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The Incidence of *Alternaria* Species Associated with Infected *Sesamum indicum* L. Seeds from Fields of the Punjab, Pakistan

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Sesame (*Sesamum indicum*) is one of the oldest and most important oilseed crops, mainly grown for its oil and protein content (Johnson et al., 1979). This crop is an important source of fats, proteins, vitamins and minerals in the food of rural people, particularly children, throughout the world (Shahidi et al., 2006). Sesame seed contains oil (48 to 60%), protein (18 to 23.5%), carbohydrate (13.5%), and ash (5.3%), with a moisture content of approximately 5.2% (Obiajunwa et al., 2005; Kahyaoglu and Kaya, 2006). Sesame oil contains the antioxidants sesamoline, sesamin and sesamol (Pastorello et al., 2001). Sesame oil is used in cuisine for salad dressings and the manufacturing of margarine, and is a raw ingredient in industry for making paints, varnishes, soaps, perfumes, insecticides, and pharmaceuticals as a vehicle for drug delivery (Grubben and Denton, 2004).

Cultivation of sesame probably originated in the Harappa Valley region of the Indian subcontinent as long as ago as KP123850.1 in GenBank accessions. The pathogenicity and virulence of these isolates of *Alternaria alternata* was confirmed in inoculations of sesame plants resulting in typical symptoms of leaf blight disease. This work confirms the identity of a major source of sesame leaf blight in Pakistan which will aid in formulating effective disease management strategies.

Keywords: *Alternaria*, *Alt a 1* gene, ITS, Punjab, sesame seeds

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The aim of the work described in this paper was to determine the *Alternaria* species associated with sesame in the Punjab, Pakistan, as an example of the source of yield losses affecting production of this important crop in South Asia. To the best of our knowledge, no such studies have been carried out to date in the Punjab. This basic work on testing seeds for the presence of seed-borne pathogens is an important step in the development of disease management strategies for this crop.

**Materials and Methods**

**Plant Material and Fungal Isolation.** One hundred and five seed samples of sesame were collected from the major sesame growing areas of the Punjab, Pakistan (Fig. 1). Seeds for isolating fungi were selected randomly from within a batch and 50% of seeds were surface sterilized in 5% NaOCl for 2 mins. Surface sterilized and unsterilized seeds were placed at the rate of 25 seeds into 90 mm diameter Petri dishes with three layers of well-moistened filter paper discs (Whatman™ 1001-090 Grade 1). Petri dishes were incubated at 22 ± 2°C for seven days with an alternate cycle of light and darkness (12 h each) in a Versatile Environmental Test Chamber (Sanyo, Japan); illumination was provided by 55W fluorescent lights (Sylvania, Germany) giving a light intensity of 125-130 μmol m⁻² s⁻¹. The experiment was performed in triplicate. After incubation, emerging fungal colonies were counted and isolated onto potato dextrose agar (PDA, Oxoid, UK) amended with 100 mg L⁻¹ streptomycin (Singleton et al., 1993). Isolates were maintained on PDA and identified on the basis of morphological characters (Ellis, 1971, 1976; Simmons, 2007).

Isolation frequency (Fr) and relative density (RD) of fungi in and on seeds were calculated as follows:

\[
Fr(\%) = \left( \frac{ns}{N} \right) \times 100, \quad RD(\%) = \left( \frac{ni}{N} \right) \times 100
\]

Where, ns is the number of samples on which a fungus occurred; N is the total number of seeds sampled; ni is the number of isolates of a fungal genus/species, and Ni is the total number of fungal isolates obtained.

**Fungal DNA Extraction, PCR amplification, and Sequencing.** Genomic DNA of representative isolates of the three most frequent groups of *Alternaria* (A13, A47, and A215) was extracted by scraping the surface of 14 days old cultures grown on PDA. DNA was extracted from 50 mg mycelium per isolate using “Plant DNeasy Mini Kit” (Qiagen, UK) by homogenizing in liquid nitrogen and following the manufacturer’s protocol. DNA concentra-
Alternaria spp. Infecting Sesame Seeds

DNA concentration was measured by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE, USA) and the final concentration of DNA was adjusted to 100 ng/µl for PCR amplification. The Internal Transcribe Spacer (ITS) region of rDNA and *Alt a 1* gene were amplified using ITS1 (TCCGTAGGTGAACCTGCGG) / ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990) and Alt-for (ATGCAGTTCACCACCATCGC) / Alt-rev (ACGAGGGTGAYGTAGGCGTC) primers (Hong et al., 2005a), respectively.

PCR was performed in a 25 µl reaction mixture containing 12.5 µl BioMix (Bioline), 0.5 µl each primer, 2 µl template DNA and 9.5 µl pure water. PCR was performed in a MyCycler™ Thermal cycler (Bio-Rad, USA) with initial denaturation at 95°C for 3 mins followed by 35 cycles of 94°C for 30s, 55°C for 30 s and 72°C for 1 min and a final elongation step at 72°C for 10 min for ITS amplification. For the *Alt a 1* gene amplification, an initial denaturation at 94°C for 1 min was followed by 35 cycles of 94°C for 30 s, annealing at 57°C for 30 s, 72°C for 1 min, and a final

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**Fig. 1.** (A) Sampling sites in the Punjab, Pakistan. (B) Typical leaf blight symptoms on sesame leaves in the field and (C) pods (arrowed) during the sampling in the Punjab, Pakistan.
extension at 72°C for 10 min. Amplified fragments were loaded on an agarose gel (1% w/v) stained with SYBR® Safe DNA gel stain (Invitrogen, USA), visualized under UV light and purified with a “Qiaquick PCR Purification kit” (Qiagen, UK), following the manufacturer’s protocol. Amplicons were sequenced by Source BioScience (UK) in both directions and the sequences analyzed using MEGA 7 software (Kumar et al., 2016) and blasted against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Phylogenetic Analysis.** The phylogeny for each genus was reconstructed using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with a superior log likelihood value. Evolutionary analyses were conducted in MEGA 7 (Kumar et al., 2016).

**Pathogenicity Testing.** Spore suspensions were prepared by flooding PDA cultures with sterile distilled water amended with two drops of Tween 20 per 100 ml and the spore concentration was adjusted to 10⁶ conidia ml⁻¹ using replicate hemocytometer counts. For the pathogenicity test, sesame seeds were sown in a glasshouse under natural light conditions (14 h photoperiod, 25-32°C). Ten sterilized seeds per pot were sown in a soil - sand - farmyard manure mixture (2:1:1 w/w). Plants, 4-weeks old, were sprayed to run off with the spore suspension using a spray bottle and covered with plastic bags to maintain high humidity for 24 h. Symptoms were evaluated 7 days after inoculation. Plants were rated for the presence (+) or absence (−) of 2- to 5-mm-diameter spots of irregular shape, dark brown to grey in color and surrounded by a bright yellow margin. Three replicate pots for each isolate were tested. To verify the ability of the *Alternaria* spp. isolates to colonize the

**Fig. 2.** (A-C) Colony morphology of three most frequent *Alternaria* isolates (A13, A47, A215) obtained from sesame seeds on PDA. (D-F) Conidia of three most frequent *Alternaria* isolates (Bar = 10 µm).
host tissues, re-isolations were made from the lesions onto PDA to fulfill Koch’s postulates. In addition, disease severity (DS) was scored on a revised rating system from Zhao et al. (2016): 0 = no symptoms on leaves; 1 = 0-25% infection on leaves; 2 = 25-50% infection on leaf area. Disease severity index (DSI) was calculated as \( \sum \) (disease severity scale points \times number of plants at each scale point) / (total number of seedlings assessed \times disease severity scale of the highest scale point observed) \times 100 (Zhao et al., 2014).

Effect of Culture Filtrate on Seed Germination and Seedling Growth. Erlenmeyer flasks containing 50 ml of potato dextrose broth (Sigma Aldrich, USA; catalog number P-6685; prepared using 24 g/L and autoclaved at 121°C for 15 minutes) were inoculated with the test fungi and incubated at 28°C. After 5 days, the culture broth and fungal mycelia were carefully separated. A 50 ml volume of 3:2:1 (v/v/v) ethyl acetate: chloroform: methanol was added to each flask containing culture broth, followed by shaking overnight on a rotary shaker. Extracts were centrifuged at 5000 rpm for 30 minutes and the supernatant incubated in a water bath at 45°C for 8-10 h to concentrate the extract to a volume of 10 ml (Jaiswal et al., 2012).

Sesame seeds were surface sterilized in 5% sodium hypochlorite for two minutes followed by 3 rinses in sterile distilled water before suspending in culture filtrates (10 ml). Following incubation at 28 ± 2°C for 24 h, seeds were removed from the filtrate extract and washed once in sterile distilled water. Treated seeds were plated on 1.5% water agar in 90 mm diameter Petri dishes, with 10 seeds per dish. Control seeds were treated with distilled water prior to plating on 1.5% water agar. After 7 days of incubation, shoot and root lengths were recorded. In addition, a vigor index was calculated (Jalander and Gachande, 2012) following the formulae:

\[
\text{Germination} \% = \frac{\text{Germinated seeds of treatment}}{\text{Germinated seeds of control}} \times 100 \\
\text{Vigor index} = \frac{\text{Seed germination} \% \times \text{Seedling Length (Shoot + Root Length)}}{\text{No. of isolates}}
\]

Table 1. Morphological characterization, isolation frequency and relative density of *Alternaria* isolates from sesame seeds

| Isolate code | Name of fungi | Origin (city) | Non sterilized seeds | Surface sterilized seeds |
|--------------|---------------|---------------|---------------------|------------------------|
|              |               |               | Fr | RD | Fr | RD |
| A6           | *Alternaria dianthi* | Sialkot       | 5  | 10 | 2.14 | 4  | 6  | 2.06 |
| A13          | *Alternaria sesami* | Sialkot       | 58 | 24 | 24.79 | 32 | 40 | 16.49 |
| A19          | *Alternaria citri* | Sialkot       | 13 | 18 | 5.56  | 5  | 8  | 2.58 |
| A47          | *Alternaria longipes* | Gujranwala    | 17 | 78 | 7.26  | 19 | 40 | 9.79 |
| A91          | *Alternaria dianthicola* | Gujranwala    | 17 | 24 | 7.26  | 7  | 10 | 3.61 |
| A166         | *Alternaria brassicicola* | Gujranwala    | 6  | 8  | 2.56  | 32 | 40 | 16.49 |
| A181         | *Alternaria solani* | Gujranwala    | 7  | 10 | 2.99  | 3  | 2  | 1.55 |
| A183         | *Alternaria raphanin* | Gujranwala    | 0  | 0  | 0.00  | 1  | 2  | 0.52 |
| A196         | *Alternaria alternata* | Gujranwala    | 13 | 18 | 5.56  | 5  | 6  | 2.58 |
| A203         | *Alternaria dianthicola* | Hafizabad     | 16 | 22 | 6.84  | 4  | 6  | 2.06 |
| A215         | *Alternaria brassicae* | Hafizabad     | 18 | 26 | 7.69  | 30 | 26 | 15.46 |
| A217         | *Alternaria citri* | Hafizabad     | 2  | 2  | 0.85  | 0  | 0  | 0.00 |
| A218         | *Alternaria infectoria* | Hafizabad     | 3  | 4  | 1.28  | 0  | 0  | 0.00 |
| A220         | *Alternaria sesamicola* | Gujrat        | 17 | 24 | 7.26  | 6  | 8  | 3.09 |
| A221         | *Alternaria helianthi* | Gujrat        | 9  | 12 | 3.85  | 6  | 8  | 3.09 |
| A228         | *Alternaria longissimi* | Gujrat        | 10 | 14 | 4.27  | 3  | 4  | 1.55 |
| A236         | *Alternaria raphanin* | Gujrat        | 4  | 4  | 1.71  | 1  | 2  | 0.52 |
| A239         | *Alternaria tenaxissima* | Attock       | 7  | 10 | 2.99  | 0  | 0  | 0.00 |
| A249         | *Alternaria triticina* | Attock        | 0  | 0  | 0.00  | 19 | 26 | 9.79 |
| A261         | *Alternaria radicina* | Mandi Bahuddin | 4 | 4  | 1.71  | 4  | 8  | 2.06 |
| A263         | *Alternaria pluriseptata* | Mandi Bahuddin | 3 | 6  | 1.28  | 2  | 2  | 1.03 |
| A267         | *Alternaria cinerariae* | Mandi Bahuddin | 2 | 4  | 0.85  | 5  | 8  | 2.58 |
| A272         | *Alternaria chlamydospora* | Mandi Bahuddin | 3 | 4  | 1.28  | 6  | 6  | 3.09 |

Fr = Isolation frequency; RD = relative density.
Results

Morphological Identification of *Alternaria* species. A total of 428 isolates of *Alternaria* were recovered from sesame seeds collected in the Punjab, Pakistan. Seeds were selected from plants exhibiting blight symptoms on leaves (Fig. 1B) and pods (Fig. 1C). These isolates were cultured to homogeneity: all isolates developed loose, cottony and greyish-green to olive brown colonies on PDA after incubation at 25°C for 7 days in the dark (Fig. 2A, B, C). Isolates were grouped morphologically, based on well-established features of *Alternaria* species, focusing on colony characteristics and conidial structure (Fig. 2). Based on these features, the isolates were placed into 36 morphological groups and identified as far as possible. Isolates produced conidia as solitary or in short chains. Conidia were narrow-obclavate, ovoid or ellipsoid with 1-2 longi-septa.

The mean isolation frequency and relative density of each isolate form was determined in 100 non-surface sterilized and in 100 surface sterilized seeds. It was observed that majority of isolates were obtained from Gujranwala followed by Hafizabad, Gujrat and Mandi Bahuddin. Quantitatively three isolate groups (A13, A47, A215) were found most frequently (Table 1) and selected for further analyses.

Identification by DNA sequencing. PCR amplification of the ITS regions of rDNA yielded fragments of ~550 bp from all tested *Alternaria* isolates. PCR of the *Alt a 1* gene gave fragments of ~500 bp in length from all isolates. BLAST searches on NCBI showed the ITS sequences to be 100% identical to those of *Alternaria* isolates described as “*Alternaria* sp.” but also matched precisely with named species including *A. alternata*, *A. longipes* and *A. brassicae*. Phylogenetic analysis using ITS sequences failed to differentiate the isolates, grouping all 3 into a single clade (Fig. 3), with the reference sequences of *A. alternata* (KJ735925.1), *A. brassicae* (KJ728842.1) and *A. longipes* (KJ722535.1) obtained from GenBank. In contrast, sequences of the *Alt a 1* gene showed > 99% homology with *Alternaria alternata* and phylogenetic analysis placed the isolates in a clade close to the reference sequence of *Alternaria alternata* (KP123850.1; Fig. 4). Similarly, some morphological characters of selected isolates (A13, A47 and A215) showed homology with colony and conidial characters of *A. alternata*. However, the *Alt a 1* sequence of strain A13 indicated that it was distinct from the *A. alternata* clade. Sequences from the isolates obtained in this work were submitted to GenBank (Table 2).

Pathogenicity Tests. Seven days after inoculation with a spore suspension of the *Alternaria* isolates, brown lesions surrounded by yellow haloes began to develop on sesame leaves; disease symptoms gradually spread from leaf margins to the midveins, similar to symptoms observed in the sesame fields during the sample collection. In a later stage, blight extended to the center of the leaves and plants be-
came defoliated (Fig. 5). Extended necrosis surrounded by yellowing was usually observed on diseased leaves. Pathogenicity tests confirmed that all tested isolates were virulent leaf blight pathogens, the most virulent being A13 (Table 3). No symptoms developed on control plants. The Alternaria isolates were reisolated from symptomatic leaves of inoculated plants. The maximum likelihood phylogenetic tree obtained from consensus sequences of Alternaria isolates from sesame seeds using Altfoward and reverse primers. Numbers above the branches are bootstrap values from 500 replicates. The scale indicates the genetic distance between the species.

Table 2. Molecular Identification of three most frequent Alternaria isolates obtained from sesame seeds

| Isolate code | Origin (city) | Morphological identification | Molecular identification | NCBI accession no. |
|--------------|---------------|------------------------------|--------------------------|-------------------|
| A13          | Sialkot       | Alternaria sesami            | Alternaria sp.            | KY190101          |
|              |               |                              | Alternaria alternata      | KY124234          |
| A47          | Gujranwala    | Alternaria longipes          | Alternaria sp.            | KY190102          |
|              |               |                              | Alternaria alternata      | KY124235          |
| A215         | Hafizabad     | Alternaria brassicae         | Alternaria sp.            | KY190103          |
|              |               |                              | Alternaria alternata      | KY124236          |
lated plants, but not from any of the control plants.

**Effect of Culture Filtrates on Seed Germination.** Seed germination and vigor of sesame seedlings were adversely affected by the culture filtrates of isolate A13 (Fig. 6). The reduction in seed germination ranged from 40-60%, while vigor index in samples treated with filtrates of this isolate was 4% compared with over 62% in control plants (Table 4).

**Discussion**

This work clearly demonstrated that *Alternaria* species are present in a high proportion of sesame seeds collected from various regions in the Punjab, Pakistan, suggesting that there is a potential source of inoculum to initiate disease outbreaks under conditions favorable to pathogen develop-
ment. The most common species of *Alternaria* found in the present work was *A. alternata*. Species delineation within the genus *Alternaria* requires careful attention in order to determine the range of species causing diseases on a given host.

Amongst the species of *Alternaria* identified morphologically, three representatives, chosen from the most commonly occurring groups (A13, A47, A215), were initially identified as *Alternaria sesami*, *Alternaria longipes* and *Alternaria brassicae*, respectively, on the basis of conidial morphology and colony characteristics. Identification of *Alternaria* species based on morphological criteria, however, remains a challenging task, leading to controversy and confusion very often accompanying classification in this genus (Maraite et al., 1998). This problem is especially relevant for the small-spored species, which share morphological characteristics such as having overlapping conidial size ranges (Simmons, 1992). Although the ITS region of rDNA is widely used as a barcode for fungi (Blaalid et al., 2013), the sequences of the *Alternaria* isolates analyzed here were 100% identical to those of “*Alternaria sp.*” in Genbank, but were also identical to those of other *Alternaria* species, including *A. alternata*, *A. longipes* or *A. brassicae*. It has been recognized that ITS sequences are not always able to differentiate at the species level in the genus *Alternaria* (Pryor and Michailides, 2002; Shipunov et al., 2008; Zur et al., 2002).

In contrast, use of the partial coding sequence of the *Alternaria* major allergen gene (*Alt a 1* gene) in PCR enabled accurate identification to the species level, confirming that all three isolates tested were *Alternaria alternata*, in agreement with other workers studying different hosts for this genus of pathogens (Guo-yin et al., 2013; Paul et al., 2015; Skóra et al., 2015). It has been suggested that the *Alt a 1* gene sequence evolved 3.8 times faster and contained 3.5 times more parsimony-informative sites than glyceraldehyde-3-phosphate dehydrogenase (*gpd*) sequences, strongly supporting the use of this part of the genome for species identification in *Alternaria* (Hong et al., 2005a).

Based on this work, therefore, it can be concluded that *Alternaria alternata* is a destructive pathogen causing leaf blight on sesame in the Punjab, Pakistan. The incidence of *Alternaria alternata* in sesame seed was high compared to other species of *Alternaria* isolated from this host plant. Most *Alternaria* spp., including *A. alternata*, exhibit considerable morphological plasticity depending on cultural conditions, including substrate, temperature, light and humidity. These fungi, therefore, in particular *A. alternata*, can be frequently misidentified when relying on morphological characteristics alone (Misagi et al., 1978; Roberts et al., 2000; Simmons, 1992). Moreover, the inoculation tests carried out with the three isolates of *Alternaria alternata* confirmed pathogenicity and virulence, with the development of leaf blight in sesame, similar to the results reported with *A. longipes* by Shoaib et al. (2014).

The wide variability among different *Alternaria* species from different hosts has been reported previously (Kumar et al., 2008; Pryor and Gilbertson 2000; Pryor and Michailides, 2002; Quayyum et al., 2005). Among the different diseases caused by species in the genus *Alternaria*, leaf blight disease is one of the most important, causing yield loss in the range of 32-57% per annum in canola, for example (Conn and Tewari, 1990). In the present study, symptoms of the disease on sesame include the presence of irregular, often circular brown to dark brown colored spots on the leaves, with concentric lines inside the spots which are in agreement with the findings of Valkonen and Koponen (1990) who reported that individual circular spots may coalesce to form large patches, resulting in leaf blight of Chinese cabbage. In some infections, small dark colored spots are formed on pods and young shoots of Chinese cabbage.

An important conclusion drawn from the work presented in this paper is that identification of *Alternaria* spp. on the basis of morphological characteristics alone is unreliable for correct determination of species. More stringent identification was possible using species-specific primers, which are required in diagnostics for sesame seed testing: ITS sequences were not sufficiently discriminatory to separate species.

**Table 4. Effects of culture filtrates from *Alternaria* isolates (A13, A47 and A215) on germination of sesame seeds and growth of seedlings**

| Treatment | Germination % | Root length (cm) | Shoot length (cm) | Vigor index |
|-----------|--------------|-----------------|-----------------|------------|
| A13       | 40.00        | 0.10            | 0.00            | 4.00       |
| A47       | 60.00        | 0.40            | 0.90            | 24.90      |
| A215      | 53.33        | 0.20            | 0.00            | 10.67      |
| Control   | 86.67        | 0.70            | 1.50            | 62.17      |

Vigor index = Seed germination (%) × Seedling Length (Shoot + Root Length).
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