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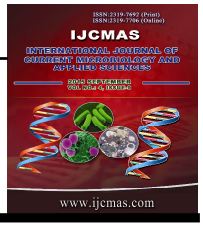

Original Research Article

Proteomics of *Alternaria* species Associated with Field and Horticultural Crops

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Keywords

Alternaria, Proteomics, 2DE, MALDI –TOF MS

A B S T R A C T

Fungal proteomics is newly emerging field to understand plant-pathogen interactions. The profiling and fingerprinting of proteins has proven to be useful for identification of virulence as well as pathogenicity factors of certain plant pathogenic fungi. In this study, analysis of proteins in 12 different plant pathogenic *Alternaria* species such as *A. solani*, *A. porri*, *A. alternata*, *A. brassicicola*, *A. brassicae*, *A. sesame*, *A. macrospora*, *A. ricini*, *A. carthami*, *A. brunsii*, *A.dauci* and *A.mali* isolated from vegetable, oil yielding and seed spice crops was carried out by using 2 dimensional electrophoresis (2DE). Proteins were extracted from 7 days old cultures grown on potato dextrose broth, separated on the basis of their isoelectric point and the identification and characterization of specific proteins was carried out through peptide sequencing of MALDI-TOF-MS. Present findings is one of the first protein fingerprinting studies on Alternaria species for the identification of marker protein(s) which can be serve as biomarker for species identification. This study can also be integrated in a polyphasic approach.

Introduction

Alternaria is a recognized plant pathogen and considered as important cosmopolitan fungi. It is impacting on agricultural and economic output due to causing considerable yield loss by affecting wide range of crops such as cereals, vegetables, ornamentals, oil-yielding, spices, and fruit crops (Srivastava *et al.*, 1964; Harne and Nerna, 1969; Rotem, 1994; Neeraj and Verma, 2010) worldwide. Several species cause post harvest crop losses and contamination of food and feed by production of mycotoxins (Montemurro and Visconti, 1992; Rotem, 1994). In addition, *Alternaria* species are as emerging human pathogen, particularly in immune compromised patients (Anaissie *et al.*, 1989; Rossmann *et al.*, 1996) and spores are as potent airborne allergens that cause asthama (Wilken-Jensen and Gravesen, 1984; Karlsson-Borga *et al.*, 1989). The species of *Alternaria* are known to produce toxic secondary metabolites with varying toxicity and that have been implicated in the development of cancer in mammals (Montemurro and Visconti, 1992; Ostry,

2008; Akimitsu *et al.*, 2014; Rossmann *et al.*, 1996; Logrieco *et al.*, 2009). Since they are widely distributed, extremely viable and have high impact on humans and crops, the precise identification was needed to formulate effective disease control measures.

The identification and classification of *Alternaria* species were primarily based on morphological characters of conidium, conidiation pattern, sporulation and pigmentation. However, because of overlapping of characters these criteria lead to taxonomical confusion and making the identification and classification difficult (Rotem, 1994).

Several molecular studies involving RAPD (Morris *et al.*, 2000; Pryor and Michailides, 2002; Roberts *et al.*, 2000), RFLP (Adachi *et al.*, 1993; Aradhya *et al.*, 2001; Pryor and Michailides, 2002), karyotype (Akamatsu *et al.*, 1999) and gene sequence analysis of ITS, 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 and Horre, 2002; Peever *et al.*, 2004; Pryor and Bigelow, 2003; Pryor *and* Gilbertson, 2000, 2002) have utilized to organize the *Alternaria* species into distinct species-groups, but species within the same species groups and closely related species are often not well separated due to lack of sufficient genetic information (Pryor and Gilbertson, 2000; Roberts *et al.*, 2000).

The chemotaxonomic approach based on profiling of secondary metabolites has been successfully implicated to differentiate morphologically similar species within a genus in Ascomycota (Smedsgaard and Frisvad, 1996; Frisvad, 1987; Frisvad *et al.*, 2008). This has been found to be useful to certain extent in distinguishing species, species-groups, and closely-related taxa in *Alternaria* (Andersen and Thrane, 1996; Andersen *et al.*, 2001, 2002, 2005, 2008).

The analysis of polypeptides/proteins using MALDI-TOF-MS has been employed successfully for 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 reports on the use of MALDI- TOF MS for identification of fungi, namely, *Antrodia*, *Candida*, *Conidiophora*, *Penicillium*, *Serpula*, and *Trichoderma* (Chen and Chen, 2005; Qian *et al.*, 2008; Schmidt and Kallow, 2005) and several proteomic studies have been done for the genera *Aspergillus* (Melin, 2002; Strom, 2005), *Cladosporium*, *Fusarium*, *Colletotrichum* and *Uromyces vignae* species and identified some pathogenecity related factors (Vadlapudi *et al.*, 2011). To the best our knowledge, no studies have been carried out using this technology for identification of *Alternaria* species. Recently, proteomic study was done on 12 different.

Alternaria species by using MALDI-TOF-intact cell mass spectrometry to differentiate individual species and it was noted that characteristic protein finger prints exists at species level (Chowdappa *et al.*, 2013).

The fungal proteomics is the newly emerging field, which will be helpful for understanding plant-fungal interactions, pathogenesis, fungal colonization and to understand virulence factors in pathogenic fungi (Bhadauria *et al.*, 2007; Kim *et al.*, 2007a). Proteomic analyses in fungi also provide an insight related to systematic metabolic flux changes (Shimizu *et al.*, 2005), These studies demonstrated the utility of proteomics to characterize systematically on the various biochemical aspects that might be utilized for the species

identification and expression studies over to changing environments (Kim *et al.*, 2007b).

The objective of present study was to assess whether analysis of proteomics using two dimensional electrophoresis and characterization of specific proteins by peptide sequencing of MALDI-TOF-MS could be used for the rapid identification of *Alternaria* species affecting field and horticultural crops.

Materials and Methods

Fungal isolates

Morphologically and genetically well characterized twelve species of *Alternaria* were collected and isolated from different vegetables, fruits, oil yielding and cole crops from different geographical locations in India were used for analysis. Isolates were cultured and maintained on slants of potato dextrose agar (PDA) at 4°C.

Extraction of soluble protein

Isolates were grown in 250 ml conical flasks containing 100 ml of potato dextrose broth. Each flask was inoculated with three mycelial disks, each of five mm diameter cut from the advancing margin of seven day old cultures grown on PDA at 24±1°C in dark. The inoculated flasks were incubated in dark at 24±1°C for seven days· Then, the mycelial mat was harvested by filtering through Whatman No. 1 filter paper, washed with phosphate buffer (pH 7·0) and damp dried and frozen overnight at -20 °C.

Sample preparation

2 g of freeze-dried mycelium of fungal cultures was ground to fine powder in liquid nitrogen and then homogenized with 1x protein buffer saline (PBS) pH 7.4 and centrifuged at 10000 rpm at 4 ºC for 10 min. The supernatant was collected and dialysis (mention details of dialysis bag) was carried out against glass distilled water. The protein concentrations were determined according to the method of Bradford (1976). 2 mg of protein was taken and added an equal volume of 10% TCA with ice-cold acetone and kept at -20 °C for overnight. Then, centrifuged at 10,000 rpm at 4 ºC for 10 min and collected only precipitate (pellet) and added 1ml of ice-cold acetone, centrifuged at 10,000 rpm at 4 ºC for 10 min and collected only precipitate (pellet).

This step was repeated for 3–4 times. The pellet was air dried (partially) and added to 200 µl of lysis buffer containing 85 mM Tris and 2% SDS pH 6.8. Final concentration was 2 mg protein/200 µl of lysis buffer.

Two dimensional electrophoresis (2DE)

First dimension (Iso electric focusing): The proteins were first separated on the basis of their isoelectric point (PI). It was carried out on the rod gel tube containing 1ml of 4% gel mix (7M urea, 133 µl of 30% acrylamide solution, 20 µl of triton-X 100, 1.5 µl of TEMED and 5 µl of 10% APS) along with carrier ampholyte of pH 3-10.

After gel polymerized, the rod gel tube was fixed to SDS-PAGE unit (ETS-6, Genei, Bengaluru). The gel was pre-focused for 1h with 2 µl of sample loading buffer at 200V. Later 250 µg of protein in 20 µl of sample loading buffer was loaded on the top of the tube gel. Later the electrophoresis was carried out for 10 hours at 250 V.

Gel equilibration: Following rod gel electrophoresis, the gels were equilibrated with buffer containing 50 mM Tris–HCl, 6M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 2% (w/v) DTT, pH 6.8 for 15-20 minutes.

Second dimension (SDS-PAGE)

It was carried out on 12 % homogeneous stacking gel. After polymerization of SDS-PAGE stacking gel, the single lane comb was placed and 1% agarose solution were added. Later, the equilibrated rod gel was placed on the polymerized stacking gel and sealed thoroughly without any air bubbles. Protein marker (97.4, 66, 43, 29, 20.1 and 14.3 kDaa) was loaded in a single well and subsequently electrophoresis was performed for 2h at 150V (ETS-6, Genei, Bengaluru). After that, the resulting gels were stained with Ezee blue solution and destained by using distilled water till the clear bands appear. The image was analysed by using Alpha Imager EP (Alpha Innotech Corporation, San Leandro, CA, USA) and mass and pI were estimated**.**

Peptide sequencing and protein identification by MALDI-TOF MS

Gel spots of 1.5 mm diameter were excised manually from the 1-mm thick gels and washed for 30 min at room temperature under vigorous shaking with 400 µl of 10 mM ammonium bicarbonate solution containing 50 % (v/v) acetonitrile. After removing the supernatant, gel pieces were dried for 15 min in a vacuum concentrator. The rehydrated gel pieces were incubated in 150 µl reduction solution (10 mm DTT, 100 mm ammonium bicarbonate) for 30 min at 50^0 C. The reduction solution was then discarded and 100 µl alkylation solutions (50 mm iodoacetamide, 100 mm ammonium bicarbonate) were added for 30 min in the dark room temperature. For digestion, 5 µl trypsin solutions (Sequencing grade modified trypsin, Promega, Madison and 10 ng/ µl in 5 mM ammonium bicarbonate/5 % acetonitrile) were added to each sample. After incubation for 5 h at 370 C, the reaction was stopped by adding 1 µl of 1 % TFA. For better extraction of peptides, the samples were stored overnight at 50 C. Without further purification, 1 µl of supernatant was mixed with 2 µl of matrix solution (5 mg α-cyano-4-hydroxycinnamic acid in 40 % [v/v] acetone, 50 % [v/v] acetonitrile, 9.9 % [v/v] water and 0.1 % [w/v] TFA in water). From this mix, 1 µl was deposited onto the MALDI target. Tryptic peptides were analysed with a MALDI-TOF mass spectrometer (Bruker-Daltonics, Germany) in positive mode. Background ions from trypsin autolysis and contamination by keratins were removed from mass lists.

The protein identification was performed by searching the resulting protein mass fingerprints against the National Center for Biotechnology Information non-redundant (NCBInr) data base in the latest version using the Mascot search engine. The following parameters were applied: Monoisotopic mass accuracy; peptide mass tolerance (0.1 Da); peptide charge state (1+); missed cleavages (1); allowed variable modifications, oxidation (Met) and fixed modification, carbamidomethyl (C). It has followed some set of criteria for confident identification (Raman *et al.,* 2005), mainly that the protein should (a) have Mascot score greater than 50 ($p < 0.05$), (b) match within 0.05% peptide mass tolerance, (c) have at least 20% sequence coverage, and (d) match at least four peptides (except few high and low MW proteins which satisfied only 3 out of 4 criteria).

Results and Discussion

The protein spots of 12 *Alternaria* spp were recorded in the range of 14.4 to 97.4 KDa (Fig. 1; Table 1). A total of 264 spots were observed from 2DE gel and the unique protein mass was obtained for all the 12 species (Fig. 1; Table 1). The *A. solani, A.*

porri, *A. macrospora* and *A. dauci* which are known to be long spored revealed characteristic species-specific masses. *A. solani* shared the specific mass of 47.54 (16), 38.15 (22), 52.99 (29), 33.85 (32), 29.69 (36), 28 (38), 25.62 (46) and 19.44 (64) kDa, *A. porri* had 74.24 (7), 42.52 (16), 23.75 (28), 22.03 (30), 19.08 (38), 16.98 (43), 15.45 (46), and 15.89 (51) kDa specific proteins, in *A. macrospora* 35.3 (3, 4, 5), 24.81 (7), 23.42 (8) and 19.5 (10) kDa specific proteins were detected and in *A. dauci* 69 (3) and 43 (5) kDa proteins were identified as species-specific. *Alternaria* infecting oil yielding crops such as *A. sesami*, *A. ricini* and *A. carthami* also revealed species-specific proteins. In *A. sesami* three proteins were identified as species-specific such as 38.24 (1, 2) and 37.5 (3) kDa, *A. ricini* had 35.57 (2,3), 33.4 (4, 5), 28.5 (6), 26.5 (8, 9), 19.64 (12), 18.75 (13) and 15.6 (14) kDa proteins are identified as species-specific and in *A. carthami* seven proteins of 46 (4), 37 (6), 40 (8), 27 (10), 25.74 (12), 25.74 (13) and 14.3 (20) kDa are detected as specific. The cole crops such as *A. brassicae* and *A. brassicola* shared the specific mass of 97.7 (1), 64 (4), 36 (6), 39 (8), 23.56 (12), 19.1 (15) and 21.88 (5), 22 (8) kDa proteins respectively. *A. burnsii* and *A. mali* are referred as special forms of *A. alternata* revealed unique metabolite masses compared to *A. alternata* (97.4 (1) and 34.53 (5) kDa). In *A. burnsii* 42 (3, 4) and 15 (14) kDa were identified as specific markers and in *A. mali* 65 (2, 3) kDa protein were identified as species-specific.

The species-specific protein spots were identified from each species and excised from the gels as marked in the figure 1. The identification of specific proteins (individual spots) was carried out by MALDI-TOF MS (Table 2). Significant expectation values were not achieved for all the species-specific spots to give positive identification. The percentage sequence coverage ranged from 18 to 43 %. Several proteins exhibited a deviation in molecular mass to the theoretical mass suggesting the possible occurrence of post-translational modification.

Five proteins were identified as ATP-binding cassette transporter from *A. solani*, 3-ketoacyl- thiolase from *A. alternata*, L-threonine 3-dehydrogenase from *A. brassicae*, CAP1 [*Cryptococcus neoformans* var. *grubii*] from *A. dauci*, ATP synthase subunit alpha, mitochondrial from *A. mali* and seven hypothetical proteins from *A. porri*, *A. brassicicola*, *A. sesami*, *A. macrospora*, *A. ricini*, *A. carthami* and *A. burnsii*. The identified proteins NCBI accession number, experimental as well as theoretical mass and pI, species name, % of matched peptides and sequence and mascot score were shown in the table 2.

The precise and reliable identification of plant pathogenic *Alternaria* species is of major concern for executing effective disease management strategies. 2D gel electrophoresis and MALDI-TOF MS are highly effective techniques has been designed to analyse and identify specific protein fingerprint and are most efficient methods in identification of novel proteins. The proteomic studies played an important role in identification and characterization of pathogenicity as well as virulent factors from filamentous fungi (Stephenson *et al.*, 2000; Gonzalez-Fernandez *et al.*, 2010). The present work attempted for the profiling and identification of species-specific proteins from the 12 different species of pathogenic fungi *Alternaria* using 2DE and MALDI-TOF-MS techniques for the first time. However, the proteomic map and analysis of intracellular proteins were successfully done for the *Aspergillus* species through 2DE and MALDI-TOF (Stephen *et al.*, 2006; Kim *et*

al., 2007) and other filamentous fungi such as *Antrodia, Candida, Conidiophora, Penicillium, Serpula,* and *Trichoderma, Cladosporium, Fusarium, Colletotrichum* and *Uromycesvignae* species (Vadlapudi *et al.*, 2011; Stephenson *et al.* 2000; Chen *and* Chen 2005; Qian *et al.* 2008; Schmidt *and* Kallow 2005).

Table.1 Protein mass of 12 Alternaria species elucidated by 2D Gel Electrophoresis analysis

Alternaria spp.	Mass in KDa (spot number)									
A.solani	**84.12 (3)**	**66 (4)**	**59.64 (5)**	**66 (6)**	**64.18 (7)**	**59.95 (8)**	**59.04 (9)**	**63.58 (10)**	**58.74 (11)**	**59.04 (12)**
	56.32 (13)	**52.68 (14)**	**48.45 (15)**	**47.54 (16)***	**44.82 (17)**	**45.42 (18)**	**42.31 (19)**	**48.14 (20)**	**39.67 (21)**	**38.15 (22)***
		38.43 (24)	**38.84 (25)**	**38.98 (26)**	**38.84 (27)**	**39.12 (28)**	**52.99 (29)***	**36.76 (30)**	**36.21 (31)**	**33.85 (32)***
	33.57 (33)	**29.83 (34)**	**33.3 (35)**	**29.69 (36)***	**28 (37)**	**28 (38)***	**27.16 (39)**	**26.93 (40)**	**26.93 (41)**	**28 942)**
	28.54 (43)	**27.77 (44)**	**27.16 (45)**	**25.62 (46)***	**28.92 (47)**	**28.54 (48)**	**25.62 (49)**	**26.31 (50)**	**24.86 (51)**	**24.47 (52)**
	22.48 (53)	**22.63 (54)**	**22.56 (55)**	**23.17 (56)**	**23.78 (57)**	**22.94 (58)**	**24.09 (59)**	**25.32 (60)**	**20.94 (61)**	**20.64 (62)**
	19.44 (63)	**19.44 (64)***	**20.18 (65)**	**18.85 (66)**	**17.68 (67)**	**16.43 (68)**	**19.22 (69)**	**17.9 (70)**		
A.porri		**67.03 (4)**	**64.74 (5)**	**63.73 (6)**	**74.24 (7)***	**65.75 (8)**	**63.73 (9)**	**59.18 (10)**	**54.63 (11)**	**54.63 (12)**
	42.4 (13)	**49.57 (14)**	**47.8 (15)**	**42.52 (16)***	**42.52 (17)**	**39.89 (18)**	**40.13 (19)**	**38.93 (20)**	**37.14 (21)**	**32.35 (22)**
	27.47 (23)	**25.94 (24)**	**31.63 (25)**	**26.74 (26)**	**24.75 (27)**	**23.75 (28)***	**24.28 (29)**	**22.03 (30)***	**24.15 (31)**	**21.96 (32)**
	20.43 (33)	**23.69 (34)**	**20.9 (35)**	**20.37 (36)**	**19.59 (37)**	**19.08 (38)***	**18.06 (39)**	**20.76 (40)**	**20.23 (41)**	**17.87 (42)**
	16.98 (43)*	**16.91 (44)**	**17.68 (45)**	**15.45 (46)***	**15.89 (47)**	**15.77 (48)**	**14.87 (49)**	**15.7 (50)**	**15.89 (51)***	**16.53 (52)**
	17.81 (53)	**17.55 (54)**	**16.15 (55)**	**16.72 (56)**						
A.alternata	**97.4 (1)***	**66 (2)**	**45 (3)**	**34.5 (4)**	**34.53 (5)***	**38.93 (6)**	**38.93 (7)**	**38.93 (8)**	**38.93 (9)**	**24.55 (10)**
	24.55 (11)									
A.brassicae	**97.7 (1)***	**66 (2)**	**65 (3)**	**64 (4)***	**44 (5)**	**36 (6)***	**39 (7)**	**39 (8)***	**25.79 (9)**	**25.79 (10)**
	24.37 (11)									
	23.56 (12)*	**21 (13)**	**20.1 (14)**	**19.1 (15)***	**18.5 (16)**	**20.1 (17)**	**16.4 (18)**	**16.4 (19)**	**16.1 (20)**	**16.1 (21)**
A.brassicicola	**35 (1)**	**35 (2)**	**29 (3)**	**29 (4)**	**21.88 (5)***	**24 (6)**	**24 (7)**	**22 (8)***	**19 (9)**	
A.sesami	**38.24 (1)***	**38.24 (2)***	**37.5 (3)***	**29 (4)**	**35 (5)**	**33 (6)**	**24.55 (7)**	**24.55 (8)**	**24.55 (9)**	**20.1 (10)**
	21 (11)	**20.1 (12)**								
A.macrospora	**81.7 (1)**	**58 (2)**	**35.3 (3)***	**35.3 (4)***	**35.3 (5)***	**24.81 (6)**	**24.81 (7)***	**23.42 (8)***	**19 (9)**	**19.5 (10)***
	18 (11)									
A.ricini	**34 (1)**	**35.57 (2)***	**35.57 (3)***	**33.4 (4)***	**33.4 (5)***	**28.5 (6)***	**26 (7)**	**26.5 (8)***	**26.5 (9)***	**24 (10)**
	24 (11)	**19.64 (12)***	**18.75 (13)***	**15.6 (14)***	**18 (15)**					
A.carthami	**68 (1)**	**58 (2)**	**45 (3)**	**46 (4)***	**45 (5)**	**37 (6)***	**38.93 (7)**	**40 (8)***	**29 9)**	**27 (10)***
	26 (11)	**25.74 (12)***	**25.74 (13)***	**20.1 (14)**	**16.1 (15)**	**18.5 (16)**	**18.5 (17)**	**16.4 (18)**	**16.1 (19)**	**14.3 (20)***
A.burnsii	**58 (1)**	**44 (2)**	**42 (3)***	**42 (4)***	**34.5 (5)**	**34.5 (6)**	**29 (7)**	**25.79 (8)**	**25.79 (9)**	**26 (10)**
	24.37 (11)	**18 (12)**	**16.1 (13)**	**15 (14)***						
A.dauci	**81.7 (1)**	**68 (2)**	**69 (3)***	**58 (4)**	**43 (5)***	**38 (6)**	**38 (7)**	**33 (8)**	**38 (9)**	**33 (10)**
	25 (11)	**25 (12)**	**21 (13)**	**19 (14)**	**20.1 (15)**	**24 (16)**	**21 (17)**	**19 (18)**	**19 (19)**	
A.mali	**97.4 (1)**	**65 (2)***	**65 (3)***	**38 (4)**	**38 (5)**	**38 (6)**	**25 (7)**	**25 (8)**		

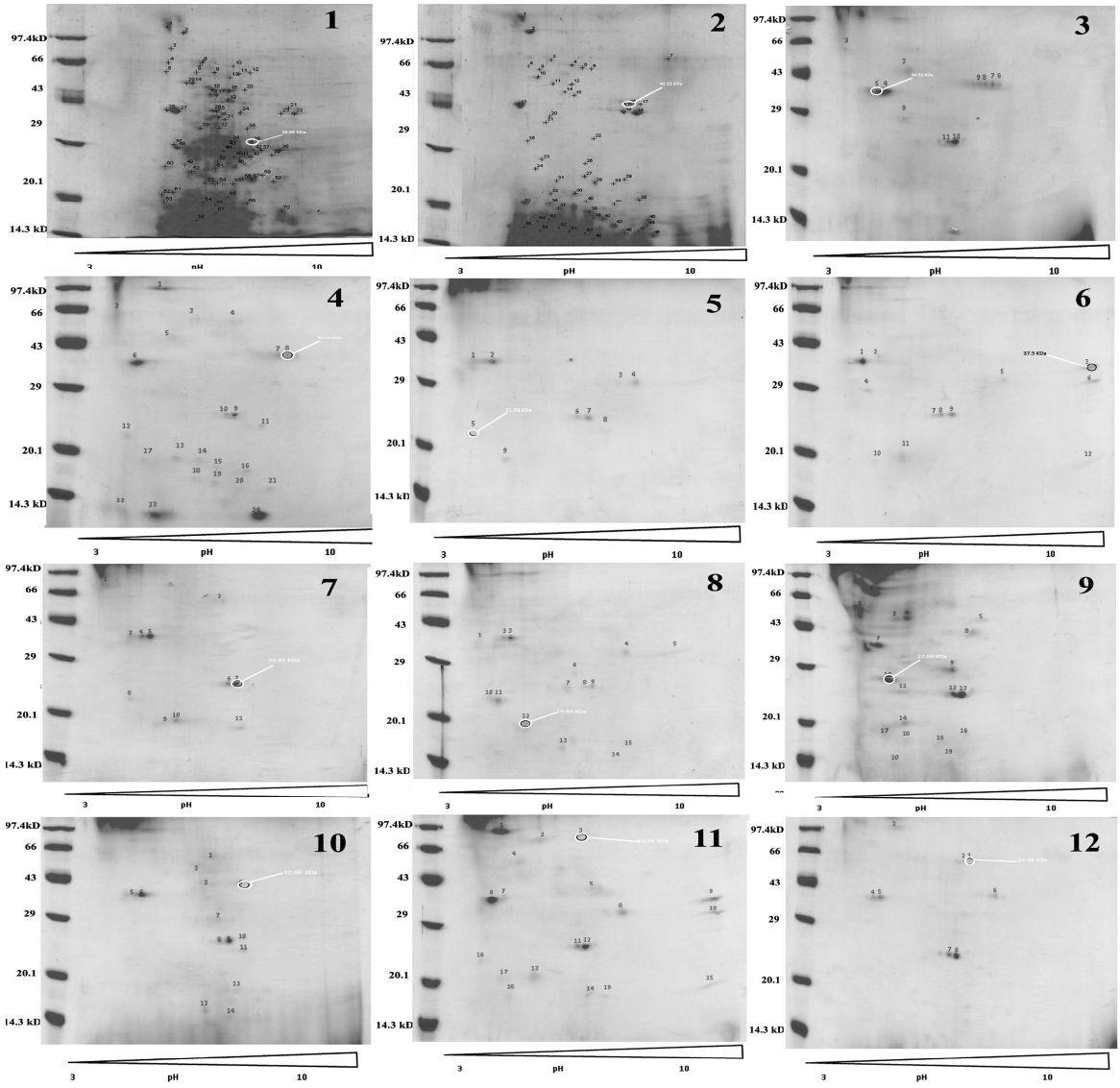
***Species-specific protein mass**

Table.2 List of Identified Species-specific Proteins of 12 Alternaria species by 2D MALDI-TOF MS

Alternaria spp.	Spot No.	Protein name	NCBI accession no.	Exp. Mr/pI	Theo. Mr/pI	Species	Mascot score*	No.of Matched peptides	MS-MS peptide sequence	SC (%)
A.solani	36	ATP-binding cassette transporter	gi|443913558 (NCBInr)	29.69/7.1	29.681/5.83	*Rhizoctonia solani*	40	5	ATVPGVDEHEHQVGRSMNER LQLLTVR QVPGTPTPSR GKINMVAGPTGCGK VGVSHSER	22
A.porri	16	hypothetical protein	gi|759307715	42.52/7.3	50.71/6.48	*Fonsecaea pedrosoi*	49	6	AEAWGGEGR YHEPGFVLTADEDKDAYLR ATGYDNVSGDHGYANFNSGWADAEACVAYALK ATGYDNVSGDHGYANFNSGWADAEACVAYALKR ICWYCDTPTGDFLITSHPR EEVIKNGGDEFIACEDGSR	21
A.alternata	5	3-ketoacyl-thiolase	gi|630204946	34.53/4.3	35.52/9.35	*Moniliophthora roreri*	32	3	DPVEVPVLLITHGYHLASEYSIPR LGLPIVGKFVAAQAVGVPPNIMGVGPTYAIPK VFITSMCIGSGTGVAGVFVTSSEIPYVLLCIFLEFK	28
A.brassicae	8	L-threonine 3-dehydrogenase	gi|754413049	39.00/8.2	40.001/6.46	*Wickerhamomyces ciferrii*	30	4	MTVQDIK QIDTISR VMFVGMGNPIQHLHIGSAALREVDLLGVFR GIENSSKAFEIAGKPFDENGNLVVK	18
A.brassicicola	5	hypothetical protein	gi|685941488	21.88/3.8	21.97/5.22	*Wallemia ichthyophag*	38	3	MLLNKIGLIFSAFAIAIAATAFAQPLVK QGTTITPIALNNTATNTTTTPPR QQLHHLYDQVCDLGDELRTSLPSLVYQQITILG	42
A.sesami	3	hypothetical protein	gi|591423198	37.5/9.65	39.40/7.5	*Fusarium oxysporum f. sp. radicis-lycopersici*	58	10	MTQALELAYKYCR TQALELAYK ASGLEILVANVNTK MINNMGSTNQK EICIFELIK EICIFELIKSTWHQQFNR LMLHHSEDK LMLHHSEDKDFWVESMPVLMELCFHFK AALDDEQASER REMLQR	28
A.macrospora	7	hypothetical protein	gi|628302732	24.81/7.0	35.69/9.4	*Capronia epimyces*	38	5	VINSDTIEAALQLPRR HYPIPDKASIYSPTVLVIR DPSQLPVISVVSAAAIYKPDTK FAAAIAGDVYAR KIVLGAFGCGAFENPLK	26
A.ricini	12	hypothetical protein	gi|662529364	19.64/4.9	24.89/9.7	*Aureobasidium pullulans*	45	5	SATPSSPDEQDVEERLDTLALPSPAVPLSEPSPHAR IVPTRDR ARHDPLR TEPIRVR TQPNGAAGPQIPTEPDVAAAQRLGLPPVR	37
A.carthami	10	hypothetical protein	gi|671181210	27/4.7	26.25/6.8	*Cladophialophora carrionii*	59	8	GVSDPVRGLAR GVSPGTYHATIR GAASTGGIWEAVKQMTGIGQPR GMFGTVQVGK DGRGSAFLDRPVSVWEIIGR SFQTDDPDTLVGVIARSAGVWDNEK TVWQER TVWQERK	43
A.burnsii	4	hypothetical protein	gi|672235455	42/7.15	34.56/9.2	*Nematocida sp*	38	3	TYCNKCIDGGLDYVYTSDIMFNK EYSPNEIVGFIGGAISNINMVGTGNAR LYALSRIESAQELFIGNDYSSHASEEWMSTELDVTGDDLVDSCSCNSK	32
A.dauci	3	CAP1 [Cryptococcus neoformans var. grubii]	gi|56566238	69/ 6.2	73.28/6.2	*Cryptococcus neoformans var. grubii*	50	9	HPSSPLDK SHSPHRGLVQWWSPLAGEEPLGVLK GWDGVYNKLK WESLLASQSKTLPQAVTEYTR YGRPPPR ETPYTSQITLSPTGPSSLYGER ETPYTSQITLSPTGPSSLYGERAHSSR YLTEEELK EDGTCDEMEHEIIFK	18
A.mali	3	ATP synthase subunit alpha, mitochondrial	gi|703994567	65/6.65	58.93/9.08	*Candida albicans*	50	12	VARPTLLTAQR VVDGLGNPIDGK AQVKAPGILPR APGILPRR ELIIGDR KLYCVYVAVGQK HALIVYDDLSK QLSLLLR QLSLLLRRPPGR LFLAQYR GERLTQLLK LTQLLKQK	17

*Mascot scores greater than 44 are statistically significant ($p \leq 0.05$) (Raman *et al.*, 2005),

Fig.1 Protein profiling of 12 *Alternaria* species by 2D Gel Electrophoresis: 1. *A.solani*, 2. *A.porri*, 3. *A.alternata*, 4. *A.brassicae*, 5. *A.brassicicola*, 6. *A.sesami*,7. *A.macrospora*, 8. *A.ricini*, 9. *A.carhami*, 10. *A.burnsii*, 11. *A.dauci*, 12. *A.mali*



Among them, cell wall degrading proteins, inhibitory proteins (van Esse *et al.* 2008), and enzymes involved in the synthesis of toxins (Friesen *et al.* 2008, Gao and Kolomiets, 2009, Kim *et al.* 2008, Lawrence, 2008; Vadlapudi *et al.*, 2011). Several studies also showed that identified proteins are involved in cellular protection (Cu, Zn-superoxide dismutase), general metabolism (malate dehydrogenase; nucleotide diphosphate kinase; and aconitase), energy production (ATP synthase, 3-isopropylmalate dehydrogenase, glyceraldehydes 3-phosphate dehydrogenase and enolase) and signal transduction pathways (cyclophilin) (Chandler *et al.*, 2008).

In the present study, all the 12 species of *Alternaria* revealed characteristic protein

fingerprints. The species-specific proteins were identified from 2D gel for each species and specific protein spots were analysed and characterized by peptide sequencing through MALDI-TOF MS.. Among them five are identified as known and seven were recognized as hypothetical proteins (Table 2), which could be used as specific biomarkers for differentiation and identification. The identified known proteins were shown to be involved in certain biological processes, for example ATP-binding cassette (ABC) transporter which utilizes the energy of ATP to transport a specific substrate across the cell membrane like ions, sugars, amino acids, phospholipids, cholesterol, peptides, polysaccharides, proteins or other ligands (Jones and George, 2004; Ponte-Sucre 2009). The 3-ketoacyl thiolase enzyme has key role in many vital biochemical pathways, including the beta oxidation pathway of fatty acid degradation and various biosynthetic pathways (Germain *et al.,* 2001). The L-threonine 3-dehydrogenase enzyme participates in glycine, serine and threonine metabolism (Green and Elliott 1964). CAP1 protein is involved in the cyclic AMP pathway and which is having divergent roles in virulence factor production in two varieties of the fungal pathogens (Julie *et al.,* 2004). ATPases (or ATP synthases) are membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and hydrolysis with the transport of protons across a membrane.

The identified fungal proteins have been shown to be located in the cell wall. Recently it was noted that three proteins such as catalase, ATP synthase (subunit β) and phosphoglycerate mutase secreted by *A. flavus* as cell wall proteins (Medina *et al.,* 2005).

However, more systematic and focused approach is required to further elucidate the identification and characterization of fungal cytoplasmic proteins and also proteomic data can serve to validate the predictive bioinformatic tools to predict intron–exon splice sites in fungal genes (Carberry *et al.,* 2006). Therefore, 2DE and peptide sequencing analysis of fungal mycelium provide a snapshot of different protein compositions of individual *Alternaria* species. Thus, the present study identified species-specific proteins, which can be serve as biomarkers for identification of *Alternaria* species.

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References

Adachi, Y., Watanabe, H., Tanabe, K., Doke, N., Nishimura, S., Tsuge, T., 1993. Nuclear ribosomal DNA as a probe for genetic variability in the Japanese pear pathotype of *Alternaria alternata*. *Appl. Env. Microbiol.,* 59: 3197–3205.

Akamatsu, H., Taga, M., Kodama, M., Johnson, R., Otani, H., Kohmoto, K., 1999. Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. *Cur. Gen.,* 35: 647–656.

Akimitsu, K., Tsuge, T., Kodama, M., Yamamoto, M. Otani, H. 2014. *Alternaria* host selective toxins: determinant factors of plant disease *J. Gen. Plant Pathol.,* 8: 109–122.

Anaissie, E. J., Bodey, G.P., Rinaldi, M. G., 1989. Emerging fungal pathogen.

European J. Clin. Microbiol. Infect. Dis., 8: 323–330.

Andersen, B., Dongi, A. Pryor, M.B. 2008. Secondary metabolic profiling of *Alternaria dauci*, *A.pori*, *A.solani* and *A. tomatoplila*. *Mycol. Res.,* 112: 241–250.

Andersen, B., Kroger, E., Roberts, G.R. 2001. Chemical and Morphological segregation of *Alternaria alternate*, *A.gaisen* and *A. longipes*. *Mycol. Res.,* 105: 291–299.

Anderson, B. Hansen, M.E., Smedsgaard J. 2005. Automated and unbiased image analysis as tool in phenotypic classification of small spored *Alternaria spp*. *Phytopathol.,* 95: 1021–1029.

Anderson, B., Kroger, E., Roberts, G.R. 2002. Chemical and morphological segregation of *Alternariaarborescens, A. infectoria*and *A. tenuissima*species-groups. *Mycol. Res.,* 106: 170–182.

Anderson, B., Thrane, U., 1996. Differentiation of *Alternaria infectoria* and *Alternaria alternata* based on morphology, metabolite profiles, and cultural characteristics. *Can. J. Microbiol.,* 42: 685–689.

Aradhya, M.K. Chan, H.M., Parfitt, D.E. 2001. Genetic variability in the pistachio late blight fungus, *Alternaria alternata*. *Mycol. Res.,* 105: 300–306.

Bhadau Logrieco, A., Moretti, A., Solfrizzo, M. 2009. *Alternaria* toxins and plant diseases: and overview of origin, occurrence and risks. *World Mycot. J.,* 2: 129–140.

Bhadauria, V., Zhao, W., Wang, L., Zhang, Y., Jun-Hua Liu Yang, J., Ling-An Kong, You, L. P. 2007. Advances in fungal proteomics. *Microbiol. Res.,* 162: 193–200.

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem.,* 72: 248–254.

Carberry, S., Neville, M. C., Kavanagh, A. K., Doyle, S. 2006. Analysis of major intracellular proteins of *Aspergillus fumigates* by MALDI mass spectrometry: Identification and characterisation of an elongation factor 1B protein with glutathione transferase activity. *Biochem. Biophys. Res. Commu.,* 341: 1096–1104.

Chandler, M.J., Treece, R.E., Trenary, R.H.,Brenneman, L.J., Flickner, J.T., Frommelt, L.J.,Zaw, M., Patterson, M.M., Rundle, T.W., Valle, V.O., Kim, D.T., Walker, R.G., Cooper, R.C. 2008. Protein profiling of the dimorphic, pathogenic fungus, *Penicillium marneffei. Proteome Sci.,* 6: 1–18.

Chen, H.Y., Chen, Y. C. 2005. Characterization of intact Penicillium spores by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spec.,* 19: 3564–3568.

Chowdappa, P., Jyothi Lakshmi, M., Madhura, S. 2013. Matrix assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry for identification of plant pathogenic *Alternaria* species. *Phytoparasitica,* 41: 169–179.

de Hoog, G.S., Horre, R. 2002. Molecular taxonomy of the *Alternaria* and *Ulocladium* species from humans and their identification in the routine laboratory. *Mycoses,* 45: 259–276.

Degand, N.E., Carbonnelle, B., Dauphin, J. L., Beretti, B.M., Le I., Sermet-Gaudelus, C. 2008. Matrix assisted laser desorption ionization–time of flight mass spectrometry for identification of non-fermenting gram-negative bacilli isolated from cystic fibrosis patients. *J. Clin. Microbiol.,* 46: 3361–3367.

Friesen, T. L., Faris, J. D., Solomon, P. S., Oliver, R. P. 2008. Host-specific toxins: effectors of necrotrophic pathogenicity. *Cellular Microbiol.*, 10(7): 1421–1428.

Frisvad, J.C. 1987. High-performance liquid-chromatographic determination of profiles of mycotoxins and other secondary metabolites. *J. Chromatogr.*, 392: 333–347.

Frisvad, J.C., Andersen B., Thrane U. 2008. The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. *Mycol. Res.*, 112: 231–240.

Gao, X., Kolomiets, M. V. 2009. Host-derived lipids and oxylipins are crucial signals in modulating mycotoxin production by fungi. *Toxin Rev.*, 28(2-3): 79–88.

Germain, V., Rylott, E.L., Larson T.R., Sherson S.M., Bechtold N., Carde J.P., Bryce J.H., Graham I.A., Smith S.M. 2001. Requirement for 3-ketoacyl-CoA thiolase-2 in peroxisome development, fatty acid beta-oxidation and breakdown of triacylglycerol in lipid bodies of Arabidopsis seedlings. *Plant J.*, 28(1): 1–12.

Gonzalez-Fernandez, R., Prats, E., Jorrin-Novo, V.J. 2010. Proteomics of Plant Pathogenic Fungi, Review. *J. Biomed. Biotech.*, Pp. 1–36.

Green, M.L., Elliott, W.H. 1964. The enzymic formation of aminoacetone from threonine and its further metabolism. *Biochem. J.*, 92 (3): 537–49.

Harne, S.M., Nerna, K.G. 1969. Seed borne fungi of some vegetables. *JNKVV Res. J.*, 3(2): 130–131.

Jones, P.M., George, A.M. 2004. The ABC transporter structure and mechanism: perspectives on recent research. *Cell Mol. Life Sci.*, 61(6): 682–99.

Julie, K., Hicks Cletus, A., D'Souza Gary, M., Cox, Joseph, H. 2004. Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans. Eukaryot Cell*, 3(1): 14–26.

Karlsson-Borga, A., Jonsson, P., Rolfsen, W. 1989. Specific IgE antibodies to 16 widespread mold genera in patients with suspected mold allergy. *Ann. Allergy, Asthma Immunol.*, 63: 521–526.

Kim, K. S., Min, J. Y., Dickman, M. B. 2008. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant-Microbe Interact.*, 21: 605–612.

Kim, Y., Nandakumar, M. P., Marten, M. R. 2007a. Proteomics of filamentous fungi. *Trends Biotech.*, 25: 395–400.

Kim, Y., Nandakumar, M.P., Marten, M.R. 2007b. Proteome map of *Aspergillus nidulans* during osmoadaptation. *Fungal Gen. Bio.*, 44: 886–895.

Lawrence, C. B., Mitchell, T. K., Craven, K. D., Cho, Y., Cramer, R. A., Kim, K. H. 2008. At death's door: *Alternaria* pathogenicity mechanisms. *Plant Pathol. J.*, 24(2): 101–111.

Lou, J., Fu, L., Peng, Y., Zhou, L. 2013. Metabolites from *Alternaria* fungi and their bioactivities. *Molecules*, 18: 5891–5935.

Marklein, G., Josten, M., Klanke, U., Muller, E., Horre, R., Maier, T. 2009. Matrix-assisted laser desorption ionization–time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. *J. Clin. Microbiol.*, 47: 2912–2917.

Medina, M.L., Haynes, P.A., Breci, L., Francisco, W.A. 2005. Analysis of secreted proteins from *Aspergillus flavus. Proteomics*, 5: 3153–3161.

Melin, P. 2002. Proteome analysis of *Aspergillus nidulans* reveals proteins associated with the response to the antibiotic concanamycin a, produced by Streptomyces species. *Mol. Genet. Genomics*, 267: 695–702.

Mellmann, A., Bimet, F., Bizet, C., Borovskaya, A. D., Drake, R. R., Eigner, U. 2009. Identification of Mycobacteria by matrix-assisted laser desorption ionization–timeof- flight mass spectrometry. *J. Clin. Microbiol.*, 44: 1963–1970.

Minan, A., Bosch, A., Lasch, P., Stammler, M., Serra, D. O., Degrossi, J. 2008. MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive Streptococcus pyogenes isolates. *FEMS Immunol. Med. Microbiol.*, 53: 333–342.

Montemurro, N., Visconti, A., 1992. *Alternaria* metabolites: chemical and biological data. In: J. Chelkowski and A. Visconti (Eds.), *Alternaria*: biology, plant diseases and metabolites. Elsevier Science Publishers, Amsterdam. Pp. 449–557.

Morris, P. F., Connolly, M. S., St. Clair, A. 2000. Genetic diversity of *Alternaria alternata* isolated from tomato in California using RAPDs. *Mycol. Res.*, 104: 286– 292.

Neeraj, Shilpi Verma, 2010. Alternaria diseases of vegetable crops and new approaches for its control. *Asian J. Exp. Biol. Sci.*, 1(3): 681–692.

Ostry, V. 2008. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.*, 1: 175–188.

Peever, T.L., Su, G., Carpenter-Boggs L. 2004. Molecular systematics of citrus-associated *Alternaria* species. *Mycologia*, 96: 119–134.

Ponte-Sucre, A. 2009. ABC transporters in microorganisms. Caister Academic Press.

Pryor B. M., Bigelow, D. M. 2003. Molecular characterization of *Embellisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium*, and *Stemphylium. Mycologia*, 95: 1141–1154.

Pryor, B. M., Gilbertson, R. L. 2000. Phylogenetic relationships among *Alternaria* and related fungi based upon analysis of nuclear internal transcribed sequences and mitochondrial small subunit ribosomal DNA sequences. *Mycol. Res.*, 104: 1312–1321.

Pryor, B. M., Gilbertson, R. L. 2002. Relationships and taxonomic status of *Alternaria radicina*, *A. carotiinculatae*, and *A. petroselini* based upon morphological, biochemical, and molecular characteristics. *Mycologia* 94: 49–61.

Pryor, B. M., Michailides, T. J. 2002. Morphological, pathogenic and molecular characterization of *Alternaria* isolate associated with Alternaria late blight of pistachio. *Phytopathology*, 92: 406–416.

Qian, J, Cutler, J.E., Cole, R.B., Cai, Y. 2008. MALDITOF mass signatures for differentiation of yeast species, strain grouping and monitoring of morphogenesis markers. *Analytical and Bioanal. Chem.*, 392: 439–449.

Raman, B., Nandakumar, M.P., Muthuvijayan, V., Marten, M.R. 2005. Proteome analysis to assess physiological changes in Escherichia coli grown under glucose-limited fed-batch conditions. *Biotechnol. Bioeng.*, 92: 384–392.

Roberts, R. G., Reymond, S. T., Andersen, B. 2000. RAPD fragment pattern analysis and morphological segregation of small spored *Alternaria* species and

species groups. *Mycol. Res.*, 104: 151–160.

Rossmann, S.N., Cernoch, P.L., Davis, J.R. 1996. Dematiaceous fungi are an increasing cause of human disease. *Clini. Infect. Dis.*, 22: 73–80.

Rotem, J. 1994. The Genus *Alternaria*: biology, epidemiology and pathogenicity. American Phytopathological Society Press, St Paul, MN.

Schmidt, O., Kallow, W. 2005. Differentiation of indoor wood decay fungi with MALDI-TOF mass spectrometry. *Holzforschung*, 59: 374–377.

Shimizu, M. 2005. Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. *Proteomics*, 5: 3919–3931.

Smedsgaard J., Frisvad J.C. 1996. Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. *J. Microbiol. Methods*, 25: 5–17.

Srivastava, M.P., Chandra, S., Tondon, R.N., 1964. Post harvest diseases of some fruits and vegetables. *Proc. Nat. Acad. Sci. India*, 34(B): 339–342.

Stephen, C., Claire, M., Neville Kevin, A., Kavanagh Sean, D. 2006. Analysis of major intracellular proteins of *Aspergillus fumigates* by MALDI mass spectrometry: Identification and characterisation of an elongation factor 1B protein with glutathione transferase activity. *Biochem. Biophys. Res. Commu.*, 341: 1096–1104.

Stephenson, S., Hatfield, J., Rusu, A.G., Maclean, D.J., Manners, J.M. 2000. CgDN3: an essential pathogenicity gene of *Colletotrichum gloeosprioides* necessary to avert a hypersensitive like response in the host *Stylosanthes guianensis*. *Mol. Plant Microb. Interact.*, 13: 929–941.

Strom, K. 2005. Co-cultivation of antifungal *Lactobacillus plantarum* MiLAB 393 and *Aspergillus nidulans*, evaluation of effects on fungal growth and protein expression. *FEMS Microbiol. Lett.*, 246: 119–124.

Vadlapudi, V., Naidu, K.C., Gudimella, R. 2011. Fungal pathogenesis: strategic prospective: a review, *EJEAFChe.*, 10(4): 2148–2152.

Van Esse, H. P., Van'tKlooster, J. W., Bolton, M. D. 2008. The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *Plant Cell*, 20(7): 1948–1963.