

Matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry for identification of plant pathogenic *Alternaria* species

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Abstract Identification of microorganisms by intact cell matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is an emerging technology. In this work, intact MALDI-TOF MS was applied for rapid identification of plant pathogenic *Alternaria* species based on analysis of protein markers. An analysis of 60 isolates belonging to 12 *Alternaria* spp. showed characteristic protein finger prints that were specific at species level. When mass fragment data were subjected to partial least squares regression (PLS-R), all these 12 species formed separate groups. The method therefore permits rapid and direct identification of *Alternaria* spp. from small quantities of fungal mycelium.

Keywords Biomarker · Intact-cell mass spectrometry · MALDI TOF mass spectrometry · Peptide

Introduction

The genus *Alternaria* is an economically important plant pathogen, causing a wide range of diseases on a variety of crops including cereals, ornamentals, oil-yielding

crops, vegetables, spices and fruits worldwide (Rotem 1994; Thomma 2003). Several species also cause post-harvest crop losses and contaminate food and feed through the production of mycotoxins (Montemurro & Visconti 1992; Rotem 1994). In addition, *Alternaria* species are gaining significance as emerging human pathogens (Rossman *et al.* 1996) and as potent airborne allergens (Karlsson-Borga *et al.* 1989). Since they are widely distributed, extremely variable and have high impact on humans and crops, a precise and correct identification of *Alternaria* species is crucial for taxonomic purposes and formulating effective control strategies (Hong *et al.* 2005; Rotem 1994).

The identification and classification of *Alternaria* species were based mainly on morphological characteristics of conidium such as size, septation, presence and/or size of beak and conidiation patterns. Because of overlap in these criteria, *Alternaria* species have been placed into one of several species groups (Simmons 1992), each of which is typified by a representative species. Due to a limited number of overlapping morphological criteria, various researchers debated the use of these morphological characters as the sole criteria for delineating species (Andersen *et al.* 2001, 2002; Kusaba & Tsuge 1995; Rotem 1994). Molecular approaches based on RAPD (Morris *et al.* 2000; Pryor & Michailides 2002; Roberts *et al.* 2000; Weir *et al.* 1998), RFLP (Adachi *et al.* 1993; Aradhya *et al.* 2001; Pryor & Michailides 2002), karyotype (Akamatsu *et al.* 1999), restriction site information from the intergenic spacer (IGS) region of nuclear rDNA (Hong *et al.* 2005), and gene sequence analysis of ITS,

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IGS, mt SSU, glyceraldehyde 3-phosphate dehydrogenase (GPD), mt LSU, β -tubulin, endo-polygalacturonase (endo-PG), anonymous genomic regions (OPA1-3 and OPA2-1) and Alt a 1 (de Hoog & Horre 2002; Peever *et al.* 2004; Pryor & Bigelow 2003; Pryor & Gilbertson 2000, 2002) have been used in *Alternaria* systematics and have supported only organization of *Alternaria* species into distinct species-groups due to lack of sufficient genetic information. The analysis of polypeptides/proteins using Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has been successfully employed for rapid and reliable identification of bacterial taxa (Degand *et al.* 2008; Mellmann *et al.* 2009; Minan *et al.* 2008) and yeasts (Marklein *et al.* 2009; Qian *et al.* 2008). There are a few publications reporting the use of MALDI-TOF MS for identification of fungi, namely, *Antrodia*, *Candida*, *Conidiophora*, *Penicillium*, *Serpula*, and *Trichoderma* (Chen & Chen 2005; Qian *et al.* 2008; Schmidt & Kallow 2005). To the best of our knowledge, no studies have been carried out using this technology for identification of *Alternaria* spp. The objective of the present study was to assess whether analysis of proteomics using MALDI-TOF MS could be used for rapid identification of *Alternaria* species affecting crop plants.

Materials and methods

Fungal isolates Sixty morphologically and genetically well characterized isolates belonging to 12 species of *Alternaria* collected from different geographical locations in India were used for analysis. Table 1 provides details of the isolates, geographical origin, host source and GenBank accession numbers for ITS region of rDNA. Isolates were maintained on slants of potato dextrose agar (PDA) at 4°C. Cultures were grown on PDA medium at 25±1°C for 5 days in the dark.

MALDI-TOF MS The matrices α -cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB) and 3,5-dimethoxy-4-hydroxycinnamic acid (SA) were used for MALDI TOF MS analyses in order to detect the maximum number of peptides or proteins in *Alternaria* isolates. Three matrices consisted of 10 mgm⁻¹ saturated solutions of CHCA (70% acetonitrile in Milli-Q grade water containing 0.1% trifluoroacetic acid TFA), DHB (50% ethanol in Milli-Q grade water containing 0.1% TFA, or SA (50%

Table 1 *Alternaria* isolates used in the present study

Fungal isolates	Host	Location/State	NCBI accession No
<i>Alternaria solani</i>			
OTA 22	Tomato	Karnataka	HQ270459
OTA 66	Tomato	Uttar Pradesh	JF796063
OTA 73	Tomato	Karnataka	JF491204
OTA 78	Tomato	Karnataka	JF491209
OTA 81	Tomato	Karnataka	JF491212
<i>Alternaria porri</i>			
OOA 2	Onion	Karnataka	JF710495
OOA 6	Onion	Karnataka	JF710488
OOA 8	Onion	Karnataka	JF710494
OOA 12	Onion	Karnataka	JF710497
OOA 13	Onion	Karnataka	JF710491
<i>Alternaria brassicicola</i>			
OCA 7	Cauliflower	Karnataka	JF710522
OCA 8	Cauliflower	Meghalaya	JF710521
OCA 11	Cauliflower	Sikkim	JF710517
OCA 10	Cauliflower	Sikkim	JF710518
OCA 12	Cauliflower	Assam	JF710519
<i>Alternaria brassicae</i>			
OCA 2	Rape seed mustard	New Delhi,	JF710515
OCA 3	Rape seed mustard	Himachal Pradesh	–
OCA 4	Rape seed mustard	Assam	JF710516
OCA 5	Rape seed mustard	Meghalaya	–
OCA 15	Rape seed mustard	Delhi	–
<i>Alternaria sesami</i>			
OSA 11	Sesame	Karnataka	JF710582
OSA 12	Sesame	Andhra Pradesh	JF710583
OSA 09	Sesame	Tamil Nadu	JF710584
OSA 08	Sesame	Karnataka	JF710585
OSA18	Sesame	Karnataka	–
<i>Alternaria ricini</i>			
OAR 1	Castor	Andhra Pradesh	JF710543
OAR 2	Castor	Karnataka	JF710544
OAR 3	Castor	Tamil Nadu	–
OAR 4	Castor	Andhra Pradesh	–
OAR 5	Castor	Andhra Pradesh	–
<i>Alternaria carthami</i>			
OAc1	Safflower	Andhra Pradesh	JF 710541
OAc2	Safflower	Andhra Pradesh	JF710542
OAc3	Safflower	Karnataka	–
OAc4	Safflower	Maharashtra	–
OAc5	Safflower	Karnataka	–
<i>Alternaria alternata</i>			
OTA29	Tomato	Karnataka	JF710500

Table 1 (continued)

Fungal isolates	Host	Location/State	NCBI accession No
OTA 11	Tomato	Andhra Pradesh	HQ270456
OTA48	Tomato	Jammu and Kashmir	JF796070
OTA56	Tomato	Himachal Pradesh	JF796071
OTA 65	Tomato	Uttar Pradesh	JF796077
<i>Alternaria burnsii</i>			
OCuAb1	Cumin	Rajasthan	—
OCuAb4	Cumin	Gujarat	—
OCuAb3	Cumin	Rajasthan	—
OCuAb4	Cumin	Rajasthan	—
OCuAb5	Cumin	Rajasthan	—
<i>Alternaria macrospora</i>			
Am 1	Cotton	Karnataka	—
Am 2	Cotton	Andhra Pradesh	—
Am 3	Cotton	Tamil Nadu	—
Am 7	Cotton	Karnataka	—
Am 9	Cotton	Andhra Pradesh	—
<i>Alternaria mali</i>			
OAAM1	Apple	Jammu and Kashmir	—
OAAM2	Apple	Jammu and Kashmir	—
OAAM3	Apple	Himachal Pradesh	—
OAAM4	Apple	Himachal Pradesh	—
OAAM5	Apple	Himachal Pradesh	—
<i>Alternaria dauci</i>			
OCAD1	Carrot	Tamil Nadu	—
OCAD2	Carrot	Tamil Nadu	—
OCAD3	Carrot	Tamil Nadu	—
OCAD4	Carrot	Tamil Nadu	—
OCAD5	Carrot	Tamil Nadu	—

acetonitrile in water containing 0.1% TFA. MALDI-TOF MS analyses of *Alternaria* isolates were carried out using 5-day-old fungal mycelium. Approximately 50 µg of fungal mycelium was scraped from the culture plates and spotted on a MALDI plate MTP 384 Ground Steel, Target Plate (Bruker Daltonics, Bremen, Germany). Then, 1 µl of matrix solution was immediately added to the sample and the sample matrix mixture was allowed to evaporate at room temperature prior to analysis. MS analyses were performed in positive linear mode in the range of 2,000–

20,000 mass-to-charge ratio m/z (higher region) using Ultraflex TOF/TOF, Flex Analysis 2.0 (Bruker) equipped with 50 Hz N₂ Laser. Averaged profile spectra fulfilling the quality criteria were collected from 600 laser shot cycles. For every sample, four to ten averaged profile spectra were stored and used for analysis.

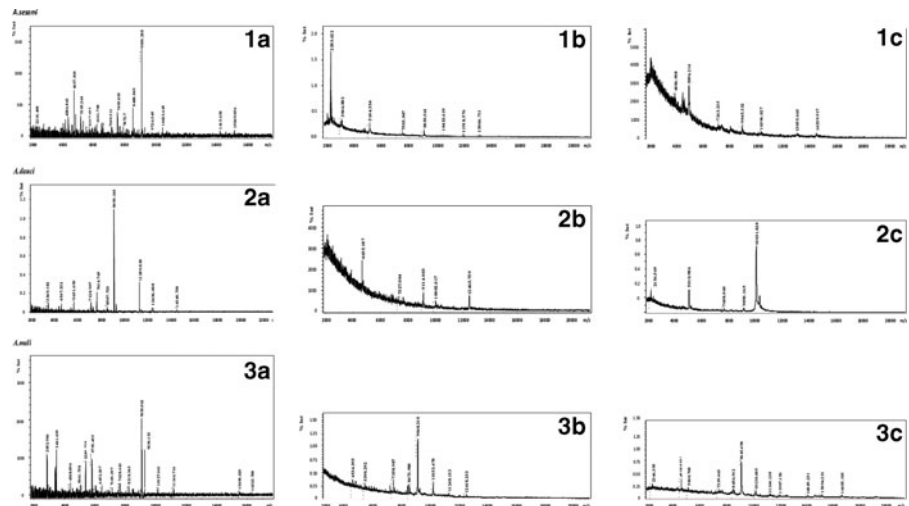
Data analysis For visual inspection of the mass spectra, Flex Analysis 2.0 software (Bruker Daltonik GmbH, Bremen, Germany) was used. Raw spectra were processed using the MALDI Biotyper 1.1 (Bonn) and 2.0 (Leipzig) software (Bruker Daltonics) with default settings. The software performs smoothing, normalization, baseline subtraction, and peak picking, thereby creating a list of the most significant peaks of the spectrum (m/z values).

Reproducibility of MALDI-TOF-MS identification Each isolate of *Alternaria* spp. was examined on five different MALDI-TOF plates run on five different days from five different batches of culture to confirm the reproducibility of MALDI-TOF-MS identification. For each culture condition, four different spots were loaded on the MALDI-TOF plate, giving a total of 20 MALDI-TOF-MS protein profiles for each isolate.

Partial Least Squares Regression (PLS-R) The binary matrix was made manually by scoring each protein mass peak as present or absent from the printed chromatograms and subjected to multivariate method using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). This full manual protein mass peak consisted of 171 x-variables and 12 y-variables (60 isolates belonging to 12 *Alternaria* species). The matrix was analyzed using PLS-R, which relates the variations in response variables (y-matrix) to the variations of several predictors (x-matrix) (Wold *et al.* 2001). By plotting the PLS components one can view main associations between x-variables (protein mass peaks) and y-variables (fungal isolates), and also interrelationships within x- and y-data.

Cluster analysis The binary matrix data were subjected to cluster analysis using DARWin version 5.0 (Perrier *et al.* 2003; Perrier & Jacquemoud-Collet 2006) using the unweighted neighbor joining method as clustering method. The distance matrix was also analyzed by Jaccard's similarity co-efficients in DARWin.

Fig. 1 The MALDI-TOF mass spectra of *Alternaria* isolates obtained with (a) SA, (b) CHCA, (c) DHB; (1) *A. sesami*, (2) *A. dauci*, (3) *A. mali*



Results

The MALDI-TOF spectra of the *Alternaria* isolates using SA, DHB or CHCA as matrices are shown in Fig. 1. When SA was used as matrix, the number and intensity of the peaks were considerably greater than when DHB or CHCA was used as matrix. The spectra were weak and generally had a more complicated background with DHB or CHCA as matrix. When

DHB or CHCA were used as matrix, MALDI TOF spectra were obtained only with three species such as *A. sesami*, *A. dauci* and *A. mali*. Thus, SA was chosen as matrix for MALDI TOF analysis of *Alternaria* isolates.

Patterns in the proteome region of 2,000 to 20,000 Da were recorded using SA as matrix (Table 2). A total of 140 different masses were observed. A unique MALDI-TOF MS profile was obtained for isolates belonging to *A.*

Table 2 Biomarker peptide masses of 12 *Alternaria* species detected by ICMS

<i>Alternaria</i> species	Mass (m/z)									
<i>A. solani</i> OTA6	2845.28	2972.77	4167.17	4753.51	6229.20	8285.54	9059.30	12462.04	—	—
<i>A. porri</i> OOA2	2050.22	4406.59	4709.02	5572.26	6174.16	9082.50	11945.16	12364.08	—	—
<i>A. brassicicola</i> OCA1	2161.48	3152.06	4552.44	4914.15	5376.13	5915.67	6589.76	7461.54	8109.46	9050.29
	12471.69	15536.69	17672.27	—	—	—	—	—	—	—
<i>A. brassicae</i> OCA3	1287.67	5377.44	9041.88	10034.85	12473.33	—	—	—	—	—
<i>A. alternata</i> Aa4	3064.01	3342.96	5026.74	10036.45	12471.31	—	—	—	—	—
<i>A. sesami</i> OSA12	2218.41	2667.69	2706.80	4286.48	4657.81	5105.26	6141.71	6554.16	7109.40	7495.06
	7872.59	8488.85	9050.29	9726.55	10423.65	14158.64	15069.06	—	—	—
<i>A. ricini</i> OAR1	2895.62	3784.84	4087.30	4267.09	4287.09	4697.52	4770.62	5102.91	5270.85	7493.59
	7855.16	8491.23	8845.33	9049.54	9923.04	12471.93	14535.09	17694.83	150766.33	—
<i>A. carthami</i> OSAA1	4288.58	4657.48	5074.88	6453.77	7496.89	9050.06	—	—	—	—
<i>A. macrospora</i> Am1	2277.83	3442.38	4286.48	4657.48	5072.63	6042.82	7110.26	7493.59	7870.76	8488.85
	9049.54	9255.03	9922.85	10425.26	10650.43	11912.25	12472.07	14156.09	14535.79	17666.34
<i>A. burnsii</i> OCuAb1	2513.99	3209.54	4071.40	4606.76	4808.76	4915.41	5103.26	5376.13	7626.14	8492.35
	8860.69	9049.05	9254.30	9570.34	12472.24	14514.90	17723.20	—	—	—
<i>A. mali</i> OAAM1	2852.91	3441.35	4368.89	5061.76	5380.24	5795.46	6046.94	6452.21	7109.40	7628.44
	8218.34	9050.06	9256.12	10127.66	11166.72	15498.03	16232.71	—	—	—
<i>A. dauci</i> OCAD1	4547.33	5651.69	7120.61	7614.77	8507.73	9099.10	11289.84	12436.01	14548.76	33699.18

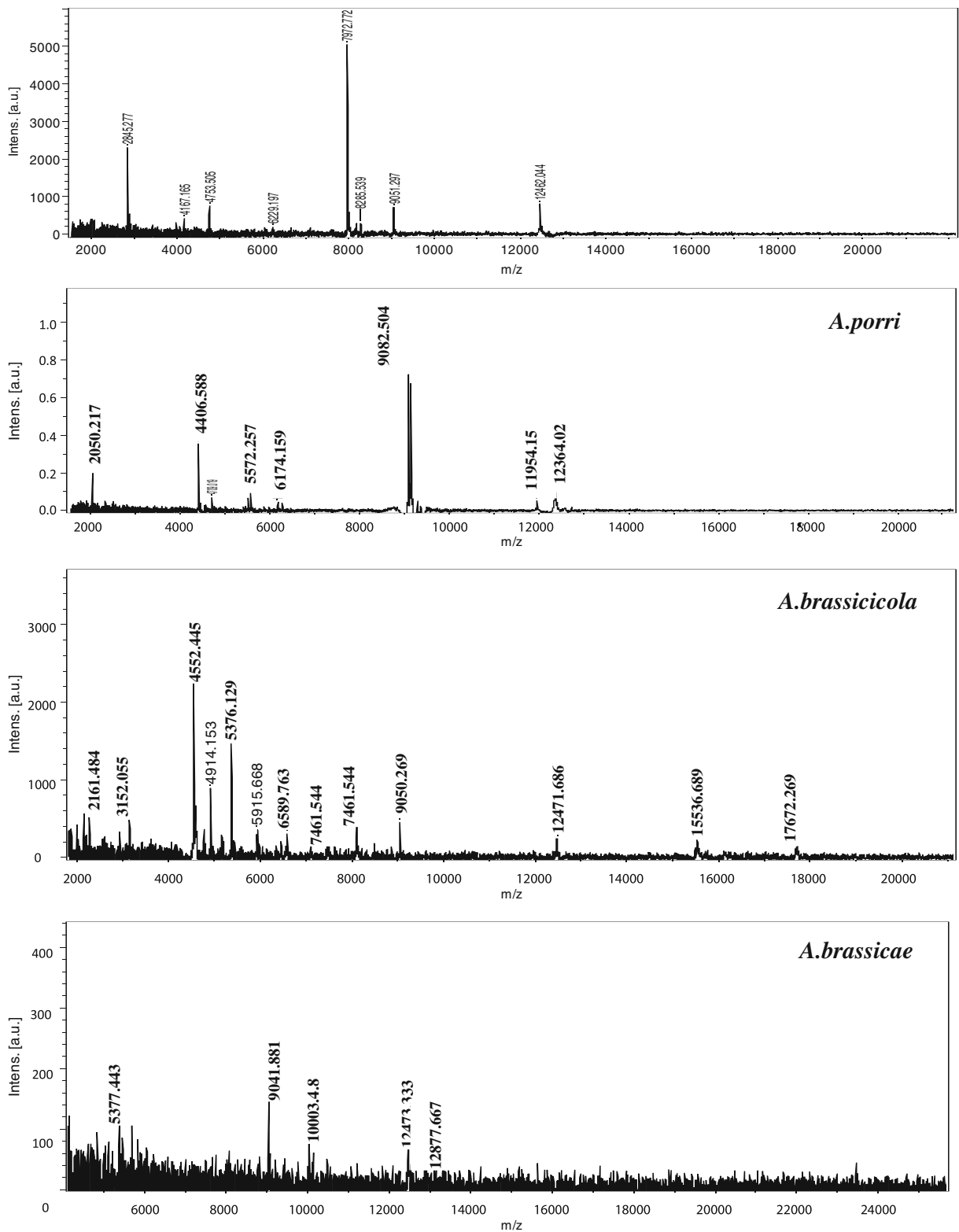


Fig. 2 Protein profile of *Alternaria* spp. generated by MALDI-TOF-MS

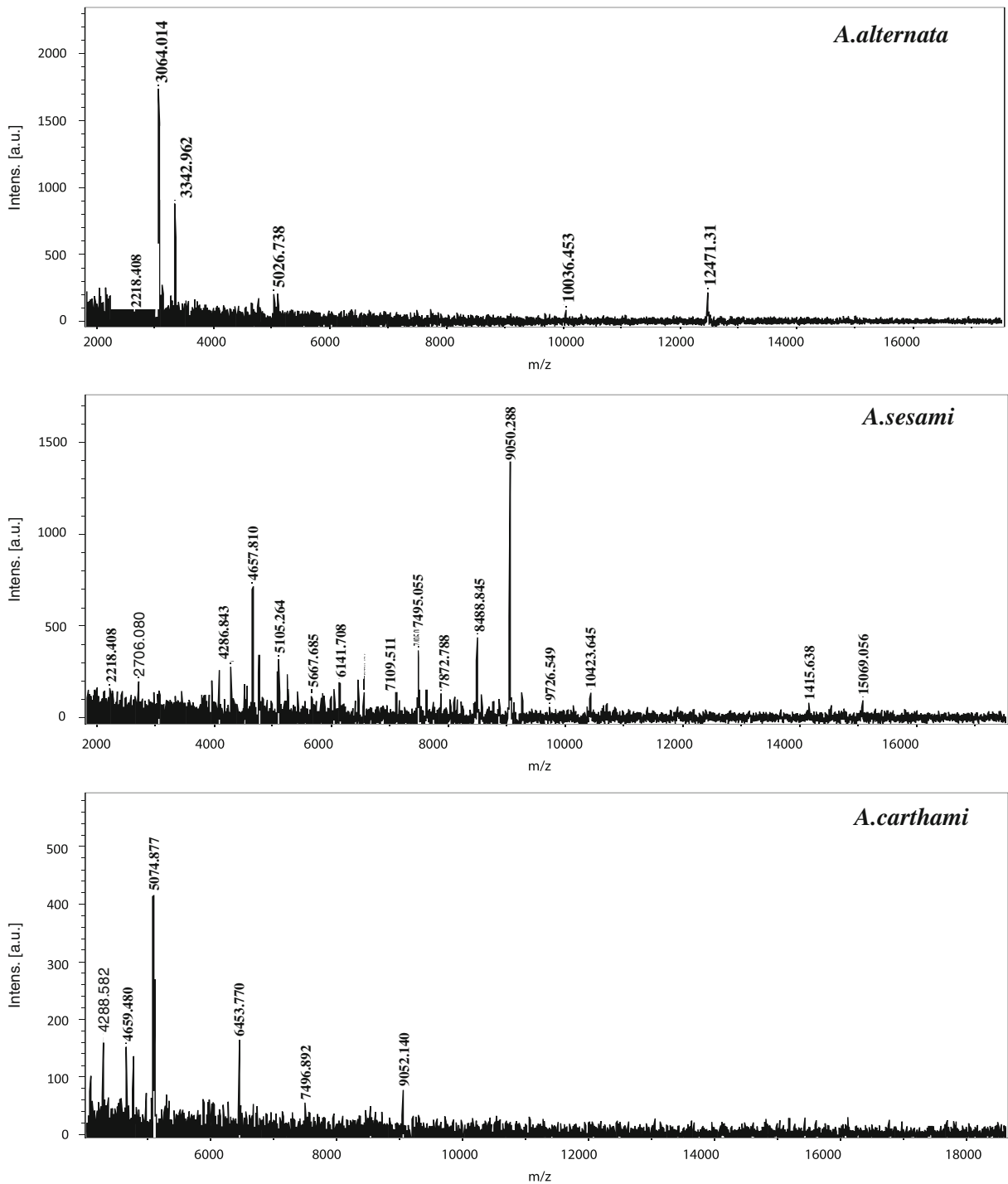


Fig. 2 (continued)

solani, *A. porri*, *A. alternata*, *A. brassicae*, *A. brassicola*, *A. sesami*, *A. macrospora*, *A. ricini*, *A. carthami*, *A. burnsii*, *A. mali* and *A. dauci* (Fig. 2, Table 2). For

example, all isolates of *A. solani* share the masses m/z 2845, 2972, 4167, 4753, 6229, 8285, 9059, which were not present in other *Alternaria* spp. including closely

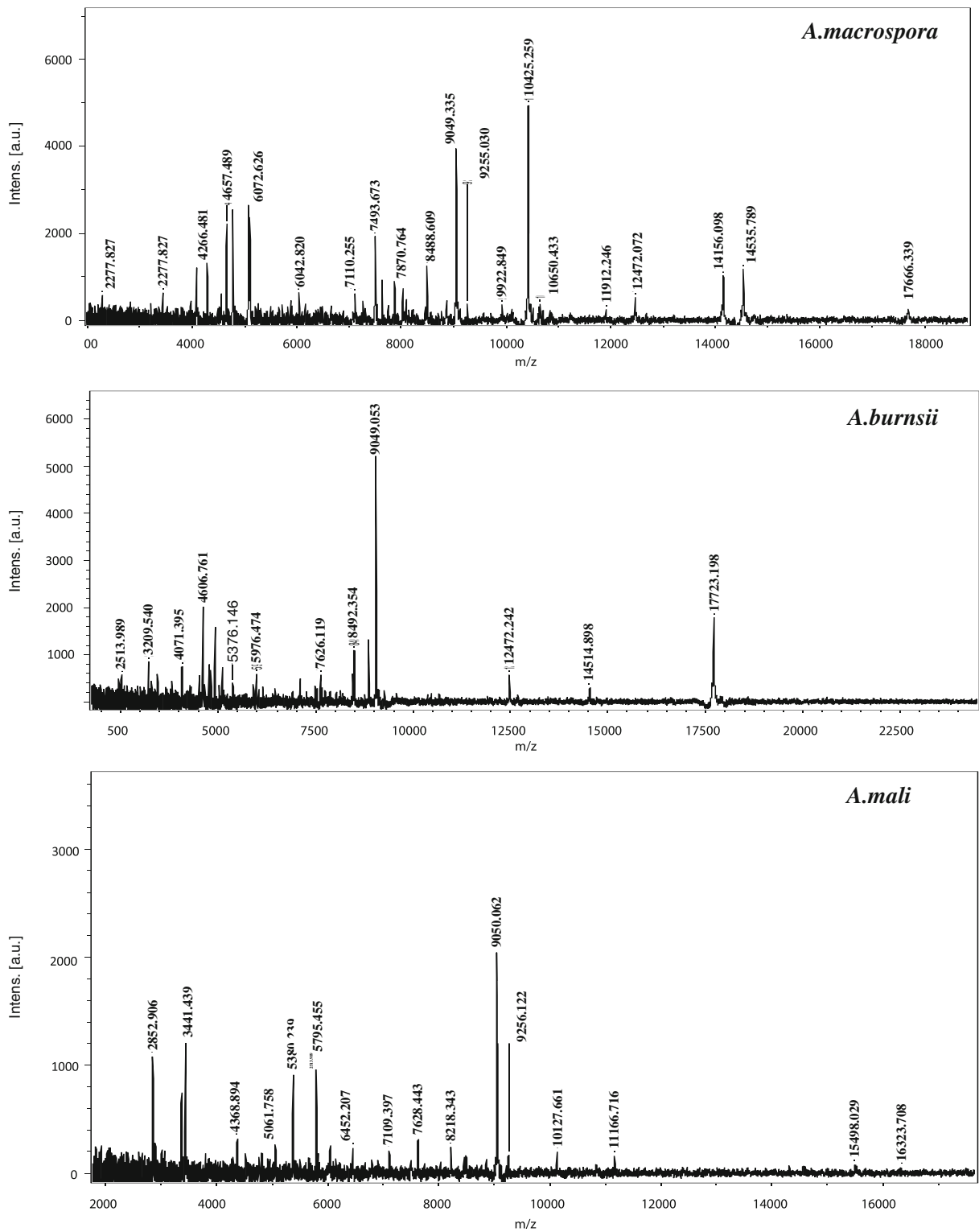


Fig. 2 (continued)

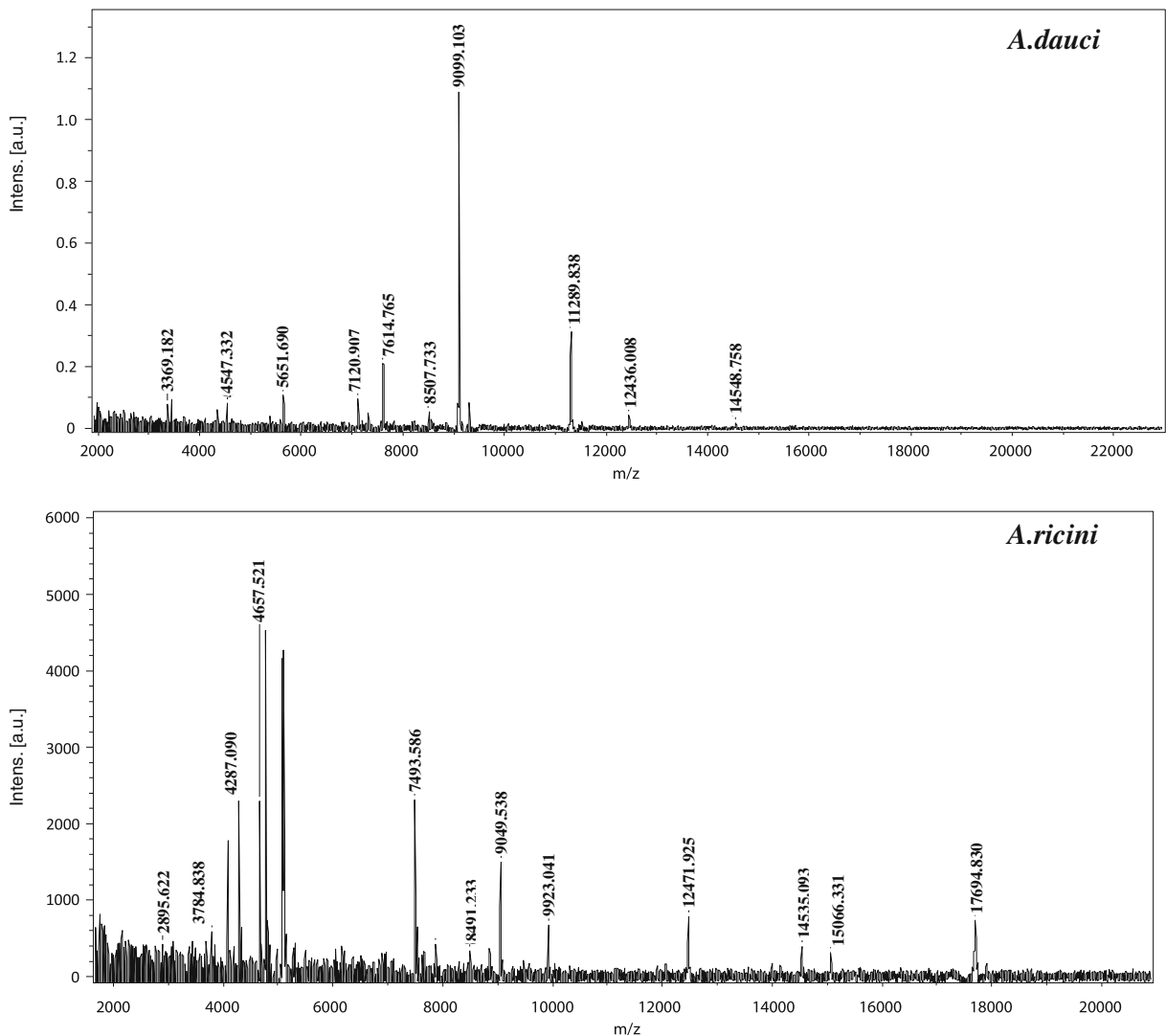


Fig. 2 (continued)

related species, namely, *A. porri* (m/z 2050, 4406, 4709, 5572, 6174, 9082, 11,945), *A. macrospora* (m/z 2277, 3442, 4286, 4657, 5072, 6042, 7493, 7870, 8488, 9255, 9922, 10425, 10650, 11912, 14156, 14536, 17666) and *A. dauci* (m/z 4547, 5651, 7120, 7614, 8507, 9099, 11290, 12436, 14549). *A. mali*, which has been referred to as a special form of *A. alternata*, has mass peaks (m/z 2852, 3441, 4368, 5061, 5380, 5795, 6046, 6452, 7109, 7628, 8218, 9256, 10128, 11167, 15498, 16232) that are distinct from *A. alternata* (m/z 3064, 3343, 5026, 10,036). *A. burnsii* had characteristic m/z 2514, 3209, 4071, 4606, 4808, 4915, 5103, 5376, 5976, 6451, 7626

8492, 8860, 9254, 9570, 14515, 17723 that were different from *A. mali* and *A. alternata*.

The isolates from oil-yielding crops, namely, *A. sesami* (m/z 2218, 2667, 2706, 4286, 4657, 5105, 6141, 6554, 7109, 7495, 7872, 8288, 9726, 10424, 14159, 15069), *A. carthami* (m/z 4288, 4657, 5074, 6453, 7496) and *A. ricini* (m/z 2895, 3784, 4087, 4267, 4287, 4697, 4770, 5102, 5270, 7493, 7855, 8491, 8845, 9049.5, 9923, 14535, 17695) had distinct finger prints. *A. brassicae* (m/z 1287, 5377, 9041) and *A. brassicicola* (m/z 2161, 3152, 4552.4914, 5376, 5916, 6590, 7462, 8109, 15537, 17672) from cole

crops also had characteristic protein profiles. These masses serve as biomarkers for these species. The MALDI-TOF MS profiles for each isolate derived from different plates and different batches of culture on different days were identical. The Partial Least Squares Regression model based on mass finger prints of *Alternaria* spp. is represented in Fig. 3. The isolates belonging to different species formed as separate clusters.

The cladogram based on ICMALDI-TOF MS resolved isolates belonging to different species of *Alternaria* into different groups (Fig. 4), indicating the potential of the method for identification and classification of *Alternaria* species according to their characteristic ICMALDI-TOF MS patterns. However, it is important to note that this kind of analysis may not establish phylogenetic relationships.

Discussion

The precise and rapid identification of *Alternaria* spp. is of major concern for implementing timely effective disease control measures. MALDI-TOF MS technology has been designed to provide a characteristic mass spectral fingerprint based on desorbed ions from the intact cell surface (Pignone *et al.* 2006). Since very few metabolites have been reported to appear in the observable molecular mass range between 2,000 and 20,000 Da these metabolites can be easily used as biomarkers (Santos *et al.* 2010). The selection of appropriate matrix for protein analysis by intact MALDI-

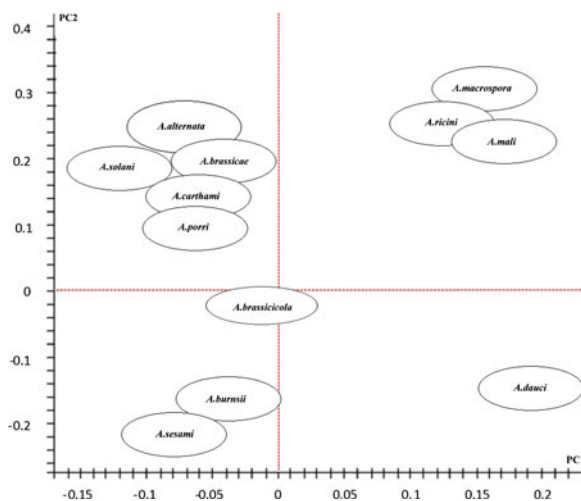


Fig. 3 PLS-R of the mass fingerprint matrix of 12 *Alternaria* spp.

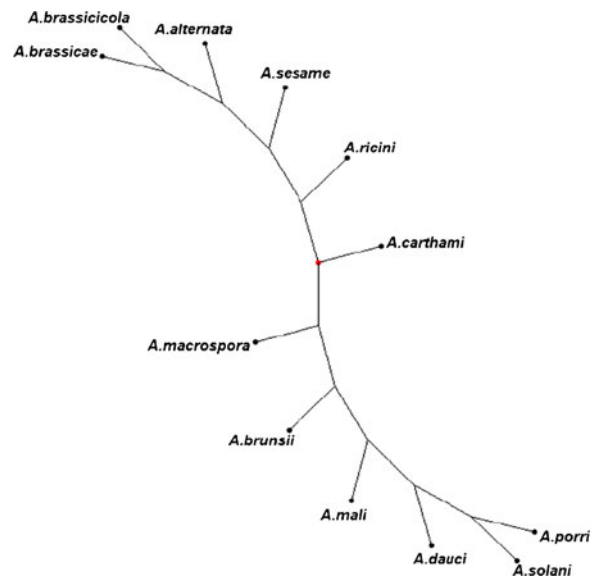


Fig. 4 Cladogram of *Alternaria* species as revealed by analysis of intact MALDI-TOF MS protein profiles

TOF MS is pre-requisite (Grassl *et al.* 2011). In the present study, for MALDI-TOF MS spectra of proteins in intact mycelium of *Alternaria* isolates, SA was the optimal matrix. Both DHB and CHCA failed to produce good signal to noise spectra for *Alternaria* isolates. Indeed, SA has been reported as the matrix of choice for MALDI-TOF MS of proteins in intact tissues (Chaurand *et al.* 2008; Deininger *et al.* 2010; Kruse & Sweedler 2003) whereas for peptides, DHB or CHCA are better suited (Deininger *et al.* 2010). Furthermore, using SA as a matrix, good reproducibility in ion intensity as well as spatial resolution was observed for analysis of intact tissues (Grassl *et al.* 2011). DHB, on the other hand, produces data with better signal-to-noise ratio; however, the large crystals produce a ring-like layer and cause non-homogeneous ionization across the spot or section (Lemaire *et al.* 2006). CHCA has been reported to produce more multiple charged ions than SA (Chaurand *et al.* 2008), which may be unfavorable for protein analysis.

In the present study, all the 12 species of *Alternaria* exhibited characteristic spectral masses, which could be used as specific biomarkers for differentiation and identification. The most important mass peaks were situated between m/z 8,000 and 10,000. Part of these proteins may be hydrophobins, as previously described (Neuhof *et al.* 2007b). Hydrophobins, small proteins of 75 to 400 aminoacid residues, are usually present on the outer surfaces of cell walls of hyphae and conidia of

filamentous fungi (Linder *et al.* 2005), probably accounting for the characteristic mass peaks of filamentous fungi. Neuhof *et al.* (2007b) showed that, in intact-cell mass spectrometry, spectra of hydrophobins were the dominating protein masses and no ribosomal proteins have been identified. Another mass spectrometry study of intact-cell with *Sepedonium* strains (Neuhof *et al.* 2007a) also confirmed that these signals could be used as specific biomarkers for differentiation below the species level. The second dominating peptides were found at about m/z 2,000–6,000. These two regions make the MALDI-TOF MS spectra quite characteristic for *Alternaria* species. Inspection of the MALDI-TOF MS spectra also revealed differences in the predominance of some masses between m/z 12,000 and 14,000, as well as the number of masses within the m/z 16,000 and 18,000 range. This suggests that not only hydrophobins, but also many other peptides and proteins can be detected by the MALDI-TOF MS system. In contrast, bacterial intact-cell MALDI-TOF spectra in the range 2–20 kDa are dominated by a set of ribosomal proteins (Dieckmann *et al.* 2005). The mass spectra of intact mycelium provide a snapshot of different protein compositions of individual *Alternaria* species and thus constitute species-specific suites of biomarkers. MALDI-TOF identification, therefore, can be used as a more reliable technique for identification of the *Alternaria* isolates. It has the advantages of being very rapid, using small samples and requiring minimal sample preparation.

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