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FIRST REPORT

First Report on the Occurrence and Morpho-Molecular Identification of *Fusarium Solani* Causing Wilt Disease in Blackgram (*Vigna Mungo* L.) In the Punjab Region of Pakistan

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A B S T R A C T

In July and August 2023 and 2024, typical wilt-disease symptoms were seen in several blackgram accessions/genotypes at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. Therefore, the present research was carried out to characterize the pathogen(s) linked with wilt-disease in blackgram using morphological and molecular approaches. Based on morpho-cultural characteristics (such as colony colour, growth-habit, number, shape and conidia and chlamydospores) and molecular identification using genus specific primer for *Fusarium* spp. and *Fusarium solani*, the associated pathogen was identified as *F. solani* from 42% samples. Pathogenicity test of fungus *F. solani* was proved on a susceptible accession "38272" planted in paper-cups under glasshouse conditions and in earthen pots under a glasshouse. Blackgram has been grown for a long time; however, this is the new report of *F. solani* causing wilt-disease in blackgram worldwide. These findings highlight the need to implement effective disease management strategies to reduce future economic losses caused by *F. solani* in blackgram-growing areas.

Keywords: Blackgram, *Fusarium solani*, morpho-molecular identification, pathogenicity, wilt disease.

INTRODUCTION

Blackgram [*Vigna mungo* (L.) Hepper] also known as Mashbean or Urdbean is closely connected to human nutrition because it is rich in easily digestible proteins and carbohydrates. Therefore, it may help to reduce protein-deficiency in peoples by offering an affordable source of protein (Vishalakshi *et al.,* 2017; Yadava *et al.,* 2022). Blackgram is estimated to be the most widely grown food legume in Pakistan after gram and mungbean. It covers about 1.5% of the total pulse-growing area and adds 1.4% to total legumes production (Qayyum *et al.,* 2019). In Pakistan, blackgram receives the least research attention, leading to a constant drop in its cultivation and yield. This decline is mainly due to various biotic and abiotic factors compared to other legumes (Hussain *et al.,* 2022).

During regular disease inspections of blackgram crops in July and August of 2023 and 2024 at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, several genotypes were found with severe wilting symptoms under field conditions (Figure-1a). Therefore, the current piece of work was carried out to detect the cause of disease following conventional and advanced techniques. Small sections (3-5mm) from roots of 50 infected plants were disinfected with a solution of Sodium hypochlorite (3%) for 5 minutes. Following drying, these

tissues were shifted onto the potato-dextrose-agar (PDA) medium supplemented with antibiotics (Streptomycin sulphate 100 mg/L and Chloramphenicol 50 ppm) and placed in an incubator at 25±28°C for 72 hours. The emerging hyphal tips of different fungi were then shifted onto PDA media for purification and future work. Of the purified colonies, 42% were tentatively identified as *Fusarium* spp. based on their morpho-cultural features. The aerial mycelium appeared fluffy and ranged in color from white to light-cream (Figure-1b). Under microscope the conidiophores were hyaline, septate and branched; macro-conidia were straight to slightly curved with 3-5 septations and micro-conidia were oval shaped having 0 to 1 septation (Figure-1c). However, no chlamydospores were observed.

To confirm the morpho-cultural identification of the *Fusarium* isolates through molecular methods, complete genomic DNA was extracted from seven days old cultures as described by Doyle and Doyle (1987) and PCR was performed with universal fungal primers (ITS1/ITS4) (Table-1) (Korabečná *et al.,* 2003; Gurjar *et al.,* 2009). All isolates recognized morphologically as *Fusarium* spp. produced a single PCR band around 550 bp, indicating that they might belong to the same species. After amplification with ITS1/ITS4 primers, these isolates were further tested using the *Fusarium*-specific (EF-1 and EF-2) primers (Table 1) (Hong *et al.,* 2010). Each tested isolate produced a single-band of a projected PCR-product size of about 700bp showing that these isolates are from genus *Fusarium* (Figure-2). To verify the *Fusarium* strains these isolates were further PCR-amplified with primer pairs specific for *F. oxysporum* (CLOX1F/R) and *F. solani* (TEF-Fs4F/R) as suggested by Haapalainen *et al.,* (2016) (Table-1). A single-band of about 658 bp was amplified using primer-pair of TEF-Fs4F/R confirming that all these isolates belongs to *F. solani* (Figure-3), while no amplification was obtained with primer pairs specific for *F. oxysporum*. The characterized culture of *F. solani* was deposited as FMB-CC-UAF 253 in the Fungal Molecular Biology Lab Culture Collection (FMB-CC) at the University of Agriculture, Faisalabad, Pakistan.

The pathogenicity of the isolated fungus *F. solani* was tested using the agar plate method in the Phytopathology laboratory (Sajjad *et al.,* 2024). To carry this out, *F. solani* was grown on PDA plates for a week. Healthy seeds of susceptible blackgram accession (38272) were surface-disinfected with Sodium hypochlorite (3%) for 10 minutes, rinsed three times with autoclaved distilled-water, and then properly air-dried. Five surface-sterilized seeds per Petri plate were placed on PDA-medium having seven-day-old *F. solani* culture, with three replicates. A negative control set with the same number of seeds on un-inoculated PDA plates was also included. These Petri plates were then shifted in an incubator having 25±2°C with 12-hour day/night cycles under fluorescent light. Experimental unit was watched daily, and final observation was recorded 15-days post inoculation following the scale suggested by Sajjad *et al.* (2024). The results showed that seed germination was 40% on PDA-plates having *F. solani* culture. The length seedling was 20.0 mm, representing a 66.8% reduction compared to healthy controls. The range of infection type due to necrosis of seedling was 4–5, the disease severity index was 4.64, and the disease response was classified as highly-susceptible. (Figure 3a-b).

To prove the ability of *F. solani* causing wilt in blackgram plants, pathogenicity test was also performed in earthen pots under a glasshouse. For this purpose seeds of a susceptible accession "38272" after surface sterilization were planted in earthen pots filled with sterilized soil under a glasshouse at 25±2°C. After 20 days of germination, the seedlings were uprooted, roots were washed and trimmed. The seedlings were then soaked in the conidial suspensions (10^6/ml) of a representative isolate of *F. solani* for 5 min and then transplanted into cups (Sun *et al.,* 2019) under a glasshouse. A same set treated with sterilized distilled water was served as a negative control. Premature leaf drop and chlorosis followed by complete wilting was observed after 15 to 20 days in all inoculated plants while the un-inoculated plants remained healthy (Figure 3c-d).

Fusarium wilt is a major threat that causes significant production losses in various crops, including legumes. This fungus is found in soils worldwide, including temperate and tropical regions, as well as extreme environments such as the Arctic and deserts. This fungus causes various plant diseases, including rots, blights, spots on leaves, rots of fruits and root, and wilts etc. However, among the plant diseases incited by Fusarium, vascular-wilt is the most-serious. Currently, there is merely one report of *F. oxysporum* inciting blackgram wilt-disease in the Western Undulated Zones of Odisha (Dhaliwal *et al.,* 2023). Additionally, in India, *F. incarnatum-equiseti*, *F. humuli*, *F. chlamydosporum*, and *F. nanum* have recently been reported to cause pod rot in blackgram (Verma *et al.,* 2023; Verma *et al.,* 2024). Although blackgram has been grown for many years around the world, including Pakistan, but this study provides the first worldwide report of fungus *F.*

solani inducing wilt disease in this crop. The appearance of fungus *F. solani* in blackgram may be linked to climate-change and could pose a potential risk to its cultivation. This study will help in developing disease-management strategies to reduce the spread of *F. solani* in blackgram-growing areas.

Table 1. Primers used to identify Fusarium species under present study.

Primer type	Sequence	Annealing Temp	Product size	Pathogen Name	Reference
ITS4a-R	TCCTCCGCTTATTGATATGC	52°C	550bp	General primer for ITS	Korabečná *et al.,* 2003; Gurjar *et al.,* 2009
ITS1a-F	CTTGGTCATTTAGAGGAAGTAA				
EF-1	ATGGGTAAGGA(A/G)GACAAGAC	57°C	700bp	*Fusarium* spp.	Hong *et al.,* 2010
EF-2	GGA(G/A)GTACCAGT(G/C)ATCATGTT				
CLOX1-F	CAGCAAAGCATCAGACCACTATAACTC	60°C	534bp	*F. oxysporum*	Haapalainen *et al.,* 2016
CLOX1-R	CTTGTCAGTAACTGGACGTTGGTACT				
TEF-Fs4F	ATCGGCCACGTCGACTCT	58°C	658bp	*F. solani*	
TEF-Fs4R	GGCGTCTGTTGATTGTTAGC				

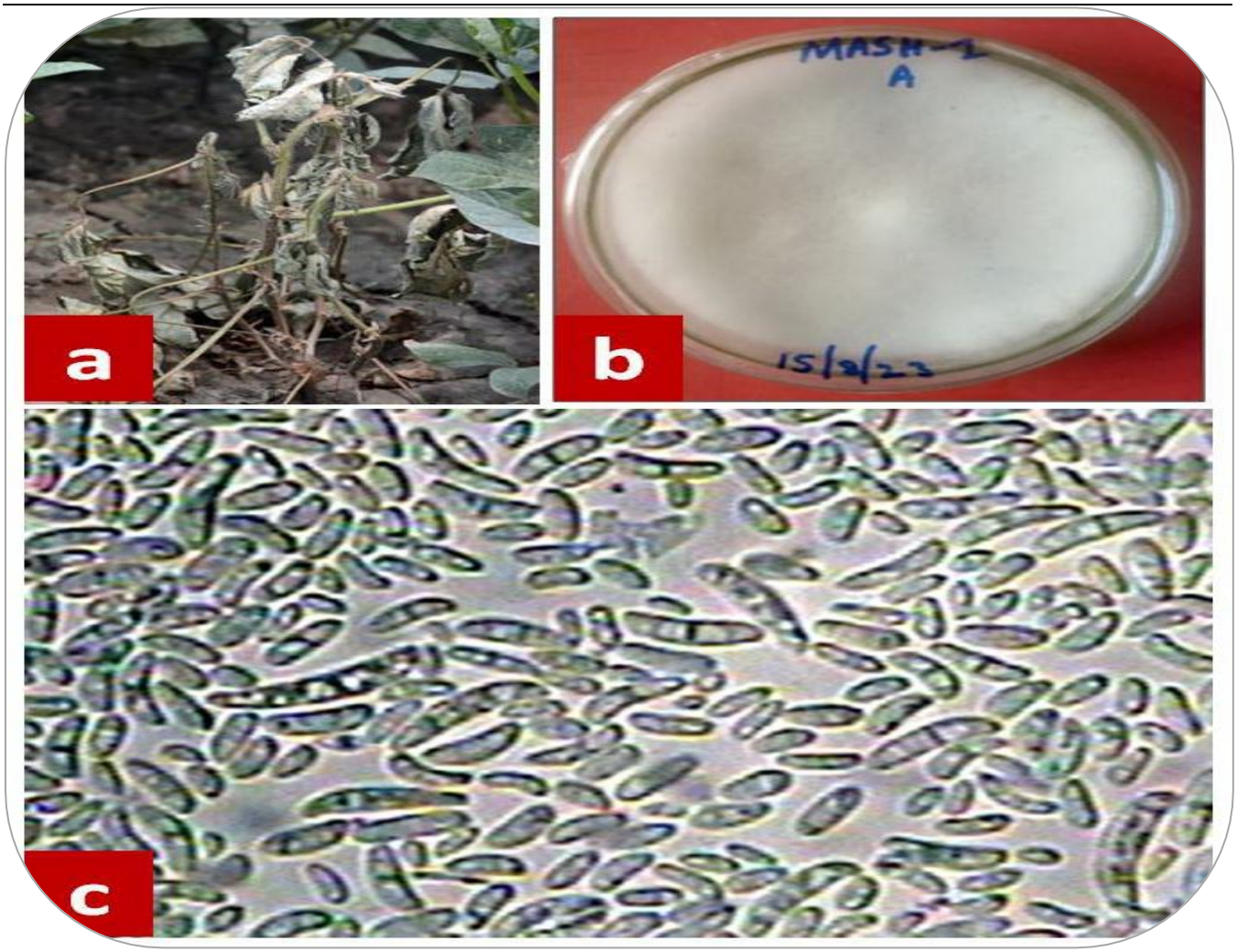

Figure 1. Symptomatological, morphological and microscopic features of *Fusarium solani*. a) Wilt disease symptoms on aerial parts in blackgram under field conditions; b) *F. solani* colony on PDA after 7days; c) Macroconidia and microconidia of *F. solani*.

Figure 2. Confirmation of *Fusarium* spp. using EF-1 and EF-2 primers: L1, L2, L3, L4, L6, L7, L8 positive for *Fusarium* spp. and L9 (+ve control)] while L5 negative control while L10 showing the DNA ladder.

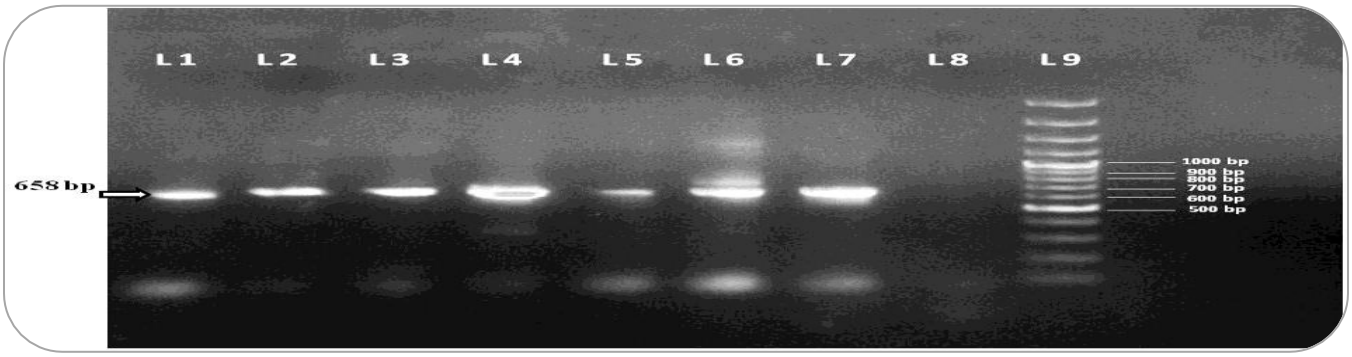

Figure 3. Confirmation of *Fusarium solani* using species specific primers (TEF-Fs4F/R): L1, L2, L3, L4, L5, L6, positive for *F. solani* while L8 negative for F. solani and L7 (+ve control)]. L10 showing the DNA ladder.

Figure 4. Pathogenicity test of *F. solani*. a) Seed germination and seedling growth on F. solani culture on PDA; b) Seed germination and seedling growth on un-inoculated PDA; c) *F. solani* inoculated blackgram plants in pots; and c) Un-inoculated blackgram plants in pots (healthy control).

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DECLARATIONS

Ethics approval: This piece of work does not contain any studies with human participants or animals performed by any of the authors.

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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Mohy U. D. Akram	:	Helped to confirm the *Fusarium* isolates through molecular methods