ISOLATION AND IDENTIFICATION OF *ASPERGILLUS TUBINGENSIS* (SCHIBER) MOSSERAY- A NOVEL LEAF SPOT PATHOGEN OF *HELIANTHUS ANNUUS* L. IN PAKISTAN

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ABSTRACT

Helianthus annuus L. (sunflower) is an annual crop and is known for its edible oil. It is attacked by many pathogenic fungi which result in low yield. In October 2018, sunflower leaf spot disease symptoms were observed in different areas of Lahore. The infected leaf samples were collected for pathogens isolation and identification. The isolated pathogen was characterized morphologically as well as genetically by nucleotide sequencing of rDNA using three different primers i.e., Internal transcribed spacer region (ITS), β-Tubulin (Bt2), and Osteomodulin (OMD). *Aspergillus tubingensis* (Schiber) Mosseray was identified as the leaf spot-causing pathogen of sunflower. To confirm the pathogenicity Koch's pathogenicity test was performed by inoculating artificial fungal suspension in pots and plate assays. The emergence of similar disease symptoms and re-isolation of the same pathogen verified Koch's postulates. This study represents the first report of *A. tubingensis* as a leaf spot pathogen of sunflower in Pakistan and provides an experimental framework to elucidate the genetic diversity and disease ecology of field populations of *A. tubingensis*.

Keywords: *Aspergillus tubingensis; Helianthus annuus;* Identification; Koch's pathogenicity test; necrosis.

INTRODUCTION

Helianthus annuus L. has great importance in the world as its seeds are used as food and edible oil. The production and expansion of sunflowers on land are fluctuating due to the invention of socio-economic constraints. The annual yield of sunflower oil is 1.3 tons/ha in Pakistan. Sunflower oil is healthy, and its seeds are nutritious for many foods (Nasir, 2003). Its seeds are rich in vitamins, minerals, magnesium, potassium, selenium, and iron (Khan, 2007; Adeleke and Babalola, 2020). They help in the improvement of brain power, digestion, and functioning of the cardiovascular system. It is used in the treatment of many diseases i.e., obesity, heart diseases, indigestion, and used to lower the level of saturated fats (Sharon and Carrie, 2004). Scientists reported 90-100 different diseases of sunflowers worldwide (Bai *et al*., 1985; Mukhtar, 2010). Mostly fungal pathogens are the main cause of deleterious diseases in sunflower and other plants. The production of crops can be increased by controlling the disease-causing pathogens with better management practices (Bowers and Locke, 2004; Tsakona *et al.*, 2012). The present work focused on the diseases of Sunflower; leaf spots and wilting caused by fungal pathogens that are indirectly affecting the economy of Pakistan. The main objective of the present study was to identify the foliar pathogen/s associated with leaf spots of sunflower plants.

MATERIALS AND METHODS

Investigation and Collection of Virulent Samples

A field survey was conducted to study and collect the samples of sunflower leaves infected with leaf spots from the fields of the Institute of Agricultural Sciences, University of the Punjab, Botanical Garden of the University of the Punjab, Canal road near University of Punjab, Model Town Park Lahore, and Jallo Park Lahore during October 2018. For the isolation of pathogens, randomly four infected leaf samples per plant from each area were taken in sterilized polythene bags and brought to the laboratory. The samples were saved at 4 °C for further experiments.

Isolation, Purification, and Identification of Pathogen

For isolation, the infected part of the leaf was cultured in an MEA medium (25-27 °C). About 4-5 spots; from infected leaf samples, were cut into 2 mm^2 pieces which also contained some healthy parts of the leaf. These leaves were surface sterilized with 1% sodium hypochlorite solution for 5 minutes followed by washing with distilled

water. About 3-4 pieces were inoculated on MEA medium under aseptic conditions. The plates were incubated at 25 ± 2 °C. Then these plates were examined regularly for fungal growth and purified. The isolated pathogen was identified on the basis of morphological characteristics. The pathogen was further identified or confirmed from nucleotide sequencing by using three primers i.e., Internal Transcribed Spacer Region ITS (ITS1; Forward, 5′-TCCGTAGGTGAACCTGCGG-3′) (ITS4; Reverse, 5′-TCCTCCGCTTAT TGATATGC-3′), Osteomodulin OMD (OMD5; Forward, 5′-CCGAGTACAAGGAGGCCTTCC-3′) (OMD6; Reverse, 5′-CCGATAGAGGTCATAACGTGG-3′) and β –tubulin (βt2a; Forward, 5′GGTAACCAAATCGGTGCTGCTTTC-3′) (βt2b; Reverse, 5′-ACCCTCAGTGTAGTGACCCTTGGC 3′). Fungus-specific universal primer pairs successfully amplified all tested genes. Using the National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) bioinformatics websites, DNA sequences were BLAST.

Pathogenicity Test

The Koch pathogenicity postulates were confirmed by *in vitro* (detached leaf assay) and *in vivo* (Pot trails) experiments. Under aseptic conditions, 10 mL of 1% saline tween (0.9% NaCl and 0.1% tween 80) were prepared in distilled water, and spores from 7-8 days old pure fungal cultures were scratched and suspended in saline tween 80. The 5 mL spore suspension (5×10^5 spores/mL) was injected with the help of a sterilized syringe in the stem nodes and also by the spraying of spore suspension in soil. Control received the same volume of distilled water. The plants were kept in shade under optimum temperature i.e. 25 – 26 ºC and watered properly. After 6-8 days of inoculation of spore suspension, disease symptoms started to appear on the leaves. The Disease rating scale was constructed on the basis of the percentage of infection.

RESULTS

The present study was designed to explore the pathogen of leaf spot disease in *H. annuus* through the isolation, and identification of pathogenic fungus followed by a pathogenicity test. A number of field surveys were conducted from June to October 2018; in different areas of Lahore i.e., Jallo Park, Botanical Garden; PU, Canal road, and Johar town, Lahore, and the infected samples were collected, observed, and analyzed on the basis of disease symptoms (Fig. 1). During the survey, the samples collected from fields of *H. annuus* were scrutinized to be infected with leaf necrosis, lesions, and dead tissues. A field survey revealed more than 50% of *H. annuus* plants were infected with leaf necrosis. In general, the symptoms observed were brown irregular lesions with a yellow halo around them on leaves (Fig. 1). The size of the spots on leaves was 2-5 mm, and about 40-50% leaf was found to be infected with such spots or lesions. Infected plants were in bad health while old infected leaves were found to be withered.

Fig. 1. Infected sample collected from field survey.

Identification of Pathogen

Pure culture of the isolated pathogen was initially studied in detail to identify the pathogen morphologically as well as molecularly and was identified as *Aspergillus tubingensis* (Schiber) Mosseray. On MEA medium *A. tubingensis* was white to yellow in the center with blackish spores with white aerial mycelia at the border (Fig. 2). The pathogen produced bi-seriate conidiophores with septate metulae and phialides. Conidial heads were dark

brown, radiated, and composed of catenulate conidia and globose vesicles. The present conidiophores were about 100 µm to 150 µm in length. The conidia were globose and phialosporous with a rough-walled surface (Fig. 2). The conidia were single-walled, radially attached at a single point, and with a double-layer ring in the center. The small, circular, globose spores were found at about 0.5 µm. Based on morphological features the species was identified to be *Aspergillus tubingensis.*

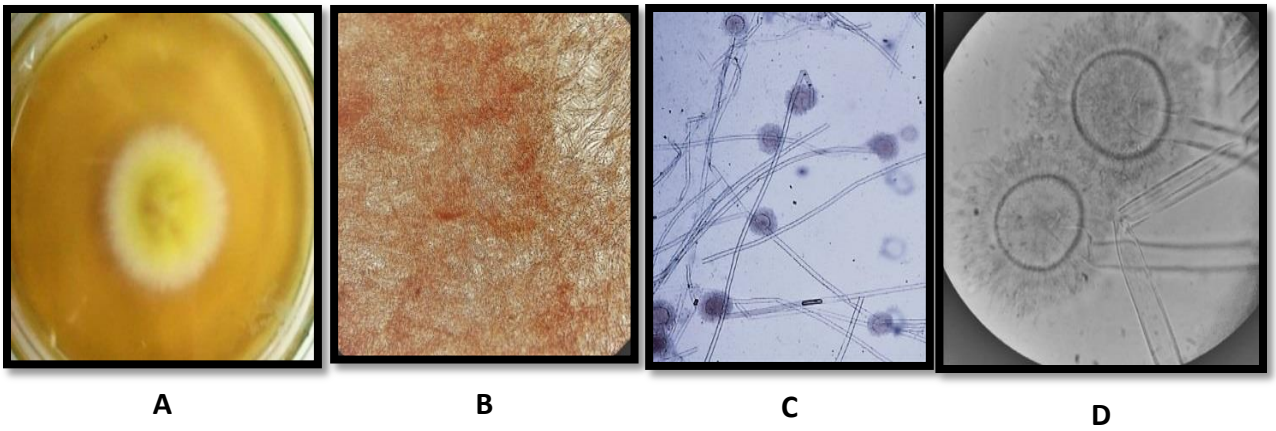


Fig. 2. *Aspergillus tubingensis* isolated from leaf spots of sunflower. (A): Front side of a colony grown on MEA; (B): Conidial heads under a stereoscope (C-D): Microphotograph of conidial heads at 10X and 40X, respectively.

Genomic Characterization of Pathogen

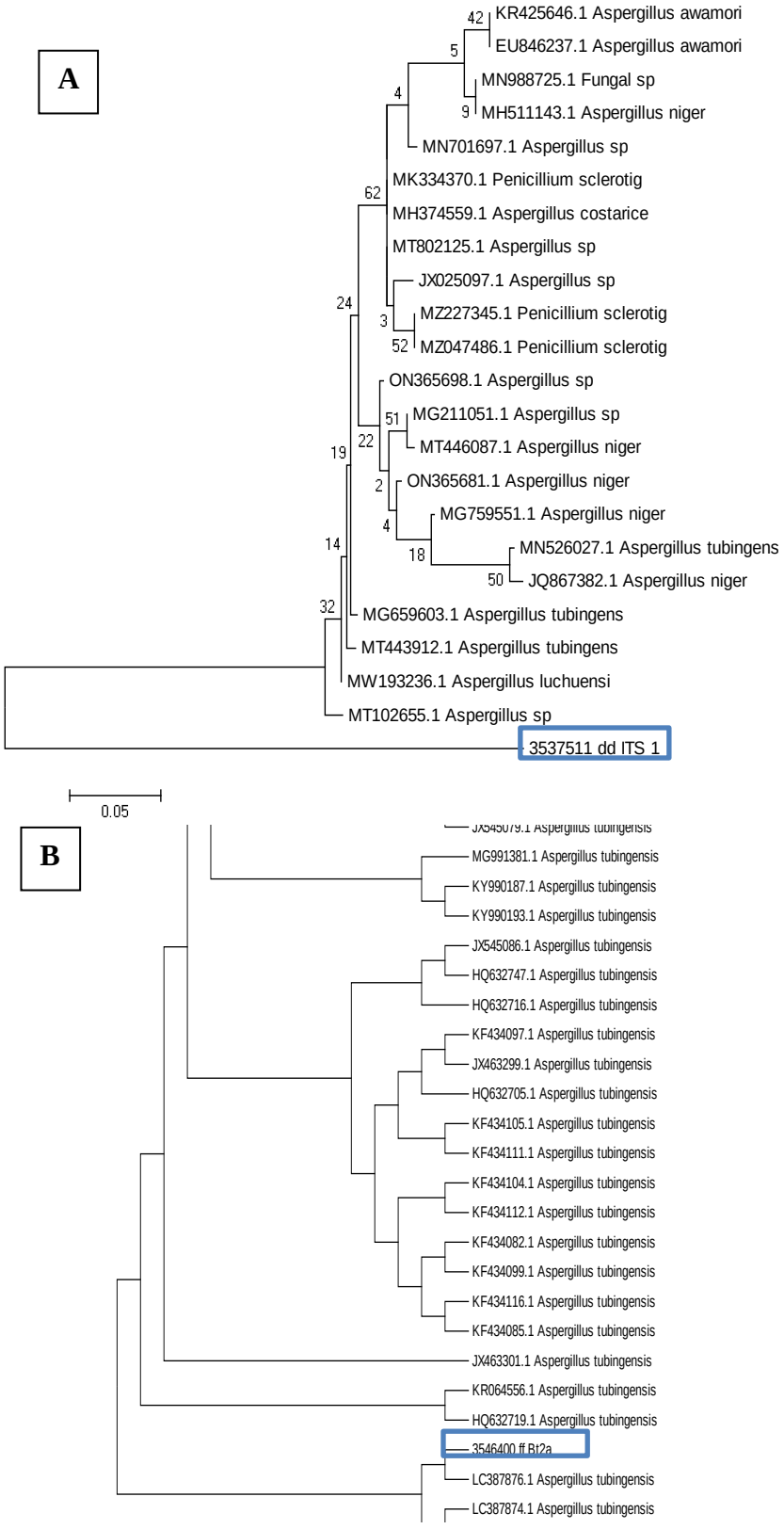
The isolated fungal strain was subjected to DNA extraction and a compact single band of about 10000-12000 bp was observed on 1% agarose gel. Fungus-specific universal primer pairs Internal Transcribed Spacer sequence (ITS), β-Tubulin, and Osteomodulin successfully amplified all tested genes, providing a single PCR product of about 500 bp - 650 bp. Purified PCR products yielded sequences of 500–650 bp in length. Using the National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) bioinformatics websites, DNA sequences were BLAST. BLAST results using ITS sequence of *A. tubingensis* as query revealed that it had 100% homology with strain (MG659603.1), 98% homology with more than 20 different strains of *A. tubingensis* in GenBank database, i.e. MK817590.1, MK817588.1, MH055393.1 & MK616376.1, provided the single PCR product of 576 bp on 1% agarose gel (Fig. 3A). The amplified ITS nucleotide sequence of *A. tubingensis* was assigned MN526027 accession ID in GenBank.

Blast analysis of *A. tubingensis* with partial Beta tubulin primer revealed that it was 99.60% homologous with *A. tubingensis* HQ632664.1 & LC467945.1, provided the single PCR product of 520 bp on 1% agarose gel (Fig. 3B). When the homologies searches were carried out for OMD sequence of *A. tubingensis*, 99.62% similarity was found with *A. tubingensis* KY612372.1, KX231823.1 and 98% similarity was noticed with KX768540.1, MH644926.1, with 564 bp band (Fig. 3C).

Koch's Pathogenicity Test

The pathogenicity test was conducted *in-vitro* and *in-vivo* to determine the pathogenic potential of the test fungus and to reconfirm the pathogen of a particular host. In the *in-Vitro* analysis after 2-3 days of inoculation chlorosis started on the leaves with the emergence of small yellow to brown irregular spots spreading on the whole leaf. Leaf spots progressed towards necrosis after 6-7 days and eventually led to the death of the whole leaf. To confirm the disease etiology the infected leaf sample was inoculated on a medium and the same pathogenic fungus was found to grow (Fig. 4).

In the *in-vivo* pathogenicity test, infection was evident 8-10 days after post-inoculation. The symptoms observed on the plants were found to be similar to the samples that were collected during the survey. Foliar symptoms visualized were: yellowing followed by chlorotic spotting of the lowermost leaflets and in later stages, complete necrosis of leaves was noticed, and eventually, death of the plant occurred (Table 1). The pathogen was thus proved virulent as it exhibited 100% mortality in the host plant.


A
42
KR425646.1 Aspergillus awamori
5
EU846237.1 Aspergillus awamori
MN988725.1 Fungal sp
4
9
MH511143.1 Aspergillus niger
MN701697.1 Aspergillus sp
MK334370.1 Penicillium sclerotig
62
MH374559.1 Aspergillus costarice
MT802125.1 Aspergillus sp
JX025097.1 Aspergillus sp
24
3
MZ227345.1 Penicillium sclerotig
52
MZ047486.1 Penicillium sclerotig
ON365698.1 Aspergillus sp
51
MG211051.1 Aspergillus sp
22
MT446087.1 Aspergillus niger
19
2
ON365681.1 Aspergillus niger
4
MG759551.1 Aspergillus niger
14
18
MN526027.1 Aspergillus tubingens
50
JQ867382.1 Aspergillus niger
32
MG659603.1 Aspergillus tubingens
MT443912.1 Aspergillus tubingens
MW193236.1 Aspergillus luchuensi
MT102655.1 Aspergillus sp
3537511 dd ITS 1
0.05
B
MG991381.1 Aspergillus tubingensis
KY990187.1 Aspergillus tubingensis
KY990193.1 Aspergillus tubingensis
JX545086.1 Aspergillus tubingensis
HQ632747.1 Aspergillus tubingensis
HQ632716.1 Aspergillus tubingensis
KF434097.1 Aspergillus tubingensis
JX463299.1 Aspergillus tubingensis
HQ632705.1 Aspergillus tubingensis
KF434105.1 Aspergillus tubingensis
KF434111.1 Aspergillus tubingensis
KF434104.1 Aspergillus tubingensis
KF434112.1 Aspergillus tubingensis
KF434082.1 Aspergillus tubingensis
KF434099.1 Aspergillus tubingensis
KF434116.1 Aspergillus tubingensis
KF434085.1 Aspergillus tubingensis
JX463301.1 Aspergillus tubingensis
KR064556.1 Aspergillus tubingensis
HQ632719.1 Aspergillus tubingensis
3546400 ff Bt2a
LC387876.1 Aspergillus tubingensis
LC387874.1 Aspergillus tubingensis

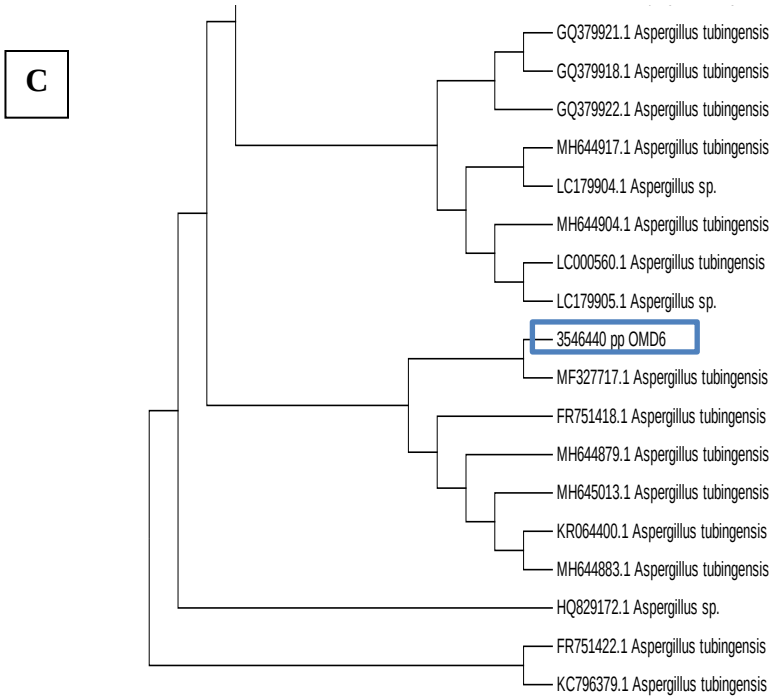


Fig. 3. Phylogenetic tree of *A. tubingensis* amplified by ITS, Partial β tubulin, and OMD Primer.

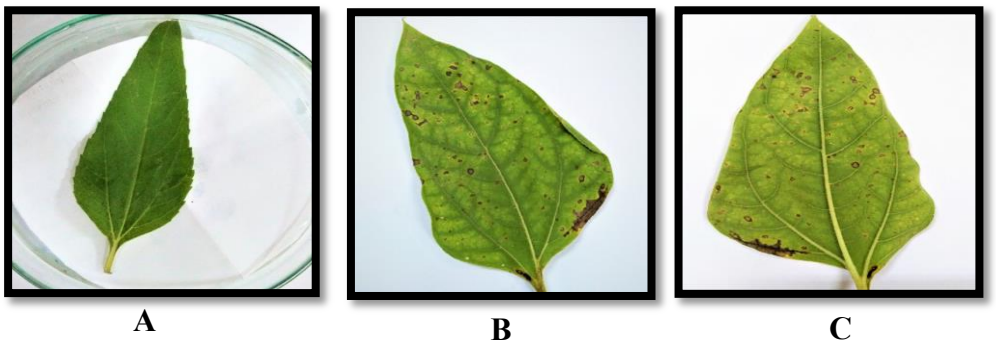


Fig. 4. Comparative analysis of disease development caused by *A. tubingensis*; (A): Control, (B & C): abaxial and adaxial side of the infected leaf, respectively.

Table 1. Pictorial representation of disease rating scale on sunflower plant by *A. tubingensis.*

0% Disease Severity	**20% Disease Severity**	**40% Disease Severity**	**60% Disease Severity**	**80% Disease Severity**	**100% Disease Severity**
					
Healthy Plant	Yellowing started on the tips of leaves	White leaf spots started to appear on the leaves surface	Brown color spots became enlarge	Leaf started to become blackish from the margin and started to drop	Complete death and dieback of whole plant

DISCUSSION

Sunflower is the most important crop for its oil seed and nutritional significance. It is affected by various fungal and bacterial diseases which result in a low yield of oil production. Among all diseases, fungal leaf spot is a serious disease that affects the productivity of plants (Kochhar, 2005; Jiskani, 2006). For the control of leaf spot fungi in sunflowers, it is necessary to accurately identify the fungi associated with leaf spot disease. This study accordingly emphasized the precise identification of the agent causing leaf spots. Presently, Sunflower leaf spots disease; caused by *A. tubingensis;* was first time reported in Pakistan. The conventional identification of pathogens is mainly based on morphological characters (Anderson *et al*., 2006). To determine the presence of pathogens, researchers need authentic tools which can cater to the increasing need of finding faster, more accurate analytical techniques for discovering agents. Therefore, molecular data i.e., the sequencing of genomic DNA and confirmation by a phylogenetic tree or by using mitochondrial small subunit rDNA sequence method in combination with morphology are used for the identification of fungi (Kretzer *et al*., 1996; Mirhendi *et al*., 2007; Porras-Alfaro *et al*., 2014; Javaid *et al*., 2018). Presently, the pathogen was isolated and identified on the basis of morphological and molecular characterization in which multi-locus sequence analysis of ITS, β-tubulin, and osteomodulin coding genes using concerned primers and PCR products were obtained. It has been widely accepted that ITS nucleotide sequences in combination with any coding gene, e.g. GADPH, elongation factor, β-tubulin, OMD, calmodulin, etc. are a useful, easy, and authentic way of fungal species identification (Schoch *et al*., 2012). In the contemporary lines, Shafique *et al*. (2019) conducted an experiment and identified *Cladosporium cladosporioides* as the causal agent of leaf spot of *S. melongena* on the basis of morphology as well as genetic characterization.

After pathogen identification; the pathogenic potential of *A. tubingensis* was assessed subsequently by applying Koch's pathogenicity postulates using the leaf detached method and pot trials where the pathogen-induced characteristic symptoms as yellowing of leaves and dark brown spots on sunflower leaves. Working in parallel lines; Sahar (2016) reported the pathogenic potential of *Setosphaeria rostrata* and *Cladosporium cladosporioides* on *Solanum melongena* using the same pathogenicity postulates. These pathogens induced almost the same symptoms on the respective plant except for a few differences. Recently in another study, Asghar (2017) evaluated the pathogenic potential of *Alternaia alternata* and *Cladosporium oxysporum* by applying Koch's postulates using the leaf detached method and pot trials and found a sharp progressive disease curve with 99% and 97% of the infected area, respectively.

The present study concludes the report on the novel isolation of leaf spot pathogen from sunflower plants. This study emphasizes the need for management of this pathogen which is responsible for yield loss of this important oil seed crop.

ACKNOWLEDGEMENTS

This work was supported by the facilities available in a fungal biotechnology research laboratory at the Institute of Agricultural Sciences, University of the Punjab, Lahore.

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(*Accepted for publication September 2022*)