Pak. J. Bot., 50(4): 1607-1614, 2018.

ALTERNARIA LEAF SPOT DISEASE OF BROCCOLI IN PAKISTAN AND MANAGEMENT OF THE PATHOGEN BY LEAF EXTRACT OF *SYZYGIUM CUMINI*

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Abstract

Broccoli (*Brassica oleracea* L. var. *italica* Plenck) is comparatively a new vegetable crop in Pakistan. Recently, a fungal leaf spot disease was noticed on broccoli plants. In the present study, causal organism of broccoli leaf spot was isolated and identified on morphological as well as molecular bases. Molecular characterization of the pathogen was carried out by nucleotide sequence analysis of rDNA ITS region. *Alternaria brassicae* (Berck.) Sacc. was identified as causal organism that also fulfilled Koch's pathogenicity postulates. Methanolic leaf extract (0, 1, 2, 3, 4, 5 mg mL^{-1}) of *Syzygium cumini* (L.) Skeels was evaluated for its antifungal activity against the isolated fungal pathogen *A. brassicae.* Various extract concentrations reduced fungal biomass by 4–46% over control. GC-MS analysis of the extract showed seven compounds in the extract. Among these, 9-Octadecenoic acid (Z)-, methyl ester (28.18%), 9, 12-Octadecadienoic acid (Z,Z)-, methyl ester (25.94%), Hexadecanoic acid, methyl ester (18.04%), and Phytol (13.46%) were major compounds.

Key words: *Alternaria brassicae*, Broccoli, Disease report, Leaf spots.

Introduction

Many plants of family Brassicaceae have high economic values being used as vegetables as well as source of edible oils, medicines and condiments (Rakow, 2004). Species of *Alternaria* affect many brassicaceous crops, including broccoli, cauliflower, turnip, mustard, celery cabbage, cabbage, rape, radish and spinach (Kirk *et al.*, 2008; Kohl *et al.*, 2010; Kumar *et al.*, 2014; Khan *et al.*, 2015; Czajka *et al.*, 2015). *Alternaria brassicicola* and *A. brassicae* are the most notorious species that cause leaf blight in cultivated or wild members of this family. *A. brassicicola* is prominent for its ability to infect vegetable crops while *A. brassicae* dominates in causing diseases to oil seed crops (Ayuke *et al.*, 2017; Manhas & Kaur, 2017). Both of these species can damage leaves, stem, pods, seeds and seedlings. Inoculum of these pathogens survives in crop debris up to several years and spread to other fields too (Jones, 1992). *Alternaria raphani* is another species that is reported to cause blight in radish (Sue *et al.*, 2005).

The taxonomic status of *Alternaria* species is principally based on phenotypic characters of the fungi and sometimes by host-pathogen relationship. *Alternaria* species namely *A. brassicae*, *A. brassicicola* and *A. raphani* that are known to infect brassicaceous plants show different spore morphology (Simmon, 2007). Recently, use of DNA sequencing in fungal identification has solved many systematic problems within genus *Alternaria*. Analysis of rDNA sequences has proved a rapid and reliable method for the identification of fungi (Akhtar *et al.*, 2015).

Broccoli, an important vegetable has a vast variety of nutritional antioxidants, and vitamins C and E (Swiglo *et al.*, 2006; Munyaka *et al.*, 2010), carotenoids, and flavanoids (Lin & Chang, 2005), polyphenols (Faller & Fialho, 2009), flavonol and hydroxycinnamoyl derivatives (Vasanthi *et al.*, 2009). Increasing toxicological concerns connected with the use of chemical pesticides, have given the thought of using alternate biological materials (Ali *et al.*, 2017; Banaras *et al.*, 2017). *Syzygium cumini* (L.) Skeels is a plant known for the high antimicrobial activity in its different plant parts such as leaves, bark, fruits and seeds (Jabeen & Javaid, 2010). Leaves of *S. cumini* have high phenolic content and also show high antioxidant activity (Paul *et al*., 2011).

The objective of the present study was to identify the causal organism of leaf spot of broccoli and to suggest the phytochemical control of this disease using *S. cumini* leaf extract. Phytochemical analysis of methanol soluble fraction of *S. cumini* leaves was also carried out for its potential use as fungicide in future.

Materials and Methods

Isolation of pathogen: Infected leaves of broccoli plants showing the severe necrotic spots were collected from the fields of University of the Punjab, Lahore, in March, 2016 (Fig. 1). For pathogen isolation, leaf spots of broccoli were cut into tiny pieces of 2 mm^2, washed thoroughly with sterilized water and then surface sterilized with sodium hypochlorite solution. Under aseptic conditions, surface disinfected pieces were inoculated onto malt extract agar (MEA) medium. Inoculated Petri plates were incubated at 25ºC until mycelium was visible coming out from the inoculated diseased leaf portions (Nayab and Akhtar, 2016). Hyphal tips from emerging fungal growth were transferred onto potato dextrose agar (PDA), vegetable juice agar (V8) and MEA growth media for purification and morphological studies (Simmons, 2007).

Identification of pathogen: Morphological identification and characterization was carried out using seven days old pure fungal cultures. Phenotypic analysis of the cultures were comprised of cultural aspects such as colony diameter, color and exudates, types of margins, texture of colony surface, sporulation and spore attachment patterns. Microscopic features included shape, color, septation and size (length and width) of conidia; septation in hyphae, branching and

attachment of conidia on conidiophores; color and size of conidiophores. Complete descriptions of all the observed features were prepared. Micro-photographs were also taken for future use and reference (Simmons, 2007).

Molecular characterization was carried out to confirm the identity of the isolated pathogen primarily identified on the basis of morphology. For this purpose nucleotide sequence analysis of Internal Transcribed Spacer Sequence (ITS) of rDNA was carried out. For DNA isolation, fungal mycelia of two weeks old pure culture were scratched with the help of a sterilized spatula; circumventing agar medium and ground in liquid nitrogen with the help of sterilized pester and mortar following the methods of Akhtar *et al.*, (2014). For quality and integrity check, isolated DNA was run on 1% agarose gel and visualized using UV transilluminator.

Approximately 650 bp was amplified using total fungal genomic DNA as a template and universal primer pair, forward ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse ITS4 primer (3′-TCCTCCGCTT ATTGATATGC-5′). PCR amplification reaction was carried out in 25 µL reaction mixture as described by Nayab & Akhtar (2016). Amplified PCR product was analyzed by gel electrophoresis and was sent for nucleotide sequencing. The nucleotide sequence of amplified PCR product was evaluated by Basic Local Alignment Search Tool (BLAST). Finally, phylogenetic relationship of the isolated pathogen with closely related strains was carried out using MEGA 6 program.

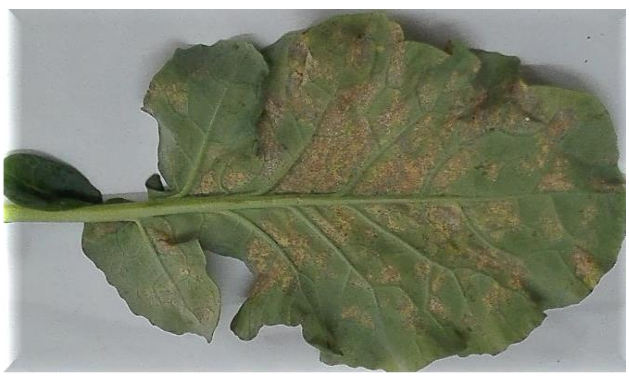

Fig. 1. Symptoms of leaf spots on the leaf of broccoli.

Pathogenicity confirmation test: Koch's pathogenicity postulates were employed to confirm the pathogenic potential of the identified fungal pathogen. Detached leaf assay (Khadka, 2016) was conducted on a layer of sterilized moisten filter papers placed in sterilized Petri plates. Three young broccoli leaves were arranged on the moist filter papers so that their petioles remained rooted in the filter paper and each leaf was inoculated with 10^5 conidia of the fungus. In control treatment, sterilized water was used instead of fungal inoculum. All the plates were covered and placed in an incubator at 25 $\pm$ 2 °C. Filter papers were moistened regularly when needed with the sterilized water and inoculated leaves were observed regularly for the emergence of disease symptoms. Re-isolation of the pathogen was carried out from the diseased portion of artificially inoculated leaves. Three independent pathogenicity tests were conducted to check the consistency of results.

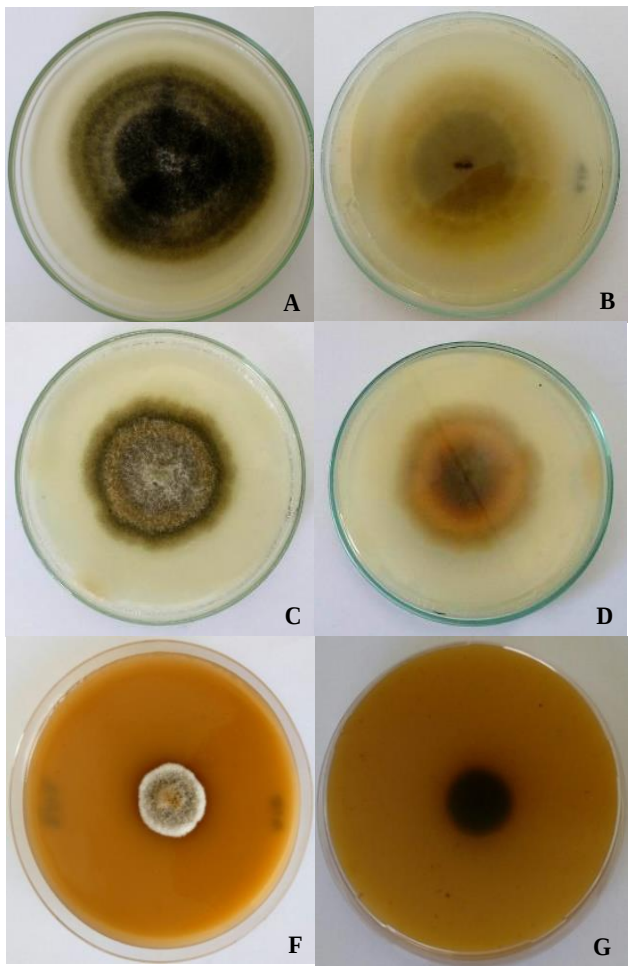

Fig. 2. Macroscopic view of *Alternaria brassicae* (FCBP 1433). Colony (A) and reverse (B) grown on malt extract agar for 7 days. Colony (C) and reverse (D) grown on potato dextrose agar for 10 days. Colony (E) and reverse (F) grown on V8 medium for 14 days.

Antifungal bioassays: Healthy mature leaves of *Syzigium cumini* were collected, first dried under shade and then in an electric oven at 45°C. For the extraction of methanol soluble compounds from the plant materials, 200 g of the thoroughly crushed leaf material was soaked in 1.0 L methanol for 15 days at room temperature in an air tight jar. The leaf material was filtered and the filtrates were evaporated at 45°C in a rotary evaporator under vacuum till 30 mL of extract left. The extract was shifted to the sterilized beaker and placed in drying oven at 45°C for complete evaporation of the solvent (Javaid & Akhtar, 2015).

To prepare the stock solution, methanol soluble fraction of leaves (9 g) was dissolved in 5 mL dimethyl sulfoxide (DMSO) and final volume of 15 mL was achieved by adding sterilized distilled water. Similarly, control solution was prepared by dissolving 5 mL DMSO in 10 mL sterilized distilled water. Experiment was conducted in 250 mL flasks each containing 55 mL of 2% sterilized malt extract broth. Five concentrations viz. 1, 2, 3, 4 and 5% were prepared by adding 1, 2, 3, 4 and 5 mL of stock solution with 4, 3, 2, 1, and 0 mL of control solution, respectively that raised the final volume of growth medium to 60 mL in each flask. For replication,

this quantity was equally divided into four portions. For control treatment, 5 mL of control solution was added to growth medium to maintain equal concentration of DMSO in control and experimental treatments. Agar plugs from 2 weeks old pure culture of *A. brassicae* were made by 2 mm diameter cork bore and transferred to each flask. Flasks were incubated at 25 ± 2°C. After 7 days of incubation, the fungal biomass in each flask was collected by filtration. Dried fungal biomass (g) was determined after drying of fungal mat in an oven at 60°C (Javaid *et al.,* 2015; Sana *et al.,* 2017).

GC-MS analysis: Chemical constituents of methanolic leaf extract of *S. cumini* were determined by Gas Chromatography Mass Spectrometry (GC-MS).

Data collection and statistical analysis: All the data of laboratory bioassays were subjected to analysis of variance followed by LSD test at p = 0.05 using Statistics 8.1. Standard errors of means of four replicates were calculated using MS Excel software.

Results

Morphology based identification: For morphology based identification, comprehensive study of the fungus culture was made macroscopically and microscopically which was then compared with authentic published literature. All the morphological characters were noted for seven days pure fungal culture grown at 25 ± 2ºC on MEA, ten days on PDA and fourteen days on V8 juice media plates.

The fungal colony on MEA was greenish-black in color from the obverse side while reverse was off-white to yellow in color. Colony was rapidly growing on MEA reaching 5–6 cm in diameter in seven days, with regular margins and immersed or partly superficial mycelia. When grown on PDA, color of the fungal colony was olive green to grayish black was yellow, colony margins were regular while concentric growth zones were present. The size of colony ranged from 4.5–5.5 cm in diameter in ten days. Colony diameter on V8 juice medium reached 2-3 cm in diameter in fourteen days, greenish olive with white regular margins from the above and black in color from lower side of plate (Fig. 2).

Microscopic studies revealed that conidiophores were branched, septate and 60–120 × 4–8 µm in size. Color of mature conidia was dull tan yellow to pale greenish, produced in chains of 4–10. Mature conidia were 120–190 × 15–20 µm, with 7–12 transverse and 2–3 longitudinal septa while juvenile conidia ranged from 50–80 × 10–15 µm in size and have 3–6 transverse septa. The spore wall was smooth, but some had geniculations (Fig. 3). Based on morphological characters, the fungus was identified as *Alternaria brassicae* (Simmons, 2007). Pure fungal culture was deposited in First Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore, Pakistan under the specific accession number FCBP 1433.

Identification based on rDNA sequence analysis: Nucleotide sequence analysis of ITS region of rDNA was carried out to characterize the fungal pathogen genetically. Total genomic DNA isolated from the fungal cells showed a very clear single compact band of DNA on agarose gel. ITS1–5.8S rDNA-ITS4 region of rDNA amplified approximately 650 bp PCR product (Fig. 4).

BLAST results of nucleotide sequence of amplicon showed 99% homology of this sequence to several different isolates of *A. brassicae* in GenBank with 100% query cover and E-Value 0 for example U2 (JF439438), DPL-1 (KU982593), BHU-LMMT14 (KX118425).

Molecular phylogenetic analysis: Maximum Likelihood method based on the Tamura-Nei model was used to infer the evolutionary history of the identified strain. The phylogenetic tree (Fig. 5) was constructed using 17 nucleotide sequences from closely related species of *Alternaria.* Analysis clearly demonstrated that *A. brassicae* was genetically distinct from the rest of the species.

Verification of pathogenicity: After 5 days of incubation, very minute spots started appearing on inoculated leaves. These spots continued to expand and after 10 days of incubation, on an average 50% area of each leaf showed disease symptoms similar with those observed during initial sampling. However, non-inoculated control leaves remained asymptomatic. *A. brassicae* was consistently re-isolated from infected lesions of all artificially inoculated leaves hence fulfilled the Koch's pathogenicity postulates.

Antifungal activity of leaf extract of *S. cumini*: Methanolic leaf extract of *S. cumini* was highly effective in suppressing growth of *A. brassicae*. All the extract concentrations significantly reduced fungal biomass over control. Affectivity of the extract was concentration dependant. A progressive decline in fungal biomass was recorded with a gradual increase in extract concentration (Fig. 6A). Different concentrations of *S. cumini* leaf extract significantly reduced biomass of *A. brassicae* by 4–46% as compared to control (Fig. 6B). A polynomial relationship between extract concentration and fungal biomass was recorded with $R^2 = 9923$ (Fig. 6C).

GC-MS analysis of *S. cumini* leaf extract: Seven compounds were found present in methanolic leaf extract of *S. cumini* (Fig. 7). The identified compounds were 9-Octadecenoic acid (Z)-, methyl ester (28.18%), 9, 12-Octadecadienoic acid (Z,Z)-, methyl ester (25.94%), Hexadecanoic acid, methyl ester (18.04%), Phytol (13.46%), Heptadecanoic acid, 16-methyl-, methyl ester (4.81%), 9, 12-Octadecadienoic acid (Z,Z)- (4.25%), Estra- 1,3,5(10)-trien- 17.beta,-ol (3.41%) (Table 1). Mass chromatograms and structures of these compounds are shown in Fig. 8.

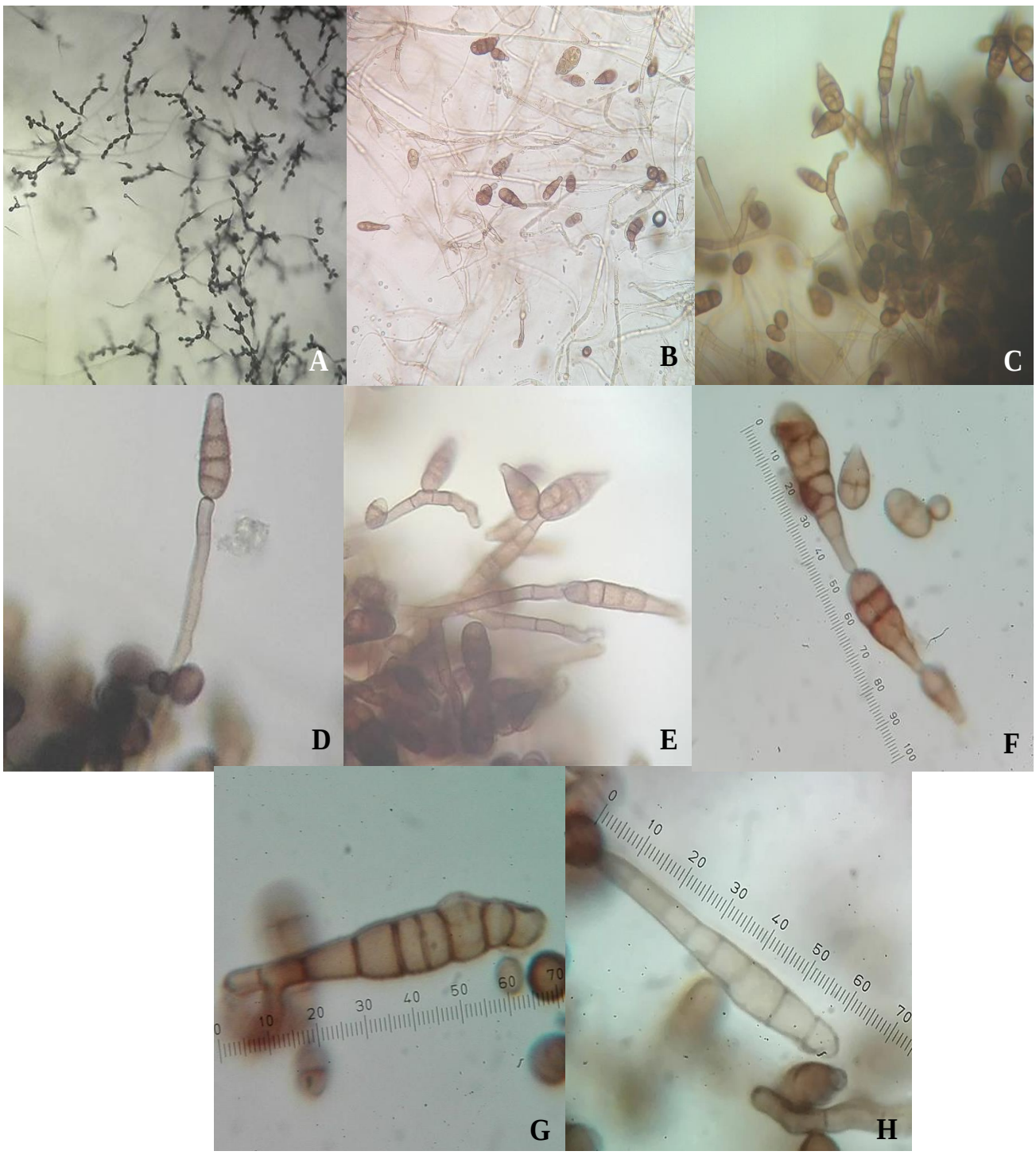

Fig. 3. Microscopic view of *Alternaria brassicae* (FCBP 1433). Spore attachment pattern on conidiophores under stereoscope (A); Appearance of spores under microscope at 10X (B and C); Spore attachment and septation of spores and conidiophores at 40X (D, E and F); Spore characteristics under the microscope at 100X magnification.

Table 1: Compounds identified from methanolic leaf extract of *Syzygium cumini* through GC-MS analysis.

No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	% of total
1.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	18.35	18.04
2.	Estra- 1,3,5(10)-trien- 17.beta,-ol	$C_{18}H_{24}O$	256	18.90	3.41
3.	9, 12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294	19.98	25.94
4.	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	20.03	28.18
5.	Phytol	$C_{20}H_{40}O$	296	20.16	13.46
6.	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	298	20.24	4.81
7.	9, 12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	20.52	4.25

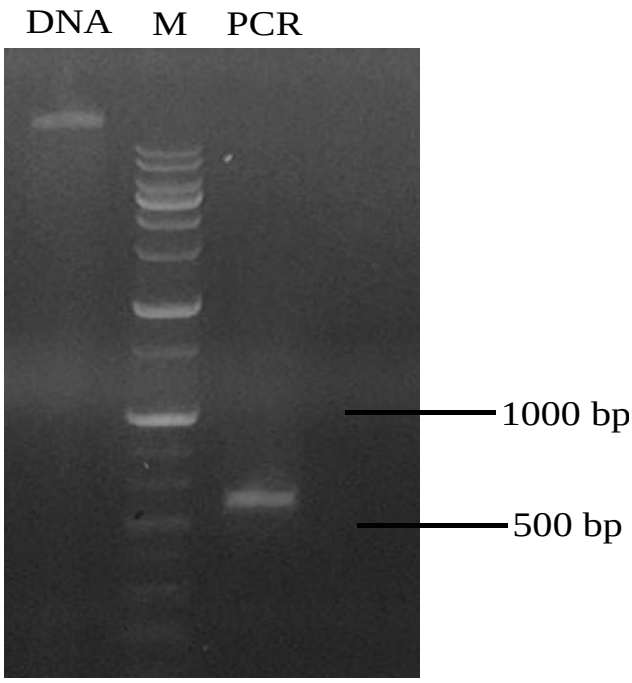

Fig. 4. *Alternaria brassicae* (FCBP 1433) (1): Genomic DNA, (M): 1 Kb DNA standard marker (2): ITS1/ITS4 amplified PCR product.

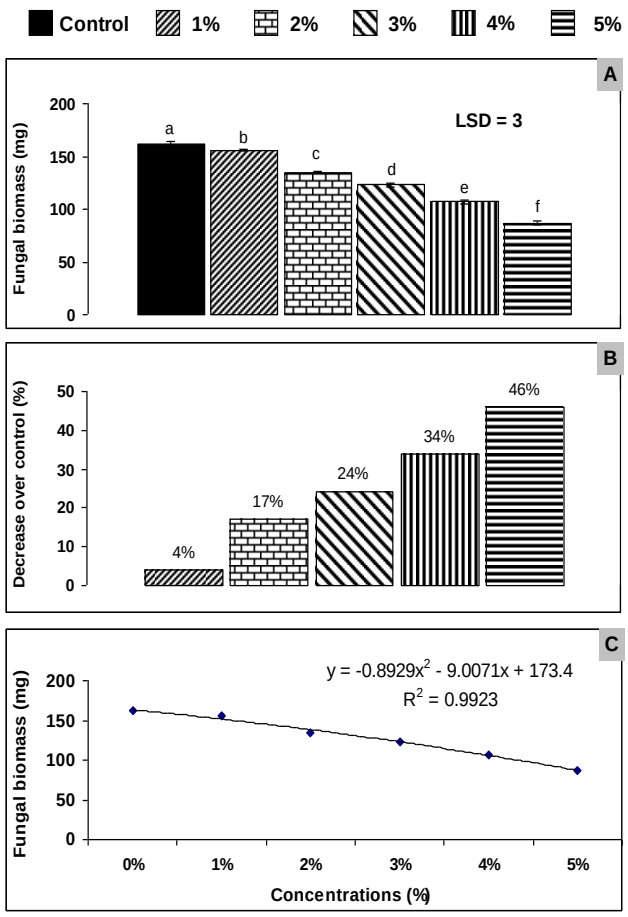

Fig. 6. Effect of different concentrations of methanolic extract of *Syzygium cumini* on biomass of *Alternaria brassicae*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($p \leq 0.05$) as determined by LSD Test.

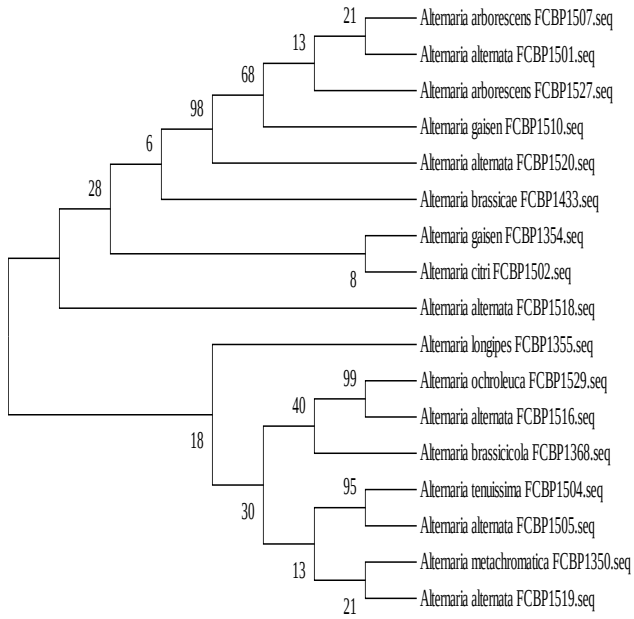

Fig. 5. Molecular Phylogenetic analysis by Maximum Likelihood method of *A. brassicae* (FCBP 1433) with other species of genus *Alternaria*. Number written on each branch is the percentage showing clustering of associated taxa and branch lengths are measured according to the number of substitutions per site.

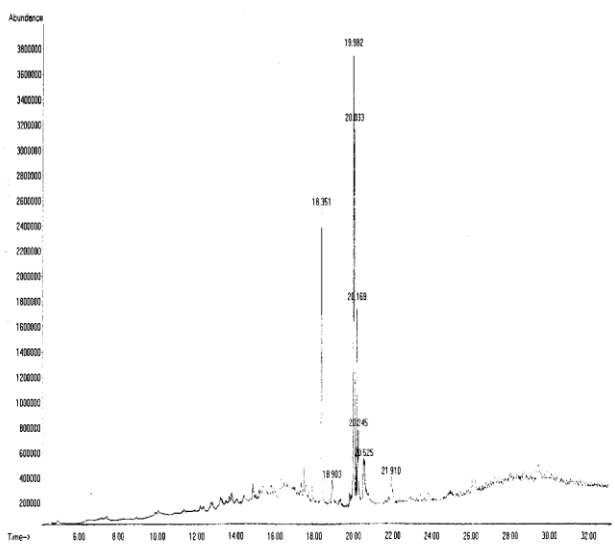

Fig. 7. GC-MS chromatogram of methanolic leaf extract of *Syzygium cumini.*

Discussion

Brassicaceae is an important plant family which includes a number of economically important vegetable crops. Foliar diseases of Brassicaceae crops act as limiting factors for the production of these nutritionally important plants (Peruch *et al.*, 2006). Like other plants, in most of the cases, species of Genus *Alternaria* are responsible for leaf necrosis in Brassicaeae (Reis & Boiteux, 2010). *Alternaria brassicae* and *A. brassicicola* are the two different species which are mostly associated with leaf blights of member of family of Brassicaceae (Ellis, 1971). Present study reports *A. brassicae* as the grey leaf spot pathogen of broccoli for the first time from Pakistan.

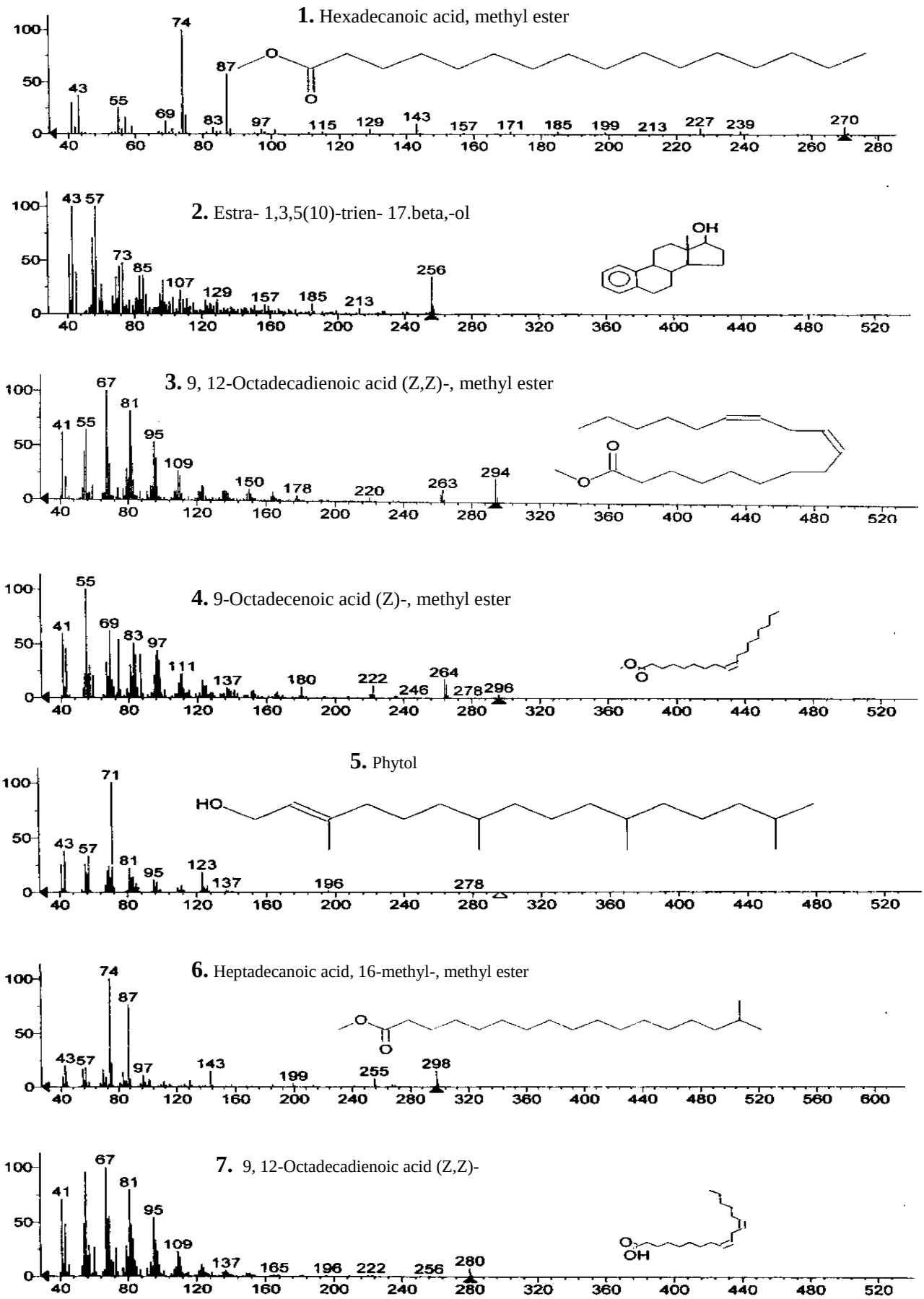

Fig. 8. Mass chromatograms and structures of compounds 1-7 identified in methanolic leaf extract of *Syzygium cumini* through GC-MS analysis.

Taxonomy of *Alternaria* species is complex. However, morphological characters are still considered as most important parameters for species differentiation within this genus. Structure and development of conidia and conidiophores are the most reliable morphological features to identify *Alternaria* at the species level (Anderson *et al.,* 2006). Consequently, polyphasic taxonomy has been established to differentiate various species (Akhtar *et al.,* 2016). BLAST and molecular phylogenetic analysis of ribosomal DNA (rDNA) sequences are the tools commonly used for species identification with success (Shoaib *et al.,* 2014; Akhtar *et al.,* 2016). Presently, *A. brassicae* was identified as causal fungus of broccoli leaf spot. Morphological features of the identified fungus were in accordance as described in published authentic literature (Ellis, 1971; Simmons, 2007). In addition, high homolgy (99%) of nucleotide sequence of amplified ITS1-5.8S-ITS4 region of rDNA strengthened the identification primarily based on morphology. Similarly, Bashir *et al.,* (2014) isolated and identified *Alternaria metachromatica* from tomato on the basis of morphology followed by ITS sequence analysis.

Methanolic leaf extract of *S. cumini* was used for management of *A. brassicae.* Use of methanolic extract is preferred over aqueous extract because of no chance of contamination while using methanol as extracting solvent. Many recent studies have shown usefulness of methanolic extracts in evaluation of antifungal activities of plant extracts against *Sclerotium rolfsii,* (Javaid & Khan, 2016; Khurshid *et al.,* 2016; Javaid *et al.,* 2017). In the present study, the highest concentration of the extract declined fungal biomass by 46%. Earlier, leaf extracts of *S. cumini* in different solvents exhibited antifungal activity against *Ascochyta rabiei* and *Macrophomina phaseolina* (Jabeen & Javaid, 2010; Javaid & Rehman, 2011). Leaf extract of *S. cumini* also known to have antifungal effects against *Candida albicans* (Santos *et al.,* 2012). The major components in leaf extract of *S. cumini* are saponins (Pereira *et al.,* 2016), which may be responsible for its antifungal activity (Coleman *et al.,* 2010). In addition, leaf extract *S. cumini* also contains tannins and other phenolic constituents as it is very rich in gallic and ellagic acid. These compounds could also be responsible for antifungal activity of leaf extract (Chattopadhyay *et al.,* 1998). In the present study, seven compounds were found present in methanolic leaf extract of *S. cumini.* The major compounds namely 9-Octadecenoic acid (Z)-, methyl ester (28.18%), 9, 12-Octadecadienoic acid (Z,Z)-, methyl ester (25.94%), Hexadecanoic acid, methyl ester (18.04%) were fatty acids methyl esters, known to exhibit antifungal activity (Agoramoorthy *et al.,* 2007; Lima *et al.,* 2011; Banaras *et al.,* 2017).

Conclusion

The present study concludes that *A. brassicae* is the cause of leaf spot of broccoli in Pakistan. Methanolic leaf extract of *S. cumini* has the potential to control growth of this fungal pathogen possibly because of fatty acid methyl esters present in the extract.

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(Received for publication 11 July 2017)