Pak. J. Bot., 56(6): 2381-2388, 2024. DOI: http://dx.doi.org/10.30848/PJB2024-6(16)

MORPHOLOGICAL AND MOLECULAR CITATIONS OF *ASPERGILLUS FUMIGATUS* ASSOCIATED WITH LEAF NECROSIS OF *HELIANTHUS ANNUUS* L. IN PAKISTAN

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Abstract

Sunflower (*Helianthus annuus* L.) is an annual edible oil produce rich in minerals, vitamins, and nutrients. Sunflowers are susceptible to many fungal diseases including rust, rotting, sclerotinia stalk, downy mildew, and leaf spots. The harshness of such diseases results in low yields. However, disease management requires the correct identification of the causal agent. In the present study, sunflower leaf spot symptoms were observed in Jallo Park, Botanical Garden; University of the Punjab, Canal Road, and fields of Johar town, Lahore. The infected leaves were sampled from fields for pathogen isolation, purification, and identification morphologically as well as genetically by nucleotide sequencing of rDNA using four primers i.e., Internal spacer region (ITS), Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-Tubulin (Bt2) and Osteomodulin (OMD). The pathogen exhibited a green colony on MEA. Conidiophores were cylindrical, with conical terminal vesicles. Conidial heads were uni-seriate and conidia were smooth-walled, globose, green, and their size was 2-4 µm. On the Base of morphological analysis, the fungus was identified as *Aspergillus fumigatus* (Fresenius, B. 1863). BLAST results executed with all primers revealed 99-100% homology of the pathogen with *A. fumigatus* strains in Genbank. To confirm the pathogenicity, Koch's pathogenicity test was performed by inoculating fungal suspension on pots and plate assays. The emergence of similar symptoms of disease and re-isolation of the identical pathogen verified Koch's postulates. The study signifies the first report of *A. fumigatus* as a pathogen inducing leaf spot of sunflower in Pakistan and explicates the need for the management of the pathogen.

Key words: *Aspergillus fumigatus*, BLAST analysis, *Helianthus annuus*, Morphology, Pathogenicity, Sequencing.

Introduction

Sunflower is famous for its beauty; healthy oil seeds and valuable nutritious aspects (Nasir, 2003). Sunflower seeds are rich in vitamins, minerals, magnesium, potassium, selenium, and iron (Khan, 2007; Adeleke & Babalola, 2020). It also contains fats and many important fatty acids i.e., linolenic acid, and oleic (90% unsaturated fatty acids) acid with remaining palmitic and steric acid (saturated fatty acid). They help in the improvement of brain power, digestion, and functioning of the cardiovascular system. It is the best remedy against obesity, heart disease, and indigestion, and is used to lower the level of saturated fats (Guo *et al*., 2017).

Sunflower crop was familiarized in Pakistan as an oilseed crop about 80 years ago when the country was in deficit in vegetable oil production (Burney *et al*., 1990). 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. Pakistan meets its requirement for oil through 28% from indigenous sources and 78% from imports. The consumption rate is increasing by 5.4% annually; if local production of edible oil is not increased the annual bills for oil will be raised to billions and affect the economy (Anon., 2008). In these circumstances, sunflower oil acts as the bridge to meet the consumption rate.

The quality and yield of sunflowers are lowered due to poor management practices (Mirza & Beg, 1983). Scientists reported 90-100 different diseases of sunflower worldwide (Bai *et al*., 1985; Mukhtar, 2010). Among all the insects, pests, viruses, fungi, and bacteria; mostly the fungal pathogens are the main cause of deleterious diseases in sunflower and other plants (Ara *et al*., 1996; Bhutta *et al*., 1997; Amin & Youssef, 1997). The major diseases caused by fungi include rust, Phoma black stem, Verticulum wilt, anthracnose, downy mildew, and leaf spot. The most destructive disease of sunflower is the *Sclerotnia sclerotiorum* sapling, stem, and head rots (Mesterhazy & Gulya, 1988). Several soil-borne pathogenic fungi reported from the sunflower seeds are *Alternaria, Aspergillus, Cladosporoium Fusarium, Drechslera*, and *Penicillium* (Kaur *et al*., 1990; Shahnaz & Ghaffar, 1991; Reddy, 1993). One of the recorded severe diseases related to sunflower is *Alternaria* blight (Mirza & Beg, 1983).

The management of the diseases is a pre-requisite to procure the increased production of sunflower. The production of crops can be increased by identifying and controlling the disease-causing pathogens with better management practices. The current research signifies the accurate identification of the causal agent associated with the sunflower diseases; leaf spots and wilting caused by fungal pathogens that are indirectly affecting the economy of Pakistan.

Material and Methods

Assortment of samples: The field survey was accompanied to know and collect samples of sunflower leaves infected with leaf spots from the fields of Jallo Park; Lahore, Botanical Garden of the University of the Punjab, Canal Road near University of the Punjab, and fields of Johar Town Lahore, during July to October 2018

and 2019. The latitude of the sampling place is 31.4 and the longitude is 74.35. For the isolation of pathogen/s, four infected leaf samples per plant were chosen from each area randomly, taken in sanitized polythene bags, and brought to the Fungal Biotechnology Lab and saved at 4°C for further experiments.

Isolation and purification of pathogen/s: Malt Extract Agar (2% MEA) was made (Sinclair & Dhingra, 1995). The leaf samples were superficially sanitized with sodium hypochlorite solution (1%) for 5 min. and rinsed subsequently using sterilized distilled water. About 4-5 spots of about $2mm^2$ were cut from infected leaf samples with some healthy parts of the leaf. Then 3-4 pieces were inoculated on MEA medium under a disinfected environment. The Petri dishes were incubated at 25±2°C and examined repeatedly for fungal growth. The Mycelium of the fungus coming out of the inoculated leaf pieces was transferred to new MEA plates to purify. The purified culture plates were preserved at 4°C for further experiments.

Identification of pathogen by morphological characters: The 7-day-old pure cultures were taken to observe their morphological characters. The colony characters observed under the stereoscope were; color, nature of culture from forward and revere side, number of growing zones, colony diameter, type of conidiophores, and chains of conidia. The microscopic characters recorded were; the conidial size, number, and place of septa, type of connection with the branch, spore size, number, spore wall, presence of beak, and shape. Photographs were taken to record complete characters. The purified fungal culture was submitted to the First Fungal Culture Bank of Pakistan (FCBP), Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore.

Identification of pathogen on the basis of rDNA sequence analysis: The mycelium (approximately 2 gm) mass of 1-week-grown pure culture was smashed and the DNA was isolated using the Nucleon reagent method. The extracted DNA was incubated at -20°C for further experiments. The quality of DNA was verified on 1% agarose gel and DNA bands were envisaged below UV light.

The fungal genome was augmented using a pair of four universal primers (Table 1) and whole fungal DNA as a template. For augmentation, the PCR reaction mixture was prepared of 30 µl. The PCR products of amplification were referred for sequence analysis and the outcomes were analyzed by nucleotide Basic Local Alignment Search Tool (BLAST) analysis. The homology of sequences was checked with corresponding strains in the GenBank database and utilized for the documentation of fungi.

Pathogenicity test: Under aseptic conditions, 10 ml of 1% saline tween (0.9%NaCl and 0.1% tween 80) was prepared in distilled water, and spores from the 7-8 days grown fungal cultures were scratched and mixed in saline tween 80. The suspension was diluted to prepare 5×10^5 spores/ml by haemocytometer and used as inoculum.

***In vitro* trials**

Detached leaf method: Pasteurized Petri plates were taken and two filter papers were kept in each plate. Double distilled water was added to moisten the filter papers. The leaves were placed on each plate in a manner that their petiole touched the surface of moistened filter paper. The inoculum (2ml) was provided to it using a micropipette under aseptic conditions and examined repeatedly for the development of symptoms. After 8-10 days, the infected part was observed, compared with the sample collected from fields, and re-isolated to confirm Koch's postulates.

***In vivo* method:** Earthen pots were washed properly and filled with sterilized soil (2 kg/pot). Then sunflower seeds were sown into pots with two seeds per hole, watered properly, and placed into the growth room at 24-30ºC.

The pathogenicity test was set up by injecting 5 ml spore suspension containing 5×10^5 spores/ml with the help of a sterilized syringe in the stem nodes of 15-day-old plants and also by the spraying of spore suspension in soil. A similar amount of distilled water was poured in Control. The plants were wrapped with polythene bags for 24 hours for the maintenance of spore germination and disease emergence. The plants were kept in shade under optimum temperature i.e., 25-26ºC and watered properly.

Disease rating scale: After 6-8 days of inoculation of spore suspension, disease symptoms started to appear on the leaves. The Disease rating scale was constructed based on the percentage of infection.

Table 1. Detail of primers used for identification of fungal pathogens in the present study.

Sr. No.	Gene	Primer Name	Sequence (5' - 3')
1.	Internal transcribed spacer region	ITS1 (Forward)	5′-TCC GTA GGT GAA CCT GCG G-3′
		ITS4 (Reverse)	5′-TCC TCC GCT TAT TGA TAT GC-3′
2.	Osteomodulin	OMD5 (Forward)	5 ′ -CCGAGTACAAGGAGGCCTTCC-3 ′
		OMD6 (Reverse)	5 ′ -CCGATAGAGGTCATAACGTGG-3 ′
3.	β –tubulin	βt2a (Forward)	5 ′ GGTAACCAAATCGGTGCTGCTTTC-3 ′
		βt2b (Reverse)	5 ′ -ACCCTCAGTGTAGTGACCCTTGGC 3 ′
4.	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH (Forward)	5′-CAA CGG CTT CGG TGG CAT TG-3′
		GAPDH (Reverse)	5′-GCC AAG CAG TTG GTT GTG C-3′

Results

During the survey, 50% of the samples collected from fields of sunflower were scrutinized to be infected with leaf necrosis, lesions, and dead tissues. In general, the symptoms witnessed were brown asymmetrical abrasions with a yellow halo around them on leaves. 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.

Identification of pathogen: To identify the pathogen; its morphological characters were distinguished on the basis of colonies, equated with standard and reliable literature. The morphological interpretations were conceded on one-week-old pure cultures grown on MEA under a compound microscope (Labomed CX22; Labo America, Inc. USA). In addition, the morphologically identified pathogen was reconfirmed on the basis of nucleotide sequence analysis based on Internal Transcribed Spacer sequence (ITS), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-Tubulin and Osteomodulin genes.

Morphological characterization of pathogens: Colony on MEA was found to spread swiftly and attained a diameter of 5-6 cm. The colony was green in color while off-white to yellow from the reverse side (Fig. 1A-B). Colony margins were even and no mycelial growth ring was detected. The colony texture was powdery because of excessive sporulation. Conidial heads were trivial and typically radiated below the stereoscope (Fig. 1C). At certain places, conidial heads seemed columnar. Hyphae were septate and hyaline. Conidiophores were undersized, elongated, tubular, and crudely roughened with a tapering terminal vesicle that was 15-25 µm in size (Fig. 1D-E). Usually, the size of conidiophores was in the range of 200-215 × 3-7 µm. Conidiophore heads formed the vesicles which support uni-seriate phialides i.e., a single row of phialides about 10-12 µm in size on the upper two third of the vesicle. Conidia were smooth-walled, globose, and green typically of 2-4 µm (Fig. 1D-F). According to morphological structures, the species was recognized to be *Aspergillus fumigatus* (Fresenius, B. 1863).

Molecular characterization: In the present study, the isolated fungal strain was subjected to DNA extraction, and a condensed band of approximately 11000-120000 bp was witnessed on 1% agarose gel. The DNA band was quantified with a standard DNA marker (Fig. IIa). Following DNA isolation, fungus-specific universal primer pairs Internal Transcribed Spacer sequence (ITS), Glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), β-Tublin, and Osteomodulin (OMD) were used for the amplification of the genes. All the specific primers were able to successfully amplify all tested genes, representing a sole PCR product of around 500 bp - 650 bp in length (Fig. 2). Using the National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) bioinformatics websites, the DNA sequences were BLAST. Fungal species were selected after the DNA sequence blast with a maximum similarity percentage (95-100%). The sequencing results of the PCR products of these genes further confirmed the identification of the isolated pathogen.

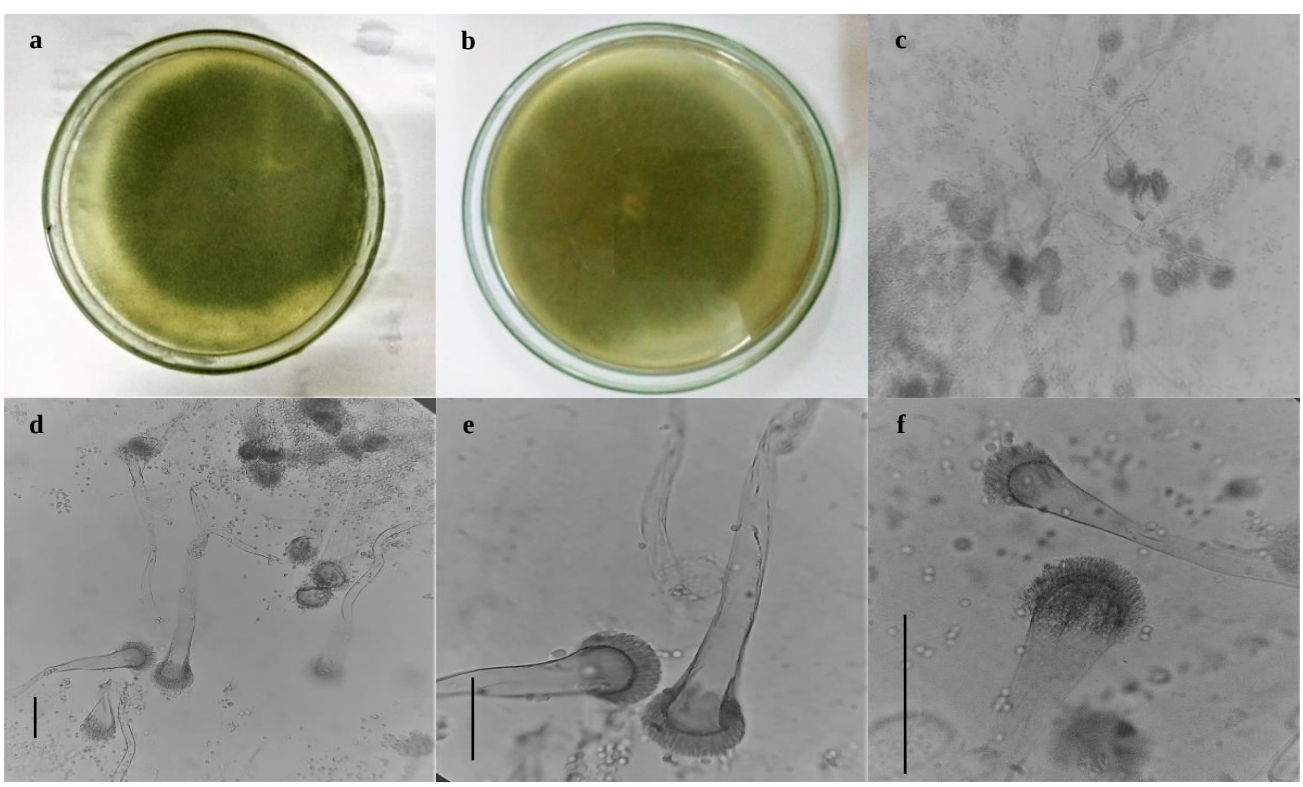

Fig. 1. *Aspergillus fumigatus* isolated from leaf spots of sunflower. (a): Front and (b): Reverse of a colony grown on MEA; (c): Conidial heads and mycelium (d-f): Microphotograph of conidia and conidial heads at 10X, 40X, and 100X magnification of a microscope, respectively. Scale bar: d = 5µm, e = 10µm and f = 20µm.

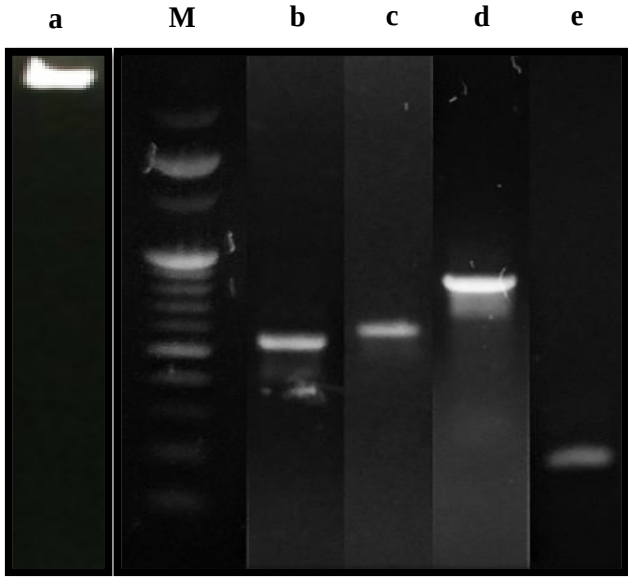

Fig. 2. Analysis of Genomic DNA and DNA fragments of isolated *A. fumigatus* strain amplified by different primers; (a): Genomic DNA; (M): DNA Marker; (b): Internal Transcribed Spacer sequence (ITS); (c): Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); (d): β-Tubulin and (e): Osteomodulin (OMD).

In the case of molecular sequence analysis of *A. fumigatus* with ITS, the consensus primers ITS1 forward and ITS4 reverse effectively augmented DNA fragments of total genomic DNA as a template. The band was visualized on 1% agarose gel having a size of 606 bp (Fig. 2b). ITS sequence alignment showed 100% homology of *A. fumigatus* with MN634637.1, MN634556.1, and MN526026.1. A phylogenetic tree representing an evolutionary relationship between the identified strain and other species was constructed (Fig. 3). The augmented ITS nucleotide sequence of *A. fumigatus* was allocated MN526026 accession ID in GenBank.

Genetic characterization performed by rDNA- partial GAPDH gene revealed a band of 614 bp on agarose gel (Fig. 2c). When the homologies searches were carried out for the GAPDH sequence of *A. fumigatus*, 98% similarity was found with *A. fumigatus* AM99976.1, CP084979.1, and 97% similarity was determined with CP097567.1. These sequences were used to build a phylogenetic tree (Fig. 4).

Molecular analysis of *A. fumigatus* with partial Beta tubulin primer provided a sole PCR product of 538 bp on 1% agarose gel (Fig. 2d). BLAST analysis of *A. fumigatus* with partial Beta tubulin primer revealed that it was 100% homologous with the *A. fumigatus* MF189897.1 & KP175503.1. The phylogenetic trees in which the related taxa grouped together are shown in (Fig. 5). All sites having gaps and missing data were excluded.

The Genotypic portrayal of morphologically known species was implemented by nucleotide sequence BLAST analysis of the rDNA- OMD gene. The primer successfully amplified the DNA fragment from total genomic DNA with a band of about 587 bp (Fig. 2e). The BLAST results performed with the OMD sequence of *A. fumigatus* revealed 99% identity of *A. fumigatus* to the strain KC701201.1, KC701200.1, LN890512.1, and MF185905.1. A phylogenetic tree was constructed to divulge the evolutionary relationships between purified strain and other species (Fig. 6).

Koch's pathogenicity test: The pathogenicity test was conducted to determine the pathogenic potential of the test pathogen and to reconfirm the pathogen of a particular host. The pathogenic potential of the isolated fungus was validated by detached leaf assay in the laboratory as well as *In vivo* by giving artificial inoculation of pathogenic fungal spores to the host plants in the seedling stage.

***In vitro* Analysis of *A. fumigatus* on sunflower plant:** The detached leaf method was conducted to determine the infectious potential of the pathogen sequestered from the fields of sunflowers. The inoculum was provided to the young leaves and the characteristics of leaf spot symptoms were observed. Initially within 3-4 days of inoculation of pathogenic spores on the leaves; similar symptoms were noticed in Petri plates that were observed in the fields. The lamina started to curl and changed color to yellowish brown, chlorosis started in the leaves with the emergence of small brown circular spots (Fig. 7). Photographs were taken at different stages of infection due to the effect of a pathogen. Confirmation of the same pathogen was done by the re-isolation of infected leaves on MEA media. Disease Severity was analyzed by using a disease rating scale. Disease progression analysis revealed that the leaf spots increased in size and joined to form larger spots, resulting in necrosis after 5-6 days and eventually to the death of the whole leaf (Fig. 8).

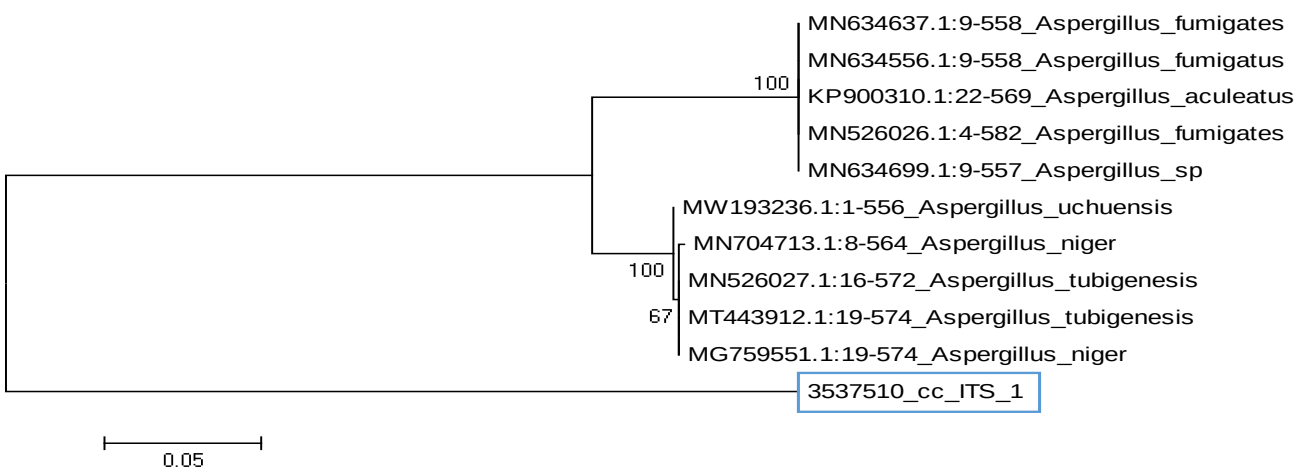

Fig. 3. Phylogenetic tree of *A. fumigatus* amplified by ITS primer by Maximum Likelihood Method.

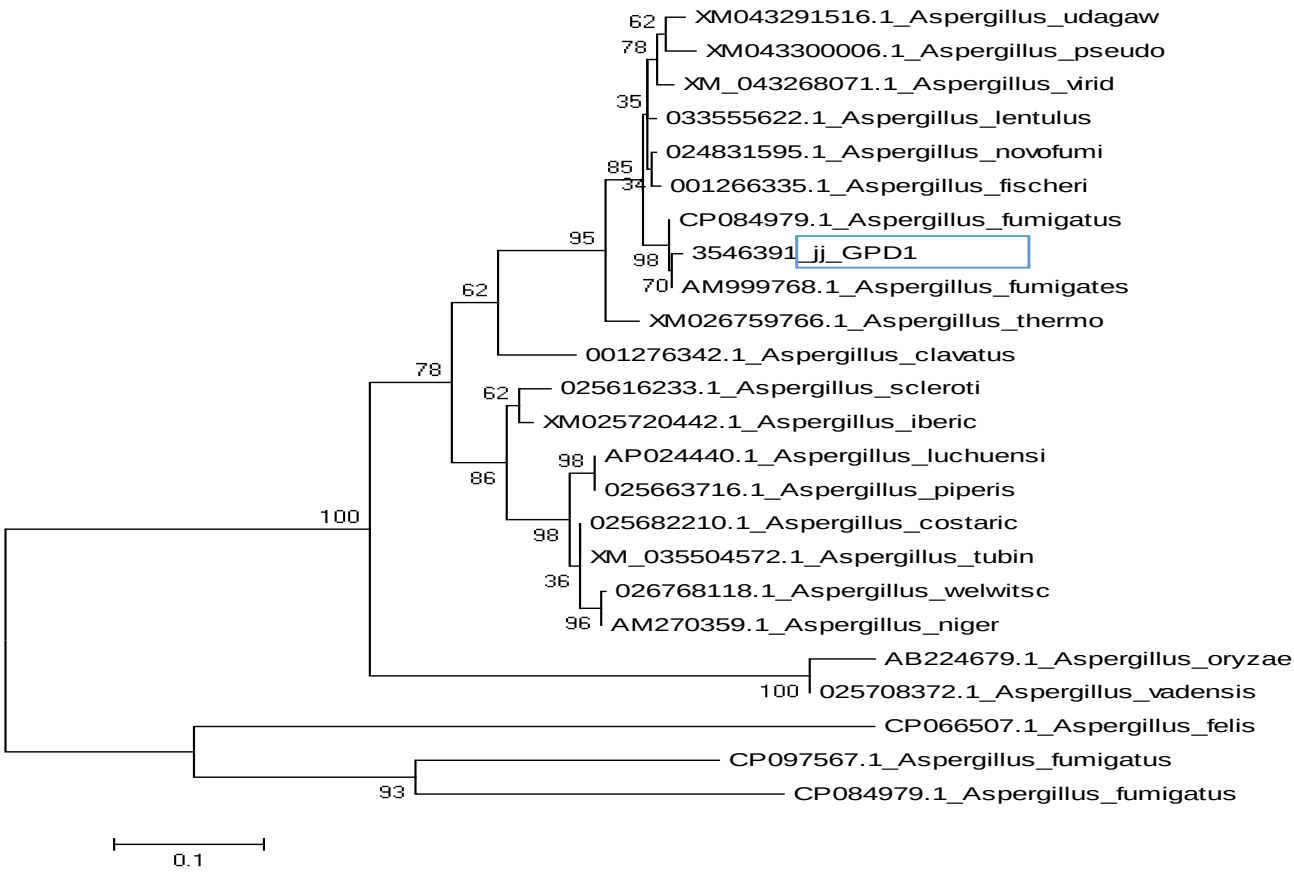

Fig. 4. Phylogenetic tree of *A. fumigatus* amplified by Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer by Maximum Likelihood Method.

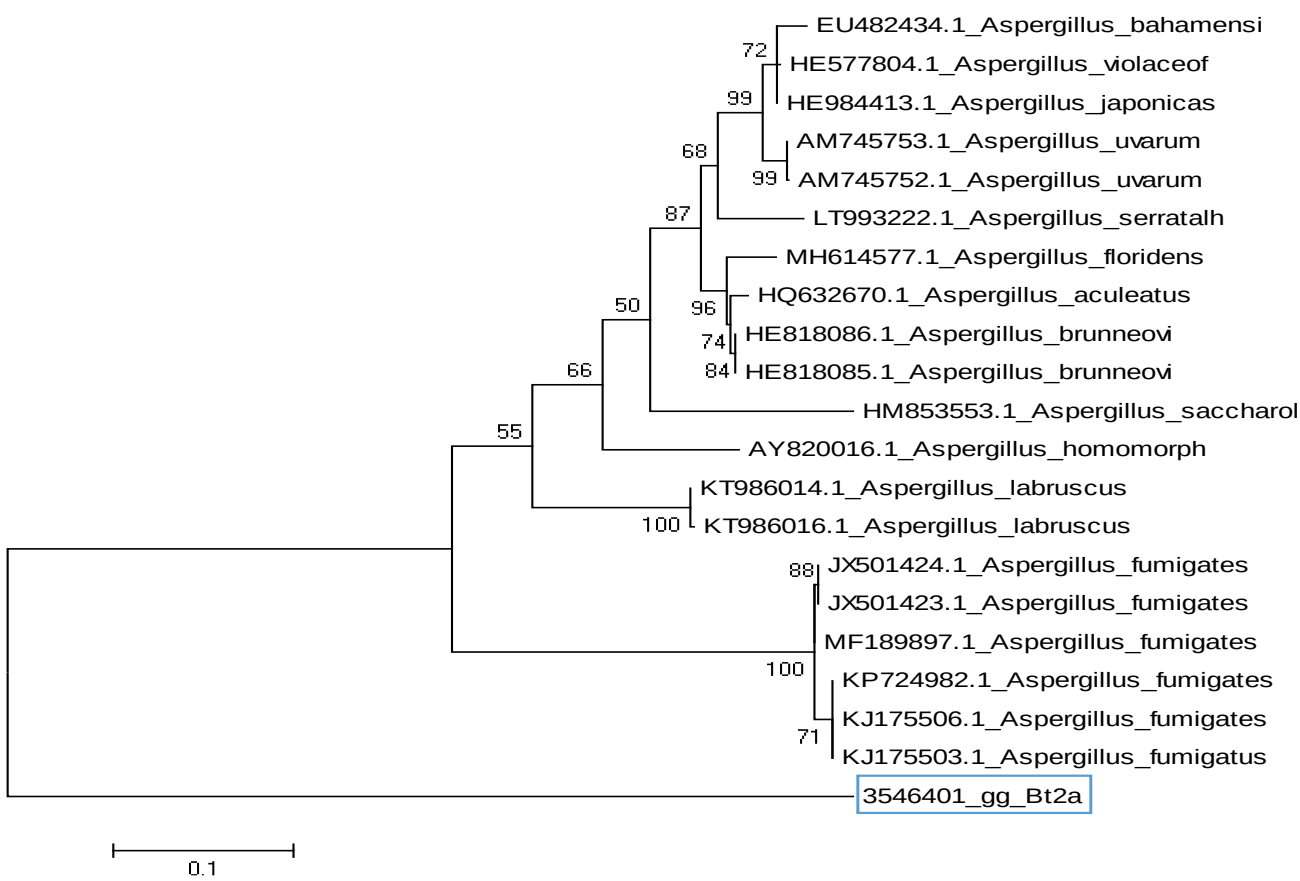

Fig. 5. Phylogenetic tree of *A. fumigatus* amplified by β-Tubulin primer by Maximum Likelihood Method.

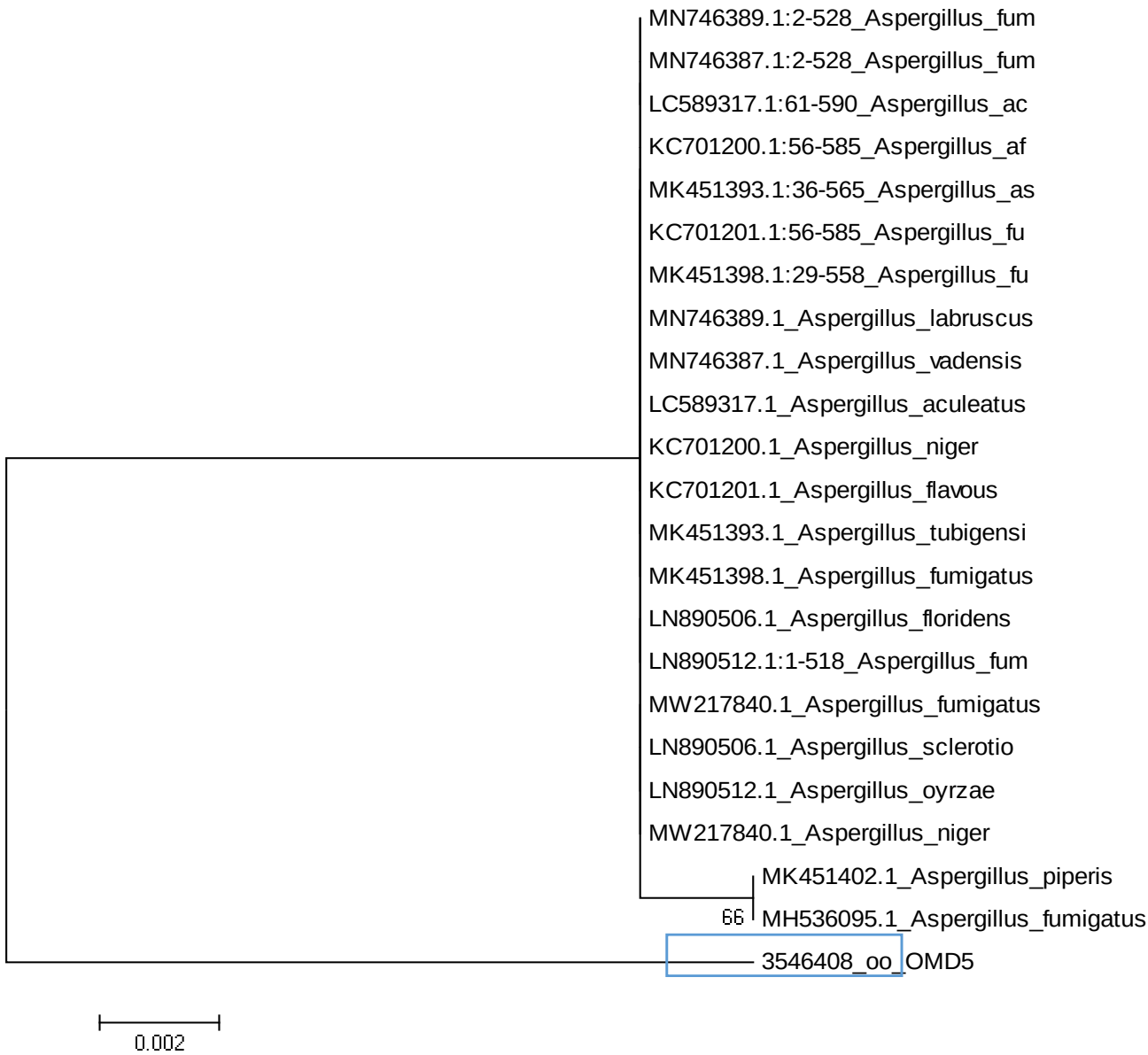

Fig. 6. Phylogenetic tree of *A. fumigatus* amplified by Osteomodulin (OMD) primer by Maximum Likelihood Method.

Fig. 7. Comparative analysis of disease development caused by *A. fumigatus*; (a): Control, (b & c): abaxial and adaxial side of the infected leaf, respectively.

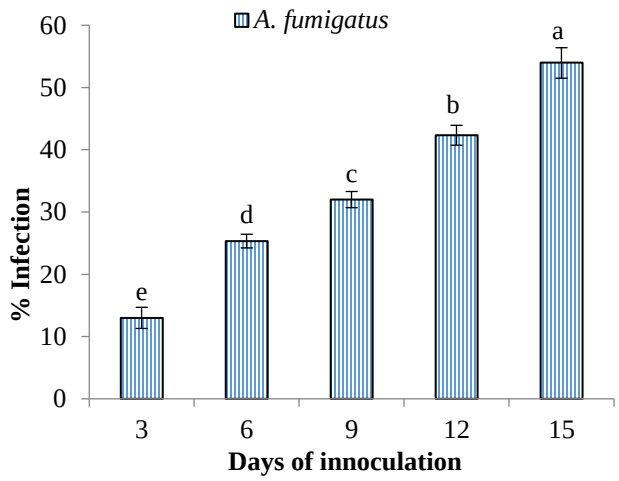

Fig. 8. Comparative analysis of Disease severity of *A. fumigatus* on sunflower plant.

Vertical bars show standard errors of the means of three replicates. Values with different letters show a significant difference by ANOVA ($p \leq 0.05$) as determined by statistix 8.1 software, HSD test at p=0.05.

***In vivo* analysis of *A. fumigatus* on sunflower plant:** The *in-vivo* pathogenicity test was performed in earthen pots. After 2 weeks of appearance, the seedlings were sprayed with 5 ml of spore suspension containing 10^4 conidia/ml. Infection was evident in 8-10 days of post-inoculation. The symptoms perceived in the plants were found to be analogous 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 2). Data analysis of pot trials revealed that the pathogenic species was very virulent and it demonstrated 100% mortality in the host plant.

Table 2. Pictorial representation of disease rating scale on sunflower plant by *A. fumigatus*.

Key scale	Disease description		Disease severity
0	Healthy Plants		0
1	The infection started i.e. leaf tips burning		20
2	Leaf spots started to appear on the leaf's surface		40
3	Burning of tips and spots became enlarged		60
4	The infection spread to all parts of the leaf		80
5	Leaves became necrotic; black and yellow.		100

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(Received for publication 20 September 2023)