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FIRST REPORT OF *CLADOSPORIUM CLADOSPORIOIDES* INSTIGATING LEAF SPOT OF *SOLANUM MELONGENA* FROM PAKISTAN

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Abstract

Solanum melongena is a popular and edible vegetable crop of Pakistan. Outbreak of irregular necrotic leaf spots with darker margins was observed. Initially the morphological identification was executed by studying the pathogen. On the bases of olive green colonies, greenish black elongated conidiophores and ellipsoidal branched conidia, the pathogen was identified as *Cladosporium cladosporioides*. In genetic characterization primers ITS1/ITS4 amplified~650bp DNA fragment of total genomic DNA. BLAST analysis of region (KY290222) exhibited 99% similarity with many strains of *C. cladosporioides* present in GenBank. The healthy leaves of *S. melongena* were further exposed to spores of *C. clasosporioides* to confirm its pathogenicity.

Key words: *Cladosporium cladosporioides,* Eggplant, Identification, Leaf spot, Pathogenicity.

Introduction

Eggplant (*Solanum melongena*) is an edible fruit crop. It has great importance among solanaceous crops in tropical regions which are sown directly into the garden. The major producers of aubergine are India, Pakistan, China, Philippines, Bangladesh, Egypt, France, Italy, Middle East, Far East and U.S.A. (Anon., 2010). It is warm-season crop needing a long growing season and favoured by 26 and 32°C (80–90°F) temperature at daytime and about 21°C (70°F) at night time (Ioannou, 2001).

Eggplants are remarkable vegetable having exclusive range of health benefits. In Pakistan, it is grown over 9,044 ha area and yields 88,148 tonnes (FAO, 2012) but its production is facing a lot of threats including diseases. The common diseases are bacterial wilt, fungal wilt and spots, phomopsis blight, mosaic, damping off. Some fungal diseases affecting eggplant are Damping Off (*Pythium* spp., *Phytophthora* spp., *Rhizoctonia* spp.) which results in huge destruction of nursery stock. The seeds and seedlings are rotted in pre-emergence damping off however, the post-emergence damping off causes infection of the young tissues of the collar at the ground level that later develop into soft and saturated area. Leaf spot is a common disease affecting the foliage of plants. Leaf spots cause defoliation of plant. Majority of the leaf spots are caused by a variety of fungal pathogens, but some are instigated by bacteria. Leaf spots by (*Cercospora melongenae*) are characterized by angular to irregular chlorotic lesions that turn greyish-brown at maturity. Severely infested leaves drop off prematurely ensuring reduced fruit yield. Alternaria spp (*Alternaria melongenae*) cause concentric rings spots on leaves that is characteristic of the genus. The irregular spots mostly coalesce and cover maximum areas of leaf blade. Severely affected leaves drop off. The infected fruits turn yellow and drop off prematurely (Pearce, 2005). Fruit Rot (*Phytophtho ranicotianae*) initially look like tiny water soaked lesions on the fruit, which later enlarges extensively. Skin of septic fruit then gets tanned with white cottony growth. Verticillium Wilt (*Verticilium dahlia*) induce dwarf and stunted internodes. Such plants are unable to produce flowers and fruits (Crop Protection Compendium, 2010). Fungal diseases affecting eggplants cause severe damage in the nursery resulting in reduced fruit yield (Crop Protection Compendium, 2010). Among these diseases, leaf spots rarely kill a crop and cause massive losses. Favorable environmental conditions continue till the product finishes. The current research work is therefore, intended to work on leaf spot causing pathogen/s; thus the study is a new report of isolation and identification of this pathogen of aubergine.

Materials and Methods

Sampling of diseased plants: A survey was conducted in March 2016 in the field of Institute of Agricultural Sciences, University of the Punjab, Lahore to collect the infected brinjal leaves. These leaves were photographed, preserved in sanitized polythene bags and used for disease study.

Isolation and purification of fungal pathogens: For the isolation of fungal pathogen, 2-3 leaf spots per leaf were selected, cut into about 3 mm sections and dipped in 1% sodium hypochlorite for 10 min for surface sterilization. After 3-4 washings leaves were plated on Malt extract agar plates aseptically and incubated at 25±2°C for 3-4 days. Hyphae from the developing colonies were sub-cultured to fresh MEA Petri plates and incubated at same conditions for purification of culture. Pure cultures were stored at 4°C.

Identification of cultures: Isolated fungal strains were identified on morphological basis primarily that were later confirmed by nucleotide sequencing Internal Transcribed Spacer Sequence (ITS) of rDNA. For morphological study seven days old pure cultures grown on MEA were used. The colony characters studied were color of culture, number of growth zones, diameter of colony (cm), presence of aerial and submerged mycelium, type of conidial chains and abundance of conidia. The microscopic characteristics recorded were color, shape,

number and position of septa (longitudinal and transverse) of conidia and their attachment with the conidiophores, ornamentation of conidial walls, presence, size and shape of conidial beak and presence of apical or basal pores. Species were identified by comparing with authentic published literature (Ellis, 1971; Simmon, 2007).

The genomic DNA of the pathogen was isolated by the revised CTAB technique of Saghai-Mroof *et al.,* (1984). The following PCR reagents with optimized concentration were used: PCR Buffer (10X) 2.5µL, $MgCl_2$ 2.5µL, dNTPs 2.5µL, *Taq* Polymerase 0.75µL, ITS I 2.5µL, ITS II 2.5µL, Template DNA 5µL, Double Distilled Deionized Water 6.75µL with total volume of 25µL. The internal transcribed coding regions of genome were amplified using the total fungal genomic DNA as template. The PCR reaction was carried out as; one cycle at 95°C for 5 min followed by 35 cycles each of denaturation at 94°C for 30 sec, annealing at 53°C for 30 second elongation at 72°C for 10 min. The PCR products were then run on 1% agarose gel and sent for nucleotide sequencing. The resulting DNA sequences were scrutinized by nucleotide BLAST. The pathogens were confirmed based on maximum homology with the species sequences in the GenBank database. Nuceotide sequences were also deposited to GenBank.

Pathogenicity test: Pathogenicity test was executed using the protocol of Grogan *et al*., (1975) for which spore suspension (5.0×10^5 conidia/mL) was made by the method of French &Herbert (1982). Detached leaf method was used to evaluate the pathogenic potential of isolated fungus. Thus two filter papers were placed in each sterilized Petri plate and moistened with 2 mL double distilled water. The detached leaves of healthy plants were arranged in Petri plates as the petiole ends remain embedded in filter paper. Then 1 mL of spore suspension was given to the healthy leaf with the help of micropipette aseptically. Then the plates were placed in incubator at 25±2°C for 7 days. Symptoms were observed after 1 week and the disease portion was re-inoculated on media plates for the confirmation of the pathogen.

In further trials, the pots were suffused with sterilized (45°C for 24 hours) soil at the rate of 180 g per pot. Two to three seeds of particular variety were propagated in pots and watered regularly; and after 20 days the plants were reduced to one per pot. The pathogenicity test was performed by spraying adjusted spore suspension of the pathogen into the pots. Control plants were treated with equal dose of sanitized water. Plants were enclosed in polythene bags for 2 days. Each treatment was replicated thrice. Plants were observed for disease appearance on regular basis. Disease rating scale was constructed by observing disease incidence and severity and disease index was calculated according to Shafique *et al.,* (2015):

$$\text{Disease severity} = \frac{\text{Area of plant part affected}}{\text{Total area}} \times 100$$

$$\text{Disease index} = \frac{\text{Number of plants in particular category}}{\text{Total number of platns}} \times 100$$

Results

Study of disease symptoms: Symptoms observed were irregular leaf spots with dark margins along with yellowish brown necrotic lesions. The preliminary symptoms are slight spherical or ovoid chlorotic spots on leaves which progress light to dark brown centers; with the abrasions expansion, they developed concentric zones; while severely infested leaves were dry out and curled then dropped from the plant.

Identification and characterization of pathogen: The colonies were effuse, olive green, velvety, reaching 3 to 4 cm in diameter with reverse greenish black color. Conidiophores elongate up to 300 µm long, 2-5 µm thick, pale to olivaceous brown with smooth surface. Conidia were produced in lengthy branched chains, 0 septate, ellipsoidal or limoniform, at 3-10X. They were 2-5 µm, pale brown, smooth to verruculose. On morphological basis, isolated pathogen was identified as *Cladosporium cladosporiodes* (Fig. 1). Pure cultures of isolated fungal strains were deposited to First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan with the number FCBP1493.

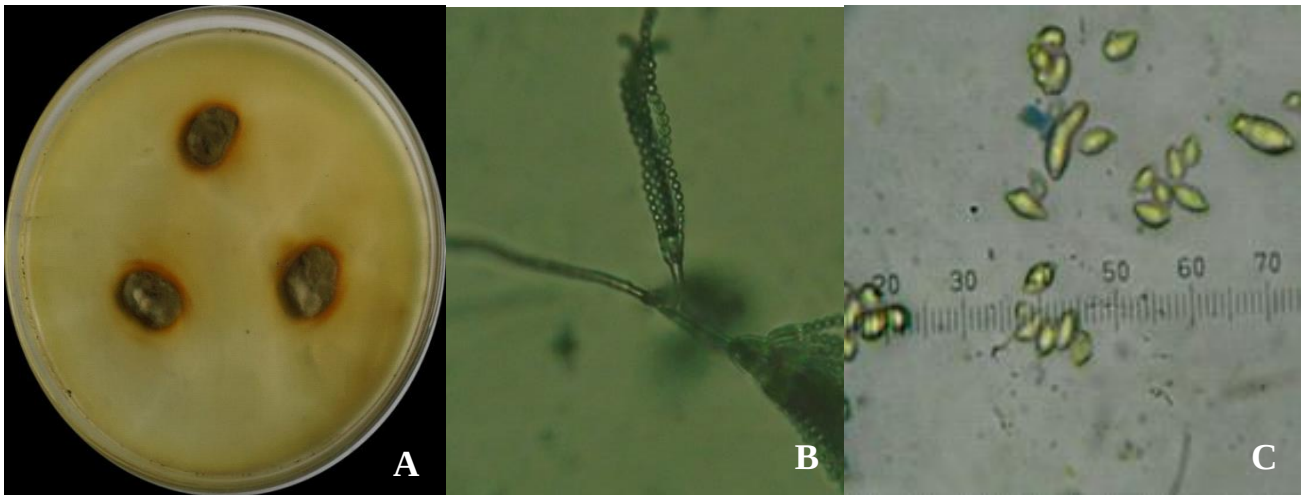

Fig. 1. *Cladosporium cladosporoides*. Colony on MEA (A), Sporulation pattern (B) and Conidia (C).

The BLAST analysis of second amplified ITS region indicated 99% similarity with the *Cladosporium cladosporoides* isolate SR10 Sequence ID: KP689250.1 and some other isolates of same species in GenBank for example strain FFJC 10, KF876823.1; strain FL20 KP689176.1; strain QTYC 15KM103316.1; strain SCSIO z015 KX258800.1; and strain WHBC21, KC880082.1. The evolutionary account of *C. cladosporioides* was concluded by the Maximum Likelihood method (Tamura & Nei 1993) to determine genetic relatedness of C. *cladosporioides* with closely related species of same genus. Two species, *Alternaria alternata* and *Curvularia lunata* were also included in this analysis due to their higher evolutionary distances (Fig. 2). Phylogenetic tree clearly showed that *C. cladosporioides* is genetically distinct from other *Cladosporium* species.

The analysis contained seven different nucleotide sequences. The clustering percentage of taxa is written beside each branch. The hierarchy is drawn to scale and branch length represents the sum of substitutions per site.

Analysis of pathogenicity assay: Pathogenicity test was prosecuted on aubergine plants to determine the pathogenicity of C. *cladosporioides* by applying Koch's postulates. Infection and characteristic visible symptoms were very evident on inoculated leaves after few days. However control leaves remained asymptomatic. Then a disease rating scale was constructed by calculating percentage of infected area (Table. 1). Initially minute spots started to appear on leaves within 2-3 days of inoculation. With the progress of time after 10 days disease progress was very sharp on the leaves in petriplates and 50% of the leaf area was observed to be infected (Fig. 3).

Table 1. Disease rating scale.

Percentage infected area (%)	Score	Grade
01-20	1-2	Highly resistant
21-40	3-4	Resistant
41-60	5-6	Moderately susceptible
61-80	7-8	Susceptible
81-100	9-10	Highly susceptible

In the *In vivo* experiment; it was revealed that the infection and evident distinctive symptoms started to appear after 15 days of inoculation. These symptoms were observed to be brownish spots and burned margins along with necrotic lesions. Firstly the plants showed yellowing that turned into chlorosis and necrosis lately, and then wilting occurred and eventually it led to the demise of the entire plant. Severely diseased plants showed arrested growth. Percentage of infected area was recorded to calculate disease severity by all the pathogens (Table. 2). The symptoms were visualized upto 35 days on the basis of disease rating scale. Disease symptoms started to develop after 1 week of inoculation with approximately 10% infected area. After 10 to 35 days, disease symptoms were very sharp and 100% plant death was observed by the pathogen.

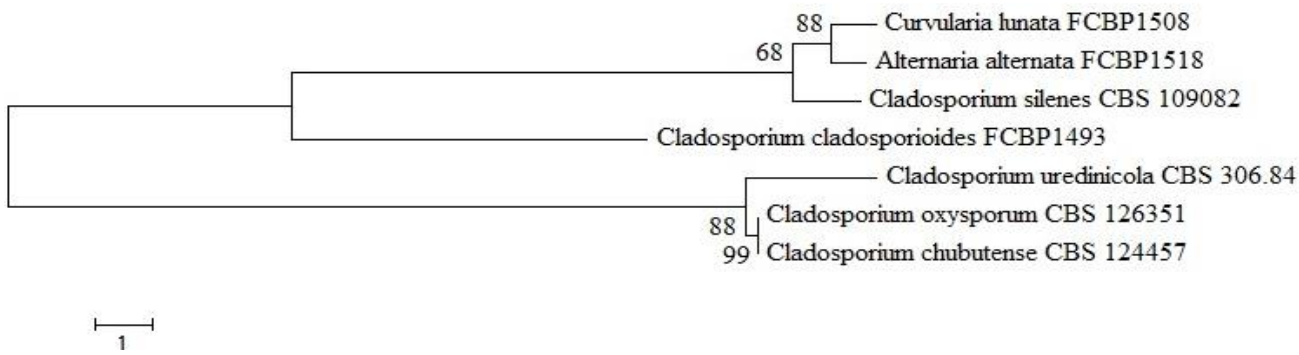

Fig. 2. Molecular phylogenetic analysis of *Cladosporium cladosporioides* (FCBP1493) by Maximum Likelihood method using MEGA 6 programme.

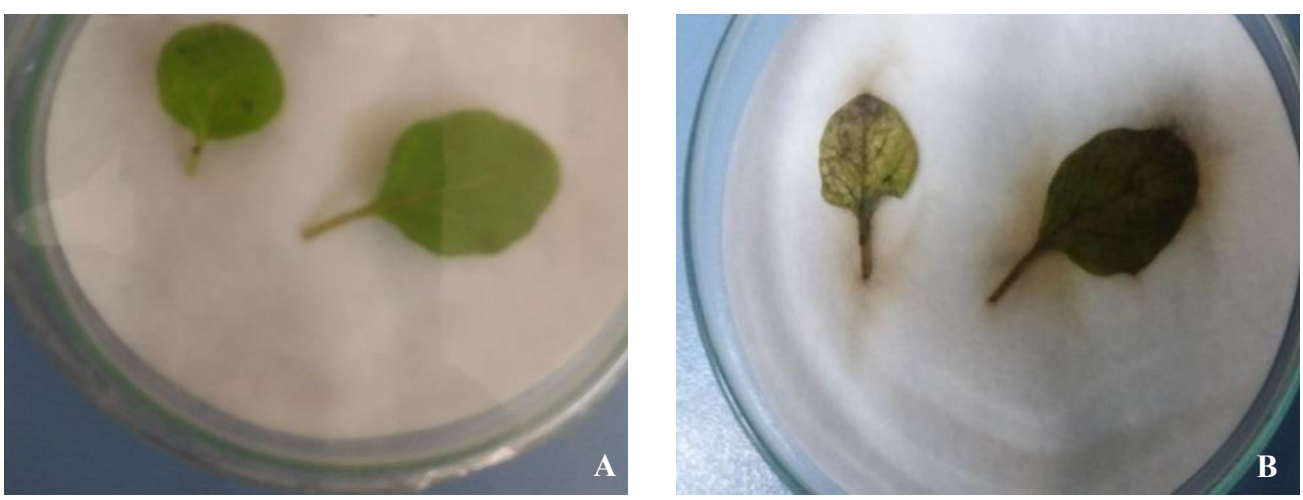

Fig. 3. Symptoms caused by *Cladosporium cladosporoides* in detached leaf assay. A: Healthy plant leaf, B: Infected plant leaf.

Table 2. Pactorial illustration of disease ranking scale on the basis of symptoms caused by *Cladosporium cladosporoides*.

Key scale	Infection symptoms		Disease severity (%)
0	No symptoms		0
1	Yellowing started on leaf margins		10
2	Apperance of brown spots at margins		20
3	Wilting started on whole plant		60
4	Plant show reduced vigor and growth		80
5	Complete death of plant		100

Discussion

Solanum melongena is an economically important edible fruit. Its common diseases are wilts, blights, mosaics, damping off, and leaf spots caused by a number of bacteria and fungi. Leaf spot of aubergine; caused by a number of fungal pathogens are characterized by small circular spots, chlorotic lesion, mostly greyish-brown and angular/irregular in shape. Highly diseased leaves drop off early thus leading to subsequent yield reduction. Correct identification of the pathogens causing leaf spots on specific host plant is important. Many leaf spot diseases have similar biology and therefore very similar management options. The work thus emphasizes the correct identification and biological control of pathogens associated with leaf spot disease of *S. melongena*. Pathogen identification is mainly based on the morphology as the morphology is still considered as the most reliable method to identify pathogen at the species level, but misidentifications are known to occur (Anderson *et al*., 2006). Consequently, a number of molecular approaches have been established to assist differentiation among species i.e., examination of ribosomal DNA (rDNA) sequences to establish molecular phylogenetic relationships within many groups of fungi (White *et al*., 1990; Mirhendi *et al.,* 2007) or by using the mitochondrial small subunit (SSU) rDNA sequence method (Kretzer *et al*., 1996). Presently, *Cladosporium cladosporioides* was identified as leaf spot causing pathogen of aubergine using morphological characterization as well as genetic analysis from nucleotide sequencing of amplified ITS1-5.8S-ITS4 region of rDNA. In the contemporary lines Bashir *et al*., (2014) isolated and identified *Alternaria metachromatica* on the basis of ample description of macro and microscopic characters followed by identification using rDNA spacer sequence and revealed that *Alternaria metachromatica* was the causal agent of leaf spot of tomato.

Further, in this study, pathogenicity test was executed for determination of pathogenic potential of the isolated pathogen causing leaf spot on aubergine. The pathogen induced almost same symptoms on the respective plant except for few differences. These upshots were in agreement with the work done by Mahmood (2010) who testified the similar drift of disease progression in tomato by *A. alternata.* In a parallel study conducted by Shafique *et al.,* (2015), they reported the pathogenic potential of 4 strains of *Fusarium oxysporum* on ten different varieties of chili plant by applying Koch's postulates using pot trials. The distinctive symptoms were obvious after 10 days of inoculation. Strain B of *F. oxysporum* was verified as the most potent pathogen as it persuaded the characteristic signs within a week. Thus the current study concludes the report of a novel isolation of leaf spot causing pathogen from aubergine plant.

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