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REPORT AND CHARACTERIZATION OF *ALTERNARIA ALTERNATA* (FR.) KEISSLER ON *AVICENNIA MARINA* (FORSK.) VIERH FORESTS OF INDUSTRIAL YANB'A CITY, SAUDI ARABIA

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Abstract

The present study was carried out to investigate the leaf spot disease of *Avicennia marina* (Forski) Vierh in Saudi Arabia. The leaf spot disease is reported for the first time in mangrove plants of SA. The symptoms of leaf spot disease and the morphological characters of the causal organism are also described. The radial growth and sporulation of *A. alternata* were variable with obvious trend on the different growth media. The optimum pH range for maximum growth was 6.0 to 6.5 and any fluctuation in pH caused significant decrease in linear growth of the mold. The chromatographic (TLC) analysis of chloroform extract of fungal culture broth indicated clearly that the mold was able to produce both alternariol and alternariol monomethyl ether. The gas liquid chromatographic analysis of mycelial fatty acids demonstrated the presence of caproic, caprylic, lauric, myristic, palmitic, margaric, stearic, arachidic, myristoleic, palmitoleic, oleic, linoleic, α linolenic, Cis-11 eicosenoic, eicosatrienoic and arachidonic fatty acids in the mycelia of the causal organism. The analysis of free amino acids in the mycelia of causal organism indicated the presence of 13 amino acids namely, alanine, threonine, valine, proline, methionine, tryptophan, tyrosine, lysin, cystin, glycine, asparatic acid, tyrosine, and phenyl alanine. The PCR product amplified with *A. alternata* DNA indicates that the 370-bp PCR product is a useful diagnostic tool to identify the causal organism in mangrove leaves. This study demonstrated that the causal organism of leaf spot disease of *A. marina* was typically similar to *Alternaria alternata* (Fr.) Keissler via classical, biochemical and molecular characterization.

Introduction

Avicennia marina (Forssk.) Vierh plants are found in the tropical and sub-tropical inter-tidal forests (Bond & Farzin, 2008) and are important plant species in Saudi Arabia (SA). The leaves of *A. marina* are known for its aroma and are also used as fodder for animals especially camels, cattle and sheep (Hamilton & Snedaker, 1984). Kathiresan, (2000) has also described the medicinal properties of the mangrove plants. Mangrove forests in SA are deteriorating due to over-exploitation, deforestation, land reclamation, pollution, occurrence of different diseases etc. Leaf spots caused by *Alternaria alternata* is a very common fungus and has been isolated from almost all habitats involving mangrove vegetation (Sivakumar & Kathiresan, 1990; Abdelmonem & Rasmy, 1996; Venkateswara *et al*., 2001; Calistru, 2004). This facultative pathogen has also been isolated from different parts of mangroves (Abdelmonem & Rasmy, 1996; Calistru *et al*., 2000; Sutherland *et al*., 2003; Calistru, 2004; Kjer *et al*., 2009).

The characterization of *A. alternata* isolates have been reported through chemo-taxonomical (Hubballi *et al*., 2010) and molecular (Mmbaga *et al*., 2011) markers as additional tool in addition to the standard morphological methods (Simmons, 2007) which are still used as the basic method. Analysis of mycelial amino acids (Aliferis & Jabaji, 2010; Alqarawi *et al*., 2012) and fatty acids (Fraga *et al*., 2008; Yang *et al*., 2010) have been used routinely to characterize, differentiate, and identify genera, species, and strains of fungi. Also, the production of cell wall hydrolytic enzymes have been used to characterize different isolates of *A. alternata* (Iram & Ahmad, 2005; El-Morsy *et al*., 2006*;* Hubballi *et al*., 2010). Molecular markers using polymerase chain reaction (PCR) were useful tool for diagnostic identification of *A. alternata* (Mmbaga *et al*., 2011; Kakvan *et al*., 2012; Nasim *et al*., 2012).

The aim of this work was to report leaf spots disease and symptoms on leaves of *A. marina* in the central industrial region of Yanb'a city (SA). Also, characterization of causal organism was carried out by chemo-taxonomical, and molecular markers in addition to the standard morphological methods.

Materials and Methods

Selection of sites: To record the foliage disease of *A. marina* in SA, one of the most important mangrove forests in Yanb'a city was selected. Systemic samples were collected from the central industrial region and 30 Km away it, towards the north in Yanb'a city (SA) as described in Fig. 1, (Google map).

Study the intensity of the disease: The intensity of the disease was recorded on the basis of whole plant, detached twigs and detached leaves.

On the basis of whole plant: To study the intensity of infection in whole plant, 3 sample plots (each sample plot comprising 10 trees) were randomly selected from each of the selected sites. Total number of healthy plants and diseased plants were counted on the basis of visual observation.

On the basis of the twig (branch): From the infected trees, 10 twigs from each of the infected plant were randomly collected. To determine the leaf infection in each twig, all the leaves were observed and total number of infected leaves were counted. The percentage infection in twigs was determined by the following formula:

$$\text{Leaf infection (\%)} = \frac{\text{Total number of infected leaves}}{\text{Total number of leaves present}} \times 100$$

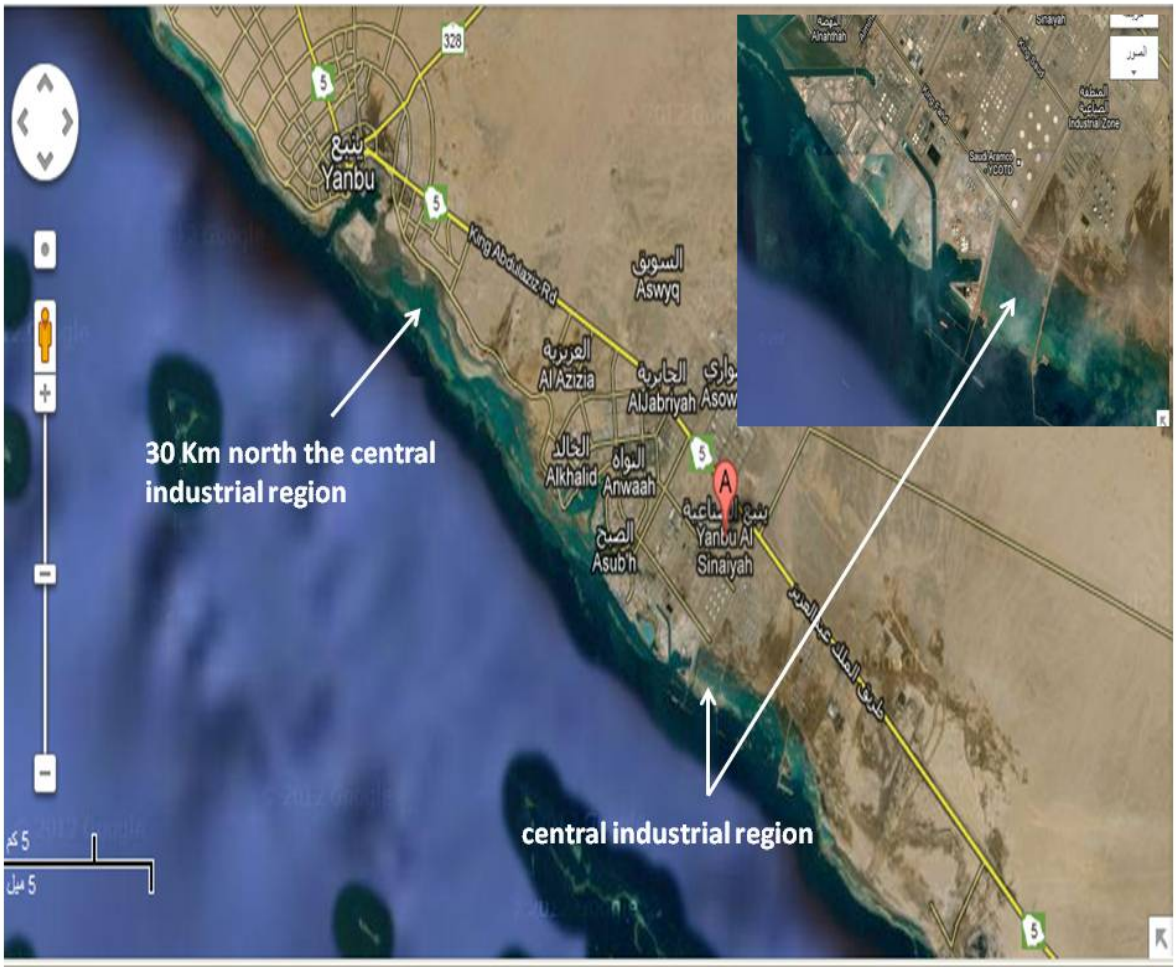

Fig. 1. Google map showing the locations of sampling areas.

On the basis of the detached leaves: After observing the total number of infected leaves in the twigs, all the infected leaves were detached from the twigs and the intensity of infection was recorded on the basis of the following five categories: zero, healthy leaves; 1, < 10% infected area of leaf; 2, < 25% infected area of leaf; 3, < 50% infected area of leaf; 4, < 75% infected area of leaf, and 5, above 75% infected area of leaf. The severity of disease in plants was determined by the following formula:

$$\text{Diseases index (\%)} = \frac{\text{Sum of all numerical ratings}}{\text{(Total number of leaves/twig) x maximum rating}} \times 100$$

Isolation of associated organism: The diseased leaves were brought to the laboratory in zipped polyethylene bags and were preserved until used at low temperature (4^oC). The isolation of associated organism was carried out by placing the diseased leaves (surface-sterlized in Na-hypochlorite for two minutes followed by washing with sterile water for 3 min.) aseptically on sterilized potato- dextrose- agar (PDA, Difco Laboratories, Detroit, MI, USA) medium containing Rose Bengal (33µg ml^{-1}, w/v) and Streptomycin (30µg ml^{-1}, w/v). The inoculated plates were incubated for 7days at 28^oC.

Preparation and maintenance of pure culture and identification: After incubation, the hyphal tips of the fungi coming out from the diseased leaves were re-cultured and from the pure colonies the spores were collected and single spore culture were prepared by following the techniques mentioned by Choi *et al*., (1999) on PDA plates. The isolated organism was identified according to Simmons, (2007).

Pathogenicity test: The pathogenicity test was carried out with the isolated fungus on detached healthy whole leaf and leaf discs. The leaves were surface-sterlized in Na-hypochlorite followed by washing with sterile distilled water, then drying the leaf with sterile filter papers. In leaf disc assay, leaf discs (10 mm) were surface-sterlized as described above. Both whole leaf and leaf disc were placed aseptically in petri dishes lined with filter paper Whatman No 2, wetted with 1 µg/ml of benzylaminopurine (Ash & Lanoiselet, 2001). They were then inoculated with 10 µl of conidial suspension (10^4 conidia/ml sterile water). A control whole leaf and leaf disc were inoculated with sterile water. The inoculated whole leaf and leaf discs were kept at 25^oC

for 6 days. Observations were made after 6 days of incubation for typical target spot development and compared with those occurred under natural condition. The pathogen was reisolated from the artificially inoculated leaves following the same procedure as mentioned earlier. The morphological characteristics of the re-isolated fungus were compared with the original isolate from which the leaves were inoculated, according to Koch's postulate. The pathogenicity test was repeated three times.

Effect of culture media: Petri plates containing 20 ml of following: Potato dextrose agar, (PDA); Czapek Dox agar, (CDA); Oatmeal agar, (OA); Richards agar, (RA) and Water agar, (WA) and were inoculated with agar disc (0.5 cm in diameter) cut from ten day old culture of *A. alternata*. The inoculated plates were incubated at 30°C ± 2 for 7 days in dark. Five replicates were maintained for each media. The sporulation of *A. alternata* on different growth media was carried out according to Masangkay *et al*., (2000). PDA medium was used to study the effect of pH value on growth of *A. alternata*. Citrate-phosphate buffer was used to adjust the pH value of the medium.

Mycotoxins analysis: Extraction of mycotoxins from the culture filtrate of *A. alternata* (10 days old culture grown on potato dextrose broth medium) was carried with ethyl acetate (Söderhäll *et al*., 1978). The organic phase was dried over anhydrous Na sulphate, concentrated to dryness under reduced pressure, and the residue dissolved in methanol. The crude extract was applied on thin-layer chromatography plates (DC-kieselgel, 60 Merck) and the plate was developed in mobile phase of benzene:dioxane:acetic acid (95:25:4, v/v/v) as described by Söderhäll *et al*., (1978). The chemical confirmatory test was carried out using *p*-aminobenzaldehyde and ferric chloride (Sigma) according to Ďuračkova *et al*., (1976). Standard of mycotoxins namely alternariol (AOH), alternariolmonomethyl ether (AME), alternariol, tenuazonic acid (TeA) and altertoxins (ATX) and altenuene (ALT) [Sigma] were used as references.

Amino acids analysis: Free amino acids were extracted from mycelial growth of *A. alternata* (10 days old culture grown on potato dextrose broth medium) with absolute ethanol and the qualitative as well as quantitative determination of amino acids was carried out using LKB 415 alpha plus Amino Acid Analyzer (AAA) according to Christias *et al*., (1975). Standard amino acids (BHD Chemicals, Poole, UK) were used as reference.

Fatty acids analysis: Total lipids were extracted with chloroform:methanol (2:1 v/v) according to the method of Fölsh *et al*., (1957). Fatty acid methyl esters were prepared by methanolysis in H_2SO_4-MeOH (Kates, 1972) and methyl esters were analyzed by gas liquid chromatography (GLC) (Perkin-Elmer Model 910, USA) equipped with a flame ionization detector (Johnson & Stocks, 1971). Qualitative and quantitative analysis of peak fatty acid methyl esters was carried via comparing their retention times with those of an authentic methyl standard (Sigma Co., St. Louis, USA).

The molecular characterization: *DNA extraction*. Fresh samples (100 mg) of artificially infected (with the causal organism, *A. alternata*) leaves and healthy leaves of mangrove were homogenized into fine powder in liquid nitrogen using a mortar and pestle. For each sample, three ml of pre-warmed (65°C) extraction buffer (100 mMTris pH=8.0; 50 mM EDTA; pH=8.0; 1.4 M NaCl; 2% (w/v) SDS; 2% (W/V) PVP-40; 0.1% (v/v) β-mercaptoethanol) was used. The mixture was thoroughly mixed with 5 µl proteinase K (10 ng/ml) and incubated at 65°C for 30 min with continuous mixing. Then, 5 µl RNase-A (10 ng/ml) was added to each sample and the mixture was incubated at 37°C for 30 min. The sample was centrifuged at 13,000x*g* for 15 min and the supernatant was transferred carefully into a new 1.5-ml eppendorf tube. Sodium acetate solution (130 µl, 5 M, pH 5.52) was added and incubated for 20 min. An equal volume of ice-cold isopropanol was added and the mixture was shaken well for 7 min at room temperature before centrifugation at 13000x*g* for 20 min. The supernatant was transferred into a new 1.5 ml tube and a 1/3 volume of potassium acetate (5M) was added. The mixture was shaken vigorously and then centrifuged at 13000x*g* for 20 min. The DNA pellet was cleaned twice with 70% ethanol (700 µl) and centrifuged at 8000x*g* for 1 min, vacuum dried and re-suspended in 300 µl warmed TE buffer (10 mMTris with 1.0 mM EDTA, pH 8.0) for 30 min, then centrifuged at 13000x*g* for 5 min at 4°C to pellet the DNA. The DNA was diluted with TE buffer to a final concentration of 100 ng/µl using Vis/UVspectrophotometer.

Microsatellite-primed PCR: The PCR reaction was carried out in 25µL reaction mixture containing: 1µ L 50 ng/µL of DNA, 2.5µL 10x PCR buffer (50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 10 mmol/LTris–HCl, pH 8.8, 0.1 % Triton X 100), 1.5µL 10 mmol/L dNTP (GH Healtcare), 0.2µL 100 mmol/L of each primer, 19.55µL MQH2O, 0.25µL (2U/µL) DyNAzymeTM II DNA Polymerase (Finnzymes) using a PTC-200 thermocycler (MJ Research, USA). PCR cycles consisted of an initial denaturation step at 94 ^oC for 5 min followed by 42 cycles of 1 min at 93^oC (denaturation), 1 min at 30 to 60^oC (annealing), and 2 min at 72^oC (extension). A final extension cycle at 72^oC for 5 min was followed by a 4^oC soak. Amplification PCR products were visualized with UV light after electrophoretically on 1.5% agarose gel in a 1X TBE buffer by loading 10µl into prepared wells. Gels were stained with ethidium bromide. ITS universal primer pair ITS1-F/ITS4 were used to amplify the ITS region in *A. alternata* by using standard PCR procedure described above (White *et al*., 1990). PCR products amplified from the primer pair ITS1-F/ITS4 were sequenced (Davis Sequencing Inc., Davis, CA, USA) and blasted to GenBank database. Sequence of *A. alternata* isolation was deposited into GenBank. The forward and reverse primers were used in this study were listed in Table 1.

Statistical analysis: The data were statistically analyzed and means were compared using significant difference values according to Daniel (1987).

Table 1. List of primers and their properties for *A. alternata* used in this study.

No.	Forward primer	Reverse primer
1.	OPA9. 5-GGGTAACGCC-3	OPA10. 5-GTGATCGCAG-3
2.	UBC 285. GGGCGCCTAG	UBC 286. CGGAGCCGGC
3.	aa-gp-f1. CGGCAACAACTACATCATCG	aa-gp-r1. CTCCTGGTCAAAAGGAGCTG
4.	SRAP10. 5-TGAGTCCAAACCGGTGC-3	SRAP 9. 5-GACTGCGTACGAATTTGA-3
5.	aa-endo-f1. GTCCCTTCAGGCACAACTTT	aa-endo-r1. GCTGGAGCCAATATCGAAAC
6.	Al-f1. CCCACCACTAGGACAAACA	Al-r1. GCTTAATGGATGCTAGACCT
7.	OPB1. 5-GTTTCGCTCC-3	OPB4. 5-GGACTGGAGT-3'

Results

Description of symptoms: Severe foliar infection was observed on *A. marina* plants in Yanb'a city mangrove forest, SA. The symptoms begin as bright to pale yellow or tan flecks, 0.5 to 1.5 mm in diameter on the upper leaf surface (Fig 2b). These small spots are surrounded by yellow halos (Fig 2b). Also at this early stage, these flecks may be surrounded by a greasy (water soaked) appearance due to cell wall breakdown within the leaf (Fig 2b). The older spots are somewhat circular to irregularly lobed and are light brown-black in color (Fig 2c). Older spots may or may not have concentric-rings. The darker bands or portions within a spot contain spores. Individual spots become brittle or blister-like and will tear and appear ragged within the darkened tissue (Fig 2c). Marked leaf spots symptoms are seen heavily in infected plants near to the water surface (Fig 2d).

Description of disease intensity and disease index of leaf spot: It is necessary to mention that, the disease severity was significantly higher in central region (adhering to factories) as compared to the other region which is far from the factories (Table 2). It was observed that the disease index was also higher in central region in comparison to other selected region. Moreover, it was observed that, the leaf infection percentage on the lower leaves near to the water surface was high as compared to upper ones (Table 2).

Description of the causal organism: The pure culture of the fungal colony appeared to be grayish white at first and became black later on (Fig. 3a). The fungus produced abundant conidia having 3-8 transverse septa and 1-2 longitudinal septa (Fig. 3c). Conidia were solitary, or in short chains, mostly ovoid with a short conical or cylindrical apical beak and smooth walled (Fig. 3b). Hyphae were branched, septate, brownish with simple olive-brown, septate conidiophores that were variable in length (Fig. 3c). Based on these characters and according to keys proposed by Simmons, (2007), the isolated fungus was identified as *Alternaria alternata*. The pathogenicity of the tested fungi was established under laboratory conditions. The re-isolation of the tested fungi was obtained from the diseased leaves of the pathogenicity experiments.

Growth of the causal organism on different media: The results in Table 3 indicated that linear growth of the causal organism (*A. alternata*) on five agar media was variable with maximum at PDA followed with OA, RA, CDA, and WA, respectively. It was observed that, the sporulation of *A. alternata* was variable with obvious trend on the different growth media. The maximum was on WA followed with RA, CDA, PDA and OA, respectively. The results in Table 4 indicated that the growth of *A. alternata* was maximum in pH range 6.0 to 6.5 (no any significant difference observed between them) and any fluctuation in pH value was accompanied with significant decrease in linear growth of the mold (Table 4). The sporulation of *A. alternata* decreased significantly with increase pH value towards alkalinity up to pH 6.0, and the further increase in pH value was accompanied with reverse increase in sporulation.

Mycotoxin production by the causal organism: The chromatographic (TLC) analysis of chloroform extract of fungal culture broth indicated clearly that the mold is able to produce both alternariol (AOH) and alternariol monomethyl ether (AME) in the present study. The mean Rf value of AOH and AME were 0.175 and 0.384, respectively.

Cellular amino acids analysis of the causal organism: The analysis of free amino acids (Fig. 4) in the mycelia of the causal organism indicated the presence of 13 amino acids namely, alanine, 2.84; threonine, 38.51; valine, 0.18; proline, 1.41; methionine, 2.04; tryptophan, 0.93; tyrosine, 25.27; lysin, 0.92; cystin, 1.12; glycine, 0.78; asparatic acid, 0.85; tyrosine, 0.15 and phenyl alanine, 29.12 (mg g dry wt^{-1}).

Cellular fatty acids analysis of the causal organism: GLC analysis of cellular fatty acids methyl ester of *A. alternata* (Fig. 5) revealed the presence of eight saturated fatty acids with different percentage namely caproic (C6, 1.57%), caprylic (C_8, 2.09%), lauric (C_{12}, 0.95%), myristic (C_{14}, 3.39%), palmitic (C_{16}, 3.69%), margaric (C_{17}, 6.98%), stearic (C_{18}, 12.89%), arachidic (C_{20}, 17.55%), and eight un-saturated fatty acids namely myristoleic ($C_{14:1}$, 3.06%), palmitoleic ($C_{16:1}$, 2.69%), oleic ($C_{18:1}$, 5.07%), linoleic ($C_{18:2}$, 30.22%), α linolenic ($C_{18:3}$, 1.43%), cis-11 eicosenoic ($C_{20:1}$, 0.14%), eicosatrienoic ($C_{20:3}$, 2.67%), arachidonic ($C_{20:4}$, 5.61) (Fig. 5).

The molecular characterization: DNA samples extracted from mangrove leaves, healthy as well as artificially infected with *A. alternata*. Seven forward and reverse primers were used in this study. As shown in Fig. 6, PCR amplification with primer Al-f1/Al-r1 expressed 370 bp amplification product in both *A. alternata* isolate and the artificially infected mangrove leaves only, however no amplified product was detected in case of healthy mangrove leaves with the diagnostic primer pair (Al-f1/Al-r1).

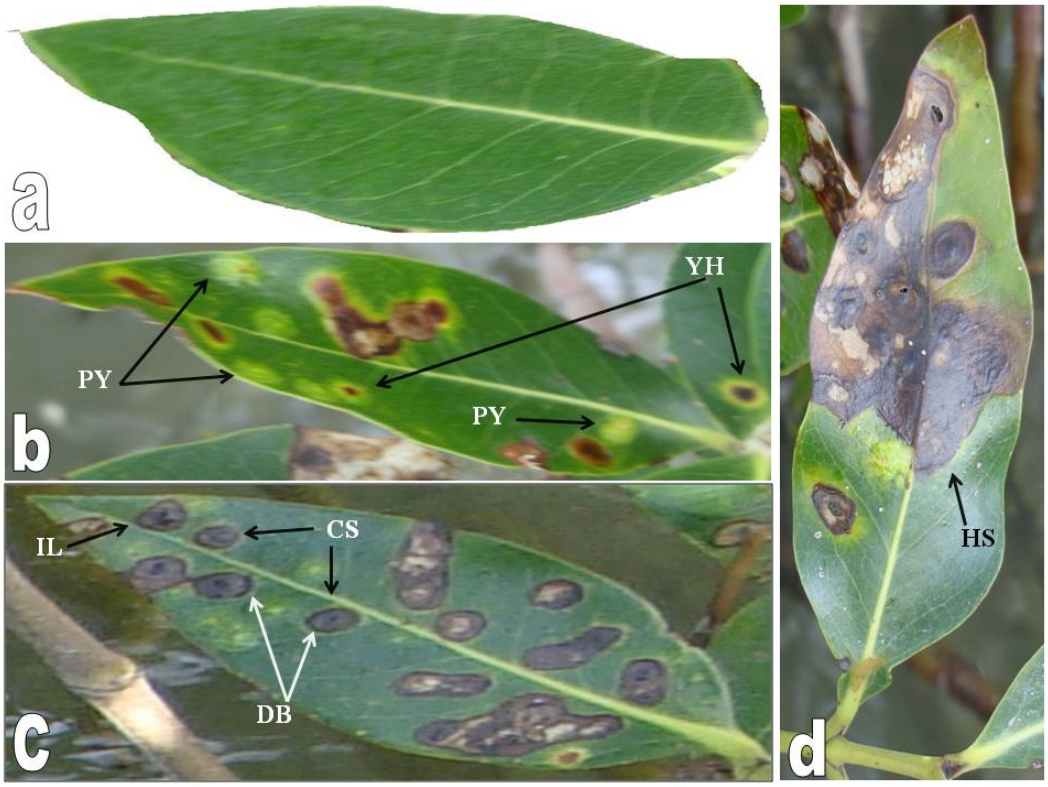

Fig. 2. The symptoms of leaf spot disease of *Avicennia marina*. a. Healthy leaf. b. Symptoms begin as bright to pale yellow and spots surrounded by yellow halos (YH). c. Older spots circular (CS) to irregularly lobed (IL) and are light brown-black in color. d. Heavily infected leaf.

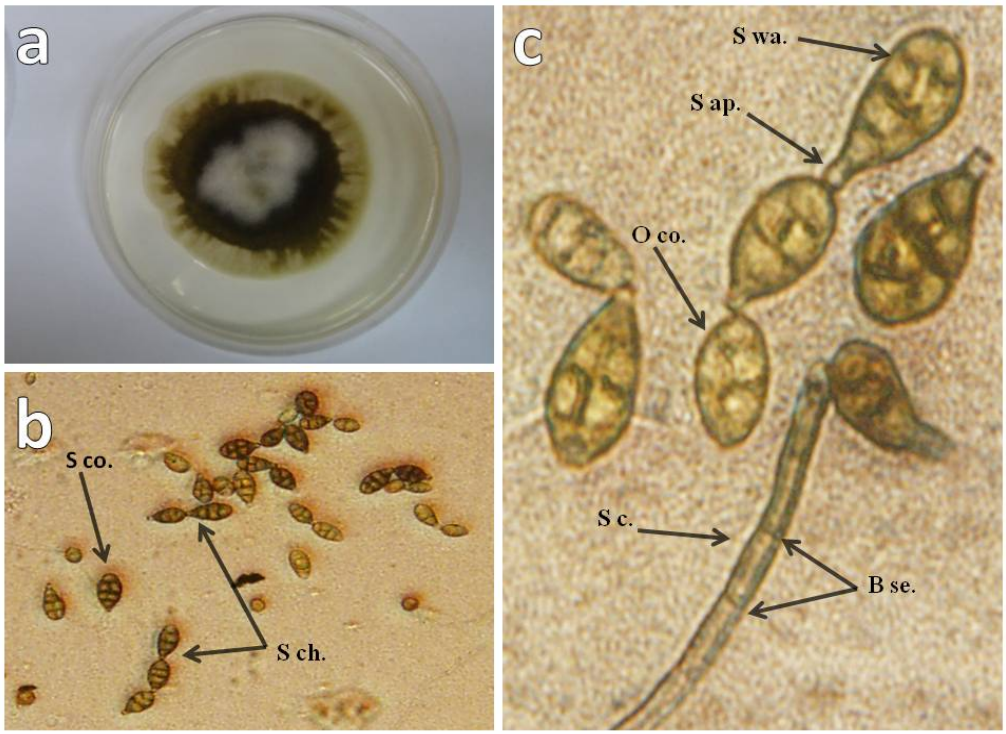

Fig. 3(a-c). Growth and morphological characters of *Alternaria alternata*. **a.** Fungal colony appeared to be grayish white at first and became black later. **b.** Conidia solitary (S co.), or in short chains (S ch.). **c.** Conidia mostly ovoid (O co.) with a short conical or cylindrical apical beak (S ap.) and smooth walled (S wa.), also brownish septate conidiophores (S c.) with simple olive-brown septate (B se.).

Table 2. The disease intensity and index of leaf spots of *Avicennia marina*.

Location	Disease intensity (%)		Disease index
	Whole plant	Twigs	Detached leaves
1	10.0 ± 0.0	5.17 ± 0.70	1.25 ± 0.06
2	93.33 ± 5.8	69.17 ± 4.89	3.99 ± 0.23
LSD at 0.05	9.2548	11.019	0.5463

1= 30 Km north the central industrial region
2= Central industrial region

Table 3. Effect of different growth media on the radial growth and sporulation of *A. alternata*.

Growth media	Radial growth (cm)	Sporulation (10^6/ cm^2)
PDA	8.67	1.27
CDA	6.52	1.60
OA	8.85	1.10
RA	6.93	2.44
WA	1.14	3.69
LSD at: 0.05	0.43	0.27

Table 4. Effect of pH value on the radial growth and sporulation of *A. alternata*.

pH value	Radial growth (cm)	Sporulation (10^6/ cm^2)
5.0	3.03	4.07
5.5	5.43	4.85
6.0	8.14	3.12
6.5	8.72	2.66
7.0	6.73	3.91
LSD at: 0.05	0.74	0.41

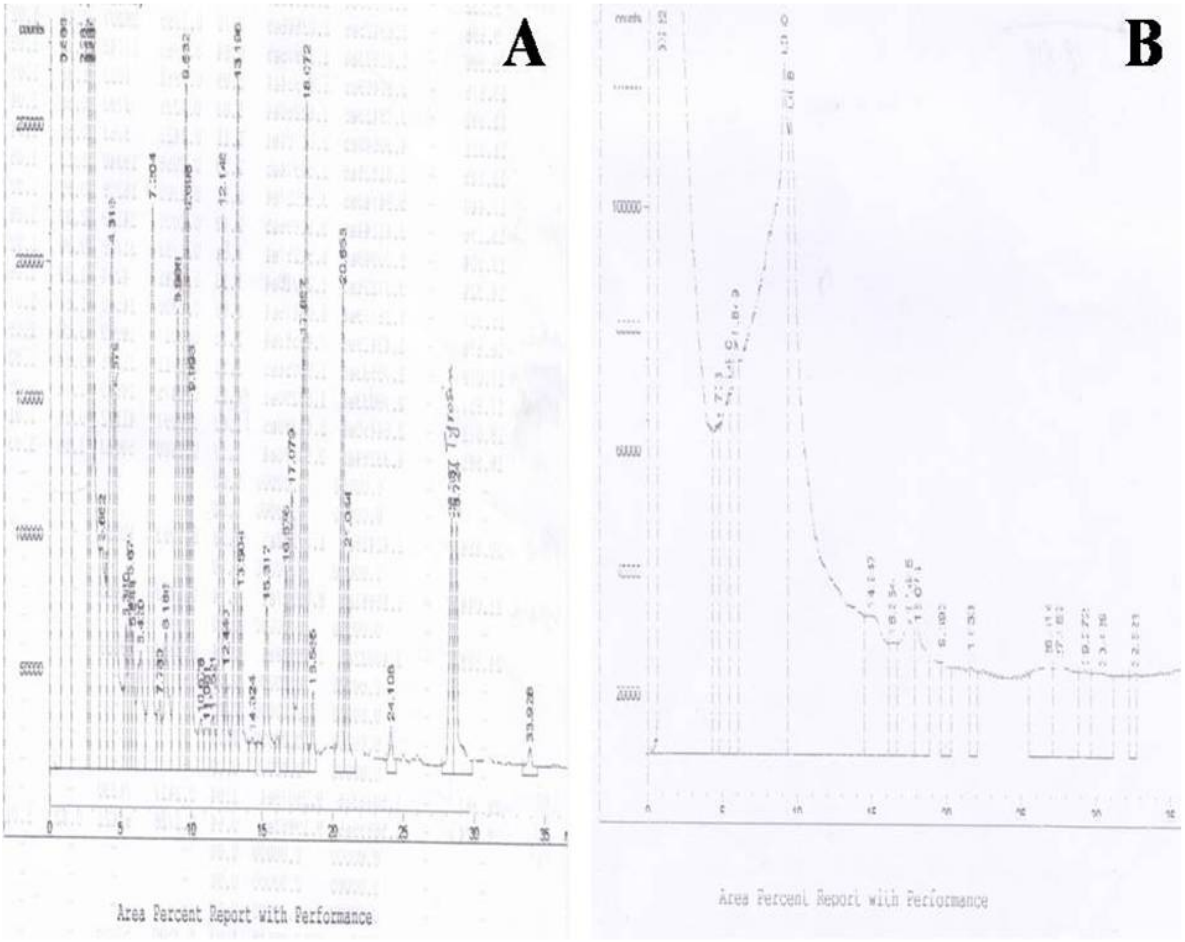

Fig. 4. Amino acids analysis. A, standard amino acids; B, amino acid of *Alternaria alternata*.

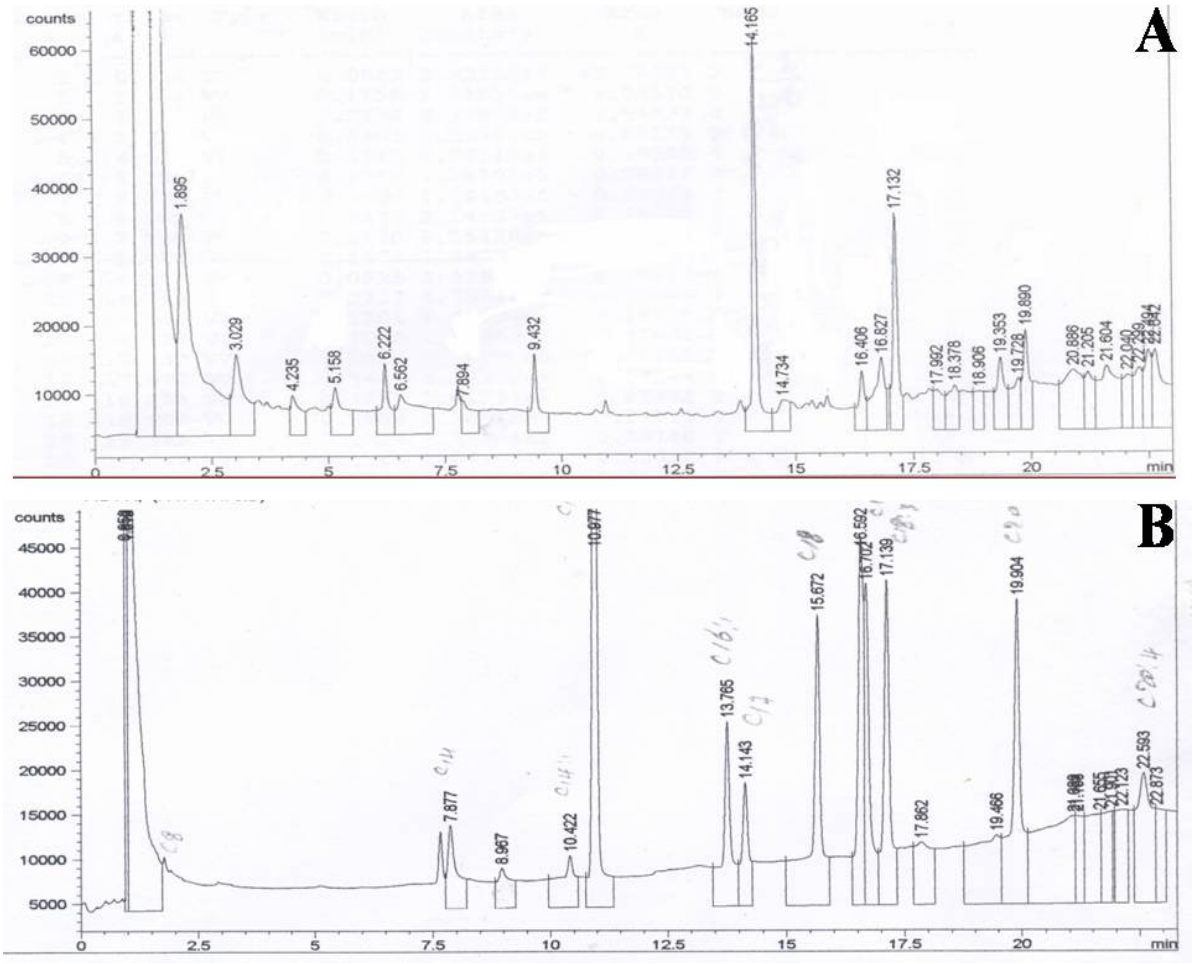

Fig. 5. Fatty acids analysis. A, standard fatty acids; B, fatty acid of *Alternaria alternata*.

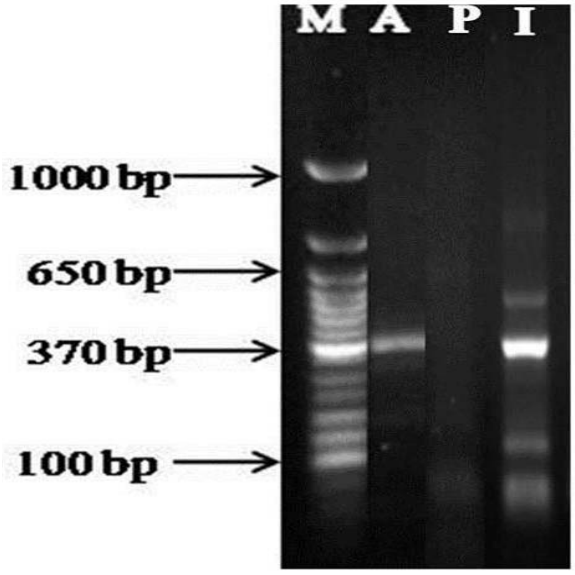

Fig. 6. Microsatellite-primed PCR amplification with primer Al-f1/Al-r1. **M**, molecular weight marker; **A**, *Alternaria alternata* isolate (the causal organism); **P**, healthy mangrove leaf and **I**, artificially infected mangrove leaf with *A. alternata*.

Discussion

The leaf spot disease by *A. alternata* has been reported on mangrove plants in China (Kjer *et al*., 2009); Republic of Panama (Gilbert *et al*., 2002); Egypt (Abdelmonem & Rasmy, 1996); Pakistan (Tariq *et al*., 2006); Australia (Chandrashekar & Bau, 1980) and India (Sivakumar & Kathiresan, 1990). This leaf spot disease on *A. marina* is reported for the first time from SA. Also, it was observed that, there was direct relationship between the percentage of diseased plant and disease severity with that of central industrial region. The disease severity index gradually decreases towards north. Higher uptake of heavy metals resulted from industrial wastewater pollution (Aki *et al*., 2009; Morsy *et al*., 2012) may inhibit physiological processes (Ben Ammar *et al*., 2007) and damage the plasma membrane (Morsy *et al*., 2012) followed with decrease in resistance of plants (Hegazy, 2008).

Our data indicated that more severe leaf infection was on old and lower leaves which were nearer to water surface. It was reported that high fungal counts on old leaf are associated with low content of tannins and sugars, high level of amino acids, low leakiness of

tannins and sugars, and high leakiness of amino acids (Sivakumar and Kathiresan, 1990; Ravikumar & Kathiresan, 1993), hence easy to infected with plant pathogenic fungi (Mamza *et al*., 2008). The high moisture content surrounding the lower leaves increases the growth and spore germination of plant pathogenic fungi (Cohen *et al*., 1971), hence increase the severity of the disease.

The optimum growth and sporulation of *A. alternata* were recorded on PDA and WA media, respectively. Our results corroborated with the findings of Ramjegathesh, (2003); Hubballi *et al*., (2010) and Ramjegathesh & Ebenezar, (2012). The optimum pH range for growth and sporulation of *A. alternata* were 6.0 to 5.5, respectively which corroborates with the results of El-Morsy *et al*., (2006) and Hubballi *et al*., (2010) who reported that the acidic side was more favorable for *A. alternata* growth.

Chemotaxonomy belonging to metabolite-biomarkers like mycotoxins, amino acids and fatty acids have been used for clustering and characterization of many fungi (Aliferis *et al*., 2013; Alqarawi *et al*., 2012; Zain, 2010; El-Morsy *et al*., 2006). Thin layer chromatographic analysis demonstrated that *A. alternata* in present study produced both AOH and AME. The production of such mycotoxins has also been reported by Ostry, (2008); Devi *et al*., (2010 a,b); Brzonkalik *et al*., (2011); Fleck *et al*., (2012). In the same connection, Centeno & Calvo, (2002) reported the same Rf value for the detected mycotoxins (AOH and AME). The amino acid characterization of myclelia of *A. alternata* indicated the presence of alanine, threonine, valine, proline, methionine, tryptophan, tyrosine, lysin, cystin, glycine, asparatic acid, tyrosine, and phenyl alanine. Such amino acids have been reported as common amino acids in many isolates of *A. alternata* (Badawy, 2002; El-Abeid, 2005) and common precursors as well as intermediates during biosynthesis and metabolism of other amino acids (Albert *et al*., 2002). Information regarding diversity within and between the amino acid compositions of *A. alternata* isolates is very important for a better understanding of their biological activities such as production of AAL-toxin (Huang, 2001). Chemotaxonomical approach includes analysis of mycelial fatty acid composition, has been used routinely to characterize, differentiate, and identify genera, species, and strains of fungi (Karliński *et al*., 2007; Fraga *et al*., 2008; Alqarawi *et al*., 2012). In this context, GLC analysis of cellular fatty acids methyl ester of *A. alternata* revealed the presence of C_6, C_8, C_{12}, C_{14}, C_{16}, C_{17}, C_{18}, C_{20},$C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:1}$, $C_{20:3}$, $C_{20:4}$ in different percentage.

The molecular characterization of mycelial DNA of *A. alternata* and artificially infected mangrove leaves with diagnostic primer pair (Al-f1/Al-r1) expressed 370 bp amplification product. Specific primer pairs developed in this study can amplify specific gene loci for the *A. alternata*. The PCR-based diagnostic detection in this study provides a fast and reliable tool to identify and detect *A. alternata* pathogenic isolates that cause lilac leaf blight. In parallel with our findings, Mmbaga *et al*., (2011) reported that the diagnostic primer pair Al-f1/Al-r1 produced a 370-bp amplification product in all isolates of *A. alternata* (the causal organism of Leaf Blight of lilac). Nasim *et al*., (2012) reported similar results in different isolates of *A. alternata* collected from First Fungal Culture Bank of Pakistan.

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References

Abdelmonem, A.M. and M.R. Rasmy. 1996. Fungi and bacteria associated with mangrove seed and measures for their control. Proceedings tree seed pathology meeting, Opocno, Czech Republic, October 9-11.

Aki, C., Güneysu, E. and O. Acar. 2009. Effect of industrial wastewater on total protein and the peroxidase activity in plants. *Afr. J. Biotech.* 8: 5445-5448.

Albert, G.M., W.F. John and P.S. Michael. 2002. Biosynthesis and metabolism of amino acids. In: *Microbial Physiology* 4th Edition, by Wiley-Liss, Inc. ISBN: 0-471-39483-1, pp. 736.

Aliferis, K.A. and S. Jabaji. 2010. Metabolite composition and bioactivity of *Rhizoctonia solani* sclerotial exudates. *J. Agric. Food Chem*. 58: 7604-7615.

Aliferis, K.A., M.A. Cubeta and S. Jabaji . 2013. Chemotaxonomy of fungi in the *Rhizoctonia solani* species complex performing GC/MS metabolite profiling. *Metabolomics* 9: S159-S169.

Al-Qarawi, A.A., A. Hashem and E.F. Abd_Allah. 2012. Seed mycoflora of *Ephedra aphylla* and amino acid profile of Seedborne *Aspergillums flavus*. *Acta Microbiol. Immunol. Hung*. 59(3): 311-320.

Ash, G.J. and V.M. Lanoiselet. 2001. First report of *Alternaria alternata* causing late blight of pistachio (*Pistacia vera*) in Australia. *Plant Pathol*., 50: 803.

Ben Ammar, W., I. Nouairi, M. Zorrouk and F. Jemal. 2007. Cadmium stress induces changes in the lipid composition and biosynthesis in tomato (*Lycopersicon esculentum* Mill.) leaves. *Plant Growth Regul*., 53: 75-85.

Badawy, S.E. 2002. Studies of tomato diseases. MSc., Faculty of Agriculture, Alazhar University, Cairo, Egypt.

Bond, C.A. and Y.H. Farzin. 2008. Alternative sustainability criteria, externalities, and welfare in a simple agroecosystem model: A Numerical analysis. *Environ. Resour. Econ*., 40: 383-399.

Brzonkalik, K., T. Herrling, C. Syldatk and A. Neumann. 2011. Influence of pH and carbon to nitrogen ratio on mycotoxin production by *Alternaria alternata* in submerged cultivation. *AMB Express*, 2(28): 1-8. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3441619/pdf/2191-0855-2-28.pdf

Calistru, C. 2004. Some implications of associated mycoflora during hydrated storage of recalcitrant seeds of *Avicennia marina* (Forssk.)Vierh. Ph. D. Thesis, Division of Biological Sciences School of Life and Environmental Sciences University of KwaZulu-Natal, Durban, South Africa.

Calistru, C., M. McLean, N.W. Pammenter and P. Berjak. 2000. The effects of mycofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Sci. Res*., 10: 341-353.

Centeno, S. and M.A. Calvo. 2002. Mycotoxins produced by fungi isolated from wine cork stoppers. *Pak. J. Nutr*., 1(6): 267-269.

Chandrashekar, M. and M.C. Bau. 1980. Leaf blight of grey mangrove in Australia caused by *Alternaria alternata*. *Trans. Br. Mycol. Soc*., 73: 413-418.

Choi, Y.W., K.D. Hyde and W.H. Ho. 1999. Single spore isolation of fungi. *Fungal Divers*. 3: 29-38.

Christias, C., C. Couvaraki; S.G. Georgopoulos; B. Macris and V. Vomvoyanni. 1975. Protein content and amino acid composition of certain fungi evaluated for microbial protein production. *Appl. Microbiol*., 29: 250-254.

Cohen, Y., M. Perl and J. Rotem. 1971.The effect of darkness and moisture on sporulation of *Pseudoperonospora cubensis* in cucumbers. *Phytopathol*., 61: 594-595.

Daniel, W.W. 1987. *Biostatistics: A Foundation for Analysis in the Health Sciences*. 4th ed. John Wiley and Sons, New York, NY. pp. 292-293.

Devi P.S., M.N. Reddy, S.D.S. Murthy, M.N. Devamma, P. Jayalakshmi and G. Revathi. 2010a. Isolation and characterization of alternariol and alternariol monomethyl ether produced by *Alternaria alternata* of groundnut. *The Bioscan*, 5(2): 323-330.

Devi, P.S., M.N. Reddy, M.N. Devamma, P. Jayalakshmi, G. Revathi and G. Narasimha. 2010b. Identification and characterization of tenuazonic acid as the causative agent of *Alternaria alternata* toxicity towards groundnut. *Afr. J. Microbiol. Res*., 4(20): 2184-2190.

Ďuračkova, Z., V. Betina and P. Nemec. 1976. Systematic analysis of mycotoxins by thin layer chromatography. *J. Chromatogr*., 116: 141-154.

El-Abeid, E.S. 2005. Variance among Alternaria spp. in relation to their pathogenic capability to some plant hosts. MSc., Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

El-Morsy, E.M., S.M. Dohlob and K.D. Hyde. 2006. Diversity of *Alternaria alternata* a common destructive pathogen of *Eichhornia crassipes* in Egypt and its potential use in biological control. *Fungal Divers*., 23: 139-158.

Fleck, S.C., B. Burkhardt, E. Pfeiffer and M. Metzler. 2012. Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. *Toxicol. Lett*., 214: 27-32.

Fölsh, J., M. Lees and G.H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*., 226: 497-509.

Fraga, M.E., D.M.N. Santana, M.J. Gatti, G.M. Direito, L.R. Cavaglieri, C.A.R. Rosa. 2008. Characterization of *Aspergillus* species based on fatty acid profiles. *Memórias do Instituto Oswaldo Cruz*, 103: 540-544.

Gilbert, G.S., M. Mejia-Chang and E. Rojas. 2002. Fungal diversity and plant disease in mangrove forests: salt excretion as a possible defense mechanism. *Oecologia*, 132: 278-285.

Hamilton, L.S. and S.C. Snedaker. 1984. Handbook for Mangrove Area Management. UNESCO, Paris, 123 p.

Hegazy, H.S. 2008. The role of heavy metals on metabolism of polyamines in bean plants (*Vicia faba*) and its susceptibility to *Fusarium* wilt. *Res. J. Agric. Biol. Sci*., 4: 198-202.

Huang, J.S. 2001. *Plant pathogenesis and resistance. biochemistry and physiology of plant-microbe interactions*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 691.

Hubballi, M., S. Nakkeeran, T. Raguchander, T. Anand and R. Samiyappan. 2010. Effect of environmental conditions on growth of *Alternaria alternata* causing leaf blight of ononi. *World J. Agric. Sci*., 6: 171-177.

Iram S. and I. Ahmad. 2005. Analysis of variation in *Alternaria alternata* by pathogenicity and RAPD study. *Pol. J. Microbiol*., 54(1): 13-19.

Johnson, A. and R. Stocks. 1971. Gas-liquid chromatography of lipids. In: *Biochemistry and Methodology of Lipids.* (Eds.): A. Johnson and J. Davenport. Wiley Interscience, New York, NY.

Kakvan, N., H. Zamanizadeh, B. Morid, H. Taheri and S. Hajmansor. 2012. Study on pathogenic and genetic diversity of *Alternaria alternata* isolated from citrus hybrids of Iran, based on RAPD-PCR technique. *Eur. J. Exp. Biol*., 2(3): 570-576.

Karliński, L., S. Ravnskov, B.K. ARokicka and J. Larsen. 2007. Fatty acid composition of various ectomycorrhizal fungi and ectomycorrhizas of Norway spruce. *Soil Biol. Biochem*., 39: 854-866.

Kates, M. 1972. Techniques of lipidology. In: *Laboratory Techniques in Biochemistry and Molecular Biology.* (Eds.): T.W. Work and E. Work. North-Holland Publishing Co., Amsterdam, the Netherlands.

Kathiresan, K. 2000. A review of studies on Pichavaram mangrove, southeastIndia. Hydrobiologia 430: 185-205.

Kjer, J., V. Wray, R.A. Edrada-Ebel, R. Ebel, A. Pretsch, W.H. Lin and P. Proksch. 2009. Xanalteric acid and related phenolic compounds from endophytic *Alternaria* sp. isolated from the mangrove plant *Sonneratia alba*. *J. Nat. Prod*., 72: 2053-2057.

Mamza, W.S., A.B. Zarafi and O. Alabi. 2008. Incidence and severity of leaf blight caused by *Fusarium pallidoroseum* on varied age of Castor (*Ricinus communis*) inoculated using different methods. *Afr. J. Gen. Agric*., 4: 119-122.

Mmbaga, M.T., A. Shi and M. Kim. 2011. Identification of *Alternaria alternata* as a causal agent for leaf blight in Syringa species. *Plant Pathol. J*., 27(2): 120-127.

Morsy, A.A., K.H.A. Salama, H.A. Kamel and M.M.F. Mansour. 2012. Effect of heavy metals on plasma membrane lipids and antioxidant enzymes of *Zygophyllum* species. *EurAsian J. Bio. Sci*., 6: 1-10.

Nasim, G., S. Khan and I. Khokhar. 2012. Molecular polymorphism and phylogenetic relationship of some *Alternaria alternata* isolates. *Pak. J. Bot*., 44(4): 1267-1270.

Ostry, V. 2008. Alternaria mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J*., 1(2): 175-188.

Ramjegathesh, R. 2003. *Studies on the eco-friendly management of the onion leaf blight disease caused by Alternaria alternata (Fr.) Keissler*. M.Sc. (Ag) Thesis, Tamil Nadu Agricultural University, Coimbatore, India.

Ramjegathesh, R. and E.G. Ebenezar. 2012. Morphological and physiological characters of *Alternaria alternata* causing leaf blight disease of onion. *Int. J. Plant Pathol*., 3(2): 34-44.

Ravikumar, S. and K. Kathiresan. 1993. Influence of tan-nins, sugars and amino acids on fungi of marine halophytes. *Mahasagar* 26: 21-24.

Simmons, E.G. 2007. Alternaria. An identification manual. CBS Biodiversity Series 6. CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.

Sivakumar, A. and K. Kathiresan. 1990. Phylloplane fungi from mangroves. *Indian J. Microbiol*., 30: 229-231.

Söderhäll, K., E. Svensson and T. Unestam. 1978. Light inhibits the production of alternariol and alternariol monomethyl ether in *Alternaria alternata*. *Appl. Environ. Microbiol*., 36: 655-657.

Sutherland, J.R., M. Diekmann and P. Berjak. 2003. Forest tree seed health testing. IPGRI, Rome, Italy.

Tariq, M., S. Dawar and F.S. Mehdi. 2006. Isolation of fungi from *Avicennia marina*. *Pak. J. Bot*., 38: 805-810.

Venkateswara, S.V., K.D. Hyde and B.P. Vittal. 2001. Frequency of occurrence of mangrove fungi from the east coast of India. *Hydrobiologia*, 455: 41-53.

White, T.J., T. Bruns, S. Lee and J. W. Taylor. 1990. Amplifi cation and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*, (Eds.): M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White. Academic Press, Inc., New York. pp. 315-322.

Yang, J.X., F. Xu, C.H. Huang, J. Li, Z.G. She, Z. Pei and Y.C. Lin. 2010. Metabolites from the mangrove endophytic fungus *Phomopsis* sp (#zsu-H76). *Eur. J. Organ. Chem*., 19: 3692-3695.

Zain, M.E. 2010. Biochemical Markers in Taxonomy of the Genus *Fusarium*. *Res. J. Agr. Biol. Sci*., 6(1): 1-7.

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