* (Fig. 2); these peaks, however, had no effects for age and strain. Other peaks showed minor effects (Figs 1–3).

The discriminant analyses performed in the first two periods revealed two significant functions (function 1, explained 56.4% of variance, Wilk's Lambda 0.283, $p < 0.001$; function 2, explained 43.6% of variance, Wilk's Lambda 0.565, $p < 0.001$) but had a low discriminant power (76.1% and 69.6% of cases correctly assigned for overall analyses and for cross-validation tests, respectively) (Fig. 4a). Peaks at 1032, 1164, 3152 and 3336 m/z were entered into the stepwise model. The discriminant analyses performed in the third and fourth periods were more useful in discriminating between species. The two discriminant functions (function 1, explained 89.1% of variance, Wilk's Lambda 0.008, $p < 0.001$; function 2, explained 10.9% of variance, Wilk's Lambda 0.233, $p < 0.001$) correctly attributed 100% of cases in the overall analyses and 93.5% in cross-validation tests with only one misidentified sample per species (Fig. 4b).

4 Discussion

Different species of *Diplodia* showed different compositions in polar metabolites detected by MALDI-TOF analyses. Indeed, there was a reduced set of peaks typical for each species, but this characterization was more evident in spectra obtained from mycelia older than 4 weeks. Some of the compounds showing species-specific differences represented high percentages of the overall amount of the detected metabolites. For instance, the peak at 3152 m/z, which was diagnostic for *D. pinea*, attained approximately 80% frequency in most samples from this species, but showed frequencies lower than 10% in the other two species. It can be concluded therefore that most variance in chemical composition of these metabolites was correlated with species membership.

The extent of phenotypic variation was unexpected in this clade. The taxonomy of the Botryosphaeriaceae is complicated by the existence of cryptic species. Several taxonomic studies on Botryosphaeriaceae used DNA sequences from the ITS rDNA gene, but focus on this single gene may result in an underestimation of the true extent of the differences between cryptic and closely related species (Slippers and Wingfield 2007). Accordingly, *D. pinea* and *D. scrobiculata* have been recognized as distinct species on the basis of multiple gene analysis from sequences of six protein-coding genes (De Wet et al. 2003). The occurrence of qualitative differences in MALDI-TOF spectra clearly confirmed the specific diversification

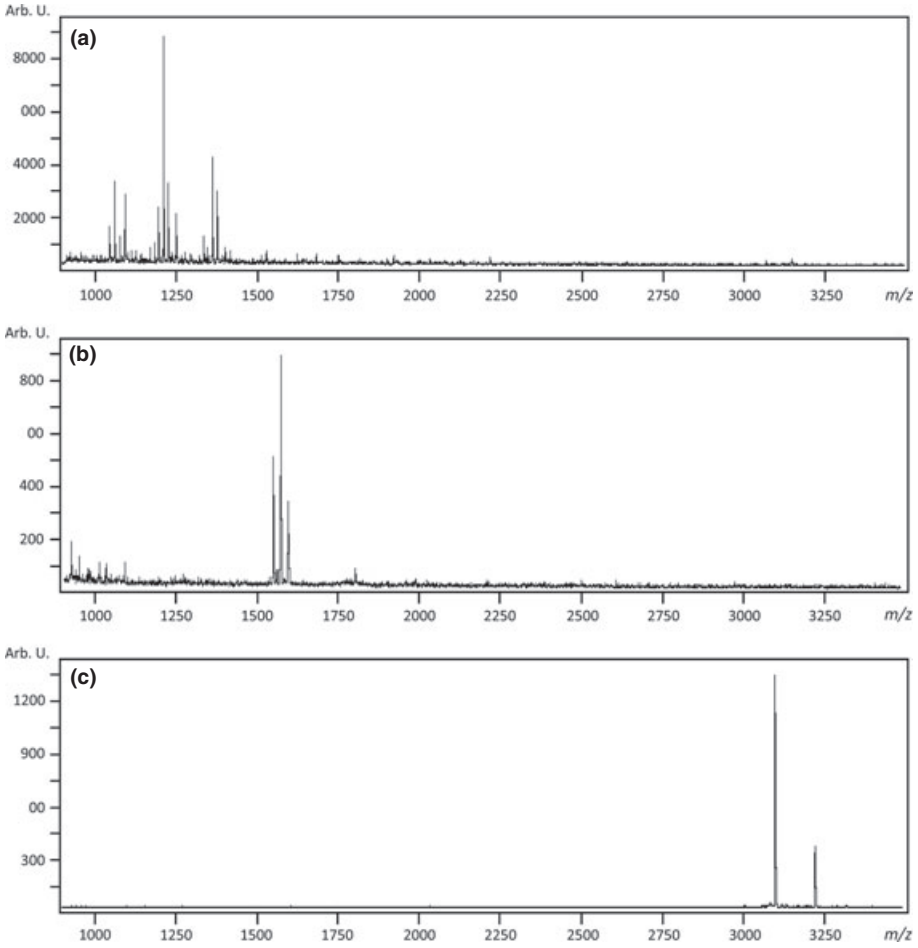

Fig. 1. MALDI-TOF reference spectra of *Diplodia seriata* (a), *Diplodia scrobiculata* (b) and *Diplodia pinea* (c); *x*-axes represent m/z values, as $z = 1$, values of m/z are equivalent to daltons; *y*-axes represent peak abundance as arbitrary units, as detected by MALDI-TOF.

Table 3. Univariate *F* and p values for species, sample and species × sample interactions obtained by the general linear model on the 12 compounds revealed by MALDI-TOF analyses. As $z = 1$, values of m/z are equivalent to daltons. Degrees of freedoms are indicated for all variables (d.f.), the d.f. for error are 48. Significant values are highlighted in bold.

Peaks m/z	*F* sample (d.f. = 1)	p sample	*F* species (d.f. = 2)	p species	*F* species × samples (d.f. = 2)	p species × samples	*F* strain (d.f. = 19)	p strain
1032	4.395	**0.041**	0.645	0.529	0.576	0.566	2.429	**0.007**
1096	10.953	**0.002**	0.007	0.992	1.070	0.351	1.514	0.123
1164	0.188	0.666	5.191	**0.009**	0.142	0.867	1.605	0.094
1541	10.344	**0.002**	10.329	**<0.001**	5.060	**0.010**	1.374	0.185
1565	10.438	**0.002**	5.034	**0.010**	3.235	**0.048**	0.663	0.835
3137	0.006	0.939	9.573	**<0.001**	0.103	0.902	2.079	**0.021**
3152	1.485	0.229	20.556	**<0.001**	1.003	0.374	2.423	**0.007**
3169	3.688	0.061	0.874	0.423	2.860	0.067	1.203	0.293
3179	4.520	**0.039**	0.666	0.518	2.288	0.112	0.967	0.512
3197	1.482	0.229	3.680	**0.033**	0.494	0.613	1.272	0.245
3282	0.064	0.801	6.314	**0.004**	0.724	0.490	1.326	0.211
3336	0.877	0.354	0.086	0.917	1.141	0.328	0.655	0.841

between these taxa and provided further evidence of the power of this technique in providing biochemical fingerprints useable for species recognition. It is also important to note that the isolates examined were collected in different continents (Table 1). Therefore, any phenotypic differences detected using MALDI-TOF were not linked to drift phenomena or local adaptations but represent constant characteristics of these taxa on a global scale.

Based on the data obtained in this work, however, the useful features of this technique only applied to extracts from cultures older than approximately 1 month. It is reasonable to assume that the physiology of mycelia changes with time due

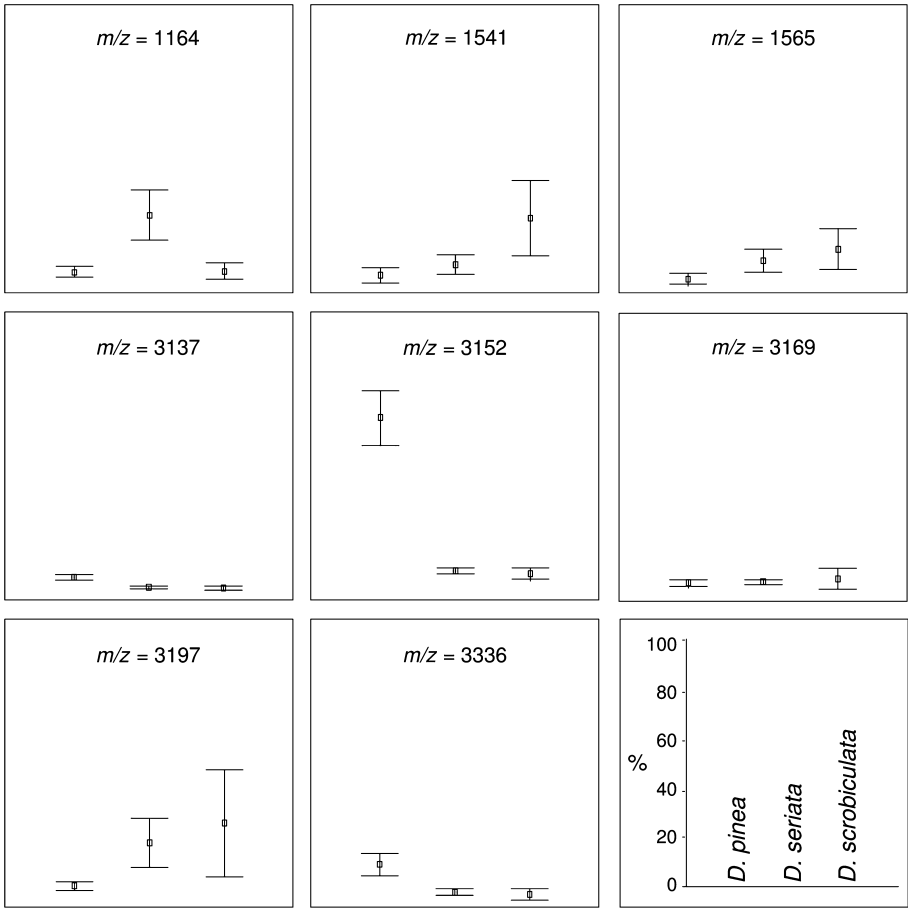

Fig. 2. Mean percentages (± SE) of compounds showing significant differences in Table 1; m/z are equivalent to daltons.

to differences in nutrient uptake, pigmentation and lysis, which could influence the metabolite contents (Schmidt and Kallow 2005). Indeed, Linder et al. (2005) found that the main MALDI-TOF peak characterizing *Ophiostoma* spp. conidia was the proteinaceous toxin cerato-ulmin, which represents both a species-specific fitness factor for the fungus and is one of the most active toxins in Dutch elm disease. The accumulation of such substances can be assumed to change according to the age of mycelia.

Moreover, as revealed by significant effects on the random factor of strains, different strains expressed different metabolite compositions. Despite the overall *F* value of the strain random factor being lower than those obtained for other determinants (Table 2), some of the peaks responsible for differences between species showed relatively high variances (Figs 2–3). This allowed a precise identification of the species only by means of a multivariate discriminant analysis: any attempt to provide precise threshold values of percentages for any diagnostic peak would have been misleading. For this reason, the percentage data of the samples analysed in this work are provided in Appendix S1, so that the data set may be re-used in the future to identify new sets of samples.

At present, there is no information on the chemical nature of the metabolites detected in this work; nevertheless, this is largely irrelevant to the objective of the study, which was to ascertain whether MALDI-TOF could be used to confirm current taxonomy with phenotypic evidence and to provide new diagnostic opportunities. Dedicated MALDI software and online libraries (e.g. MALDI typing) have been created to automate the process of acquiring mass spectra and performing library matching for the recognition of microorganisms. The great advantage of this approach is that information on peak position, intensity and frequency is sufficient to match the observed spectra with the library data.

In conclusion, the data obtained in this work provided two main pieces of information. Firstly, MALDI-TOF spectra can be used to confirm DNA-based taxonomy by revealing phenotypic differences between previously recognized taxa. In addition, the MALDI technique has the great advantage of being extremely rapid (much faster than genetic analyses) and is increasingly used in mass spectrometry and proteomics laboratories (which reduces costs). Nevertheless, the results presented here suggest that some caution should be used in applying MALDI-TOF for fungal taxonomy: different strains of a species tend to express slightly different metabolite compositions, and mycelium age is also an important factor. As these factors can certainly bias any identification based on precise thresholds, it is suggested that recognition should be made on the basis of multivariate techniques and on relatively old mycelia expressing the species-specific markers.

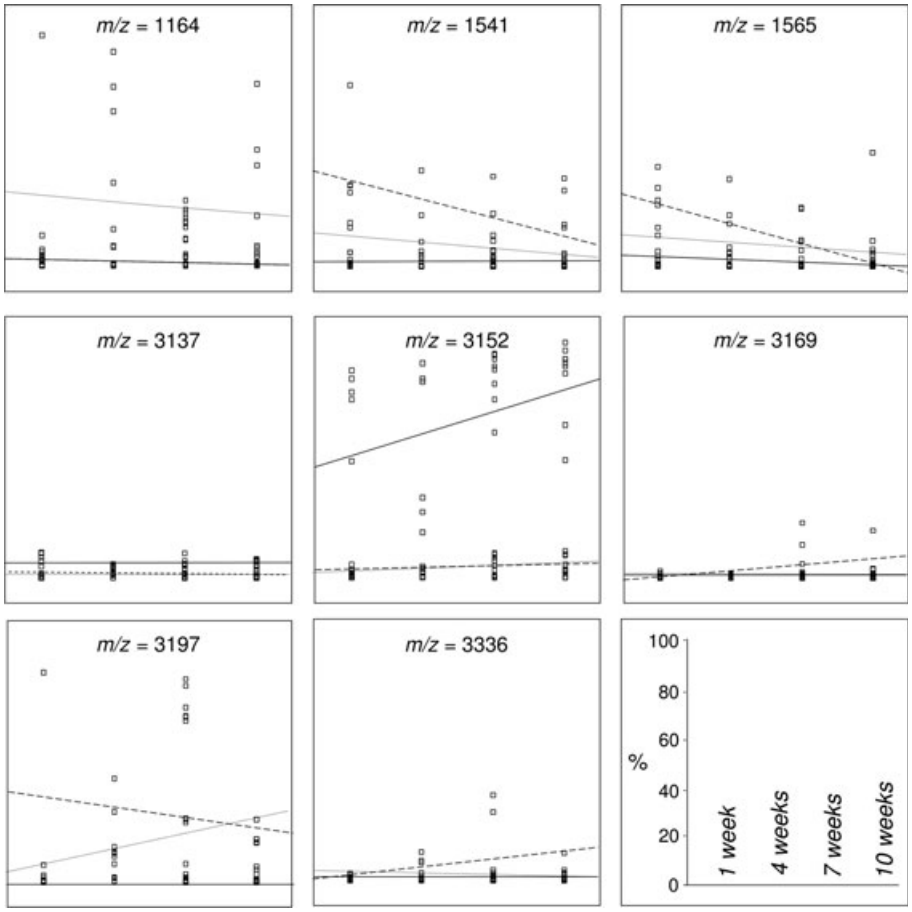

Fig. 3. Values for single analyses of compounds showing significant differences in Table 1. The lines represent individual trend lines for the three species (solid line, *Diplodia pinea*; dotted line, *Diplodia seriata*; dashed line, *Diplodia scrobiculata*); m/z are equivalent to daltons.

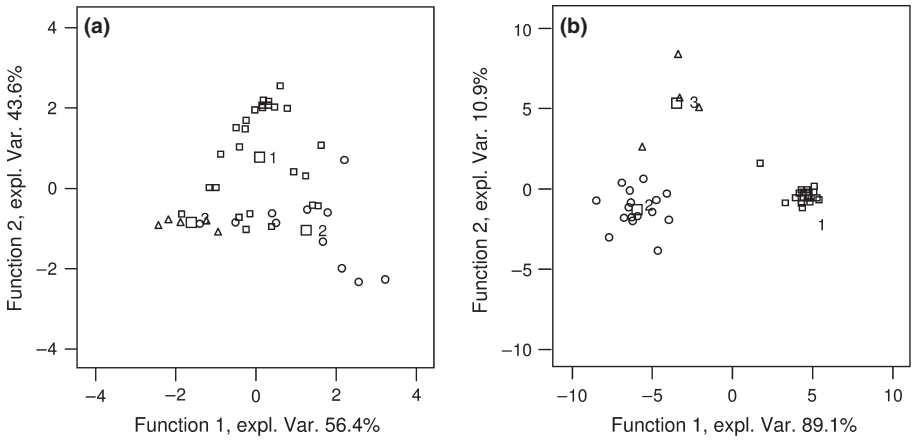

Fig. 4. Discriminant scores showing the separation based on differences in chemical composition between *Diplodia pinea* (squares), *Diplodia seriata* (circles) and *Diplodia scrobiculata* (triangles) on early (less than 1 month after culture, (a) and late (1 month or more after culture, (b) collections; expl.var =: explained variance, numbered squares represent centroids of groups (1, *D. pinea*; 2, *D. seriata*; 3, *D. scrobiculata*).

Acknowledgements

The authors thank J.R. Úrbez-Torres (Department of Plant Pathology, University of California), M. Jaspers (Faculty of Agriculture and Life Sciences, New Zealand), S. Savocchia (National Wine and Grape Industry Centre, Charles Sturt University, Australia), M.J. Wingfield (Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa), G.R. Stanosz (Department of Plant Pathology, University of

Wisconsin – Madison), P. Bonello (Department of Plant Pathology, The Ohio State University, Columbus, USA) and C. Villari (Università degli Studi di Padova) for providing us with isolates of *Diplodia* spp. VM and LD were funded through the PINITALY project. We also thank two anonymous referees for their constructive comments on an early version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Appendix S1. Percentage data for the peaks used in the present study.