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# Integrated Morphological And Molecular Approaches For Characterizing Fusarium Isolates Causing Wilt In Black Gram

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***Abstract***
*Fusarium species rank among the most harmful fungal pathogens impacting cereals and legumes, leading to significant yield reductions and posing a threat to global food security. Precise identification of these pathogens is crucial for formulating effective disease management strategies. This research focused on characterizing Fusarium isolates from farmers' fields across five different agroclimatic zones. Thirty isolates were collected from diseased, wilted black gram plants, surface-sterilized, and cultured on Potato Dextrose Agar (PDA) at a temperature of 28 ± 2 °C. Genus-level identification was achieved through colony morphology, growth patterns, and microscopic examination of conidial structures using lactophenol cotton blue staining. Malachite Green Agar 2.5 ppm (MGA 2.5) was employed for the selective recovery and enumeration of Fusarium spp. This medium was effective in both pure and mixed cultures and contaminated samples, with infected seedlings showing mortality within five days, confirming pathogenicity. Molecular characterization was conducted for species identification. Genomic DNA was extracted from pure isolates, and the internal transcribed spacer (ITS) region was amplified using universal primers ITS1 and ITS4. The resulting ~600 bp amplicons were sequenced, and BLASTn analysis against NCBI GenBank identified three predominant species: Fusarium oxysporum, Fusarium solani, and Fusarium proliferatum. This method, which combines morphological characteristics with ITS-based molecular identification, highlights the diversity of Fusarium spp. in black gram cropping systems. The results provide foundational data for pathogen monitoring, disease management, and the development of resistant varieties.*

***Keywords:*** *Fusarium oxysporum, Fusarium solani, Fusarium proliferatum, morphological characterization, ITS sequencing, crop pathogens*

## INTRODUCTION

Fungal pathogens pose a major threat to global food production, with the Fusarium genus being particularly destructive. These fungi, which thrive in soil, attack a variety of host plants such as cereals, legumes, and horticultural crops, leading to significant yield losses and deterioration after harvest (Leslie & Summerell, 2006). In tropical and subtropical regions, Fusarium diseases are especially problematic due to the warm climates and stressed soils that favor their spread, complicating management in smallholder farming systems. Black gram (Vigna mungo), a vital pulse crop grown extensively in South Asia, is highly vulnerable to Fusarium wilt, which can result in yield reductions of up to 60% during severe outbreaks (Nirmaladevi et al., 2016). With the increasing demand for pulses as a protein source in human diets, early and precise detection of Fusarium pathogens is essential for maintaining food and nutritional security.

The pathogenicity of Fusarium species stems from their ability to penetrate plant vascular systems, block xylem vessels, and produce mycotoxins like fumonisins, trichothecenes, and zearalenone (Desjardins, 2006). These compounds cause plant wilting, tissue death, and pose health risks to animals and humans through contaminated grains. In legumes like black gram, infection causes yellowing, wilting, and seedling death, affecting yield and seed quality (Ramesh et al., 2017). *F. oxysporum*, *F. solani*, and *F. proliferatum* commonly cause wilt diseases in pulses and cereals, showing their ecological adaptability.

Historically, *Fusarium spp.* identification has relied on analyzing morphological and cultural traits, including colony color, growth rate, and shapes of macroconidia and microconidia (Nelson et al., 1983). These characteristics, while useful for identifying the genus, often fail to distinguish closely related species

or formae speciales with different pathogenic impacts. Morphological variability under different growth conditions complicates accurate diagnosis (Leslie & Summerell, 2006). To address this, selective media like Malachite Green Agar (MGA) have been developed to isolate *Fusarium* spp., with low concentrations of 2.5 ppm enhancing viable colony recovery from contaminated samples (Komada, 1975).

The advent of molecular biology has revolutionized fungal taxonomy and diagnostics. The internal transcribed spacer (ITS) region of ribosomal DNA serves as the primary fungal barcoding marker, due to its high copy number and conserved flanking regions (White et al., 1990). Using primers ITS1 and ITS4, the ITS region yields ~600 bp fragments for species identification via NCBI GenBank. While ITS may not differentiate cryptic Fusarium lineages, it remains practical for routine identification (O'Donnell et al., 2009). Fusarium wilt severely impacts black gram crops in India and Southeast Asia, thriving in sandy soils. Plants show vascular browning, chlorosis, and wilting, causing significant seedling mortality (Gurjar et al., 2009). The pathogen persists as chlamydospores, reducing crop rotation effectiveness. Rising wilt prevalence requires pathogen characterization for developing resistant varieties and management strategies.

An integrative approach combining morphological, cultural, and molecular techniques reliably characterizes Fusarium pathogens. Morphological analysis serves as initial screening, while selective media like MGA help isolate field samples. Molecular tests confirm species identification, reducing misclassification risk. This strategy ensures diagnostic accuracy and reveals Fusarium species distribution and pathogenic potential across agroecological zones. Given black gram's importance in smallholder farming and wilt disease challenges, this study aimed to characterize Fusarium isolates from five agroclimatic zones. The objectives were to: (i) recover Fusarium species from diseased black gram plants; (ii) document morphological characteristics for genus identification; and (iii) use ITS-based sequencing for species identification. Integrating morphological and molecular data will inform *F. oxysporum*, *F. solani*, and *F. proliferatum* prevalence, supporting resistance breeding and disease management.

## MATERIALS AND METHODS

### Sample Collection and Isolation of Fusarium Isolates

This cross-sectional study characterized *Fusarium spp.* associated with black gram (*Vigna mungo*) wilt across five major agroclimatic zones. Symptomatic plants showing wilting, chlorosis, and vascular browning were collected from farmers' fields during cropping season. Thirty diseased samples were selected from different locations for ecological representation. Root and stem tissues were washed to remove soil particles and surface sterilized using 1% sodium hypochlorite for 2 minutes followed by three rinses in sterile water. The tissues were blot-dried and transferred to Petri plates containing Potato Dextrose Agar (PDA) with streptomycin sulfate (100 mg/L) (figure-1). Plates were incubated at 28 ± 2 °C for 5–7 days, and fungal colonies were sub-cultured through hyphal tip isolation for pure cultures.

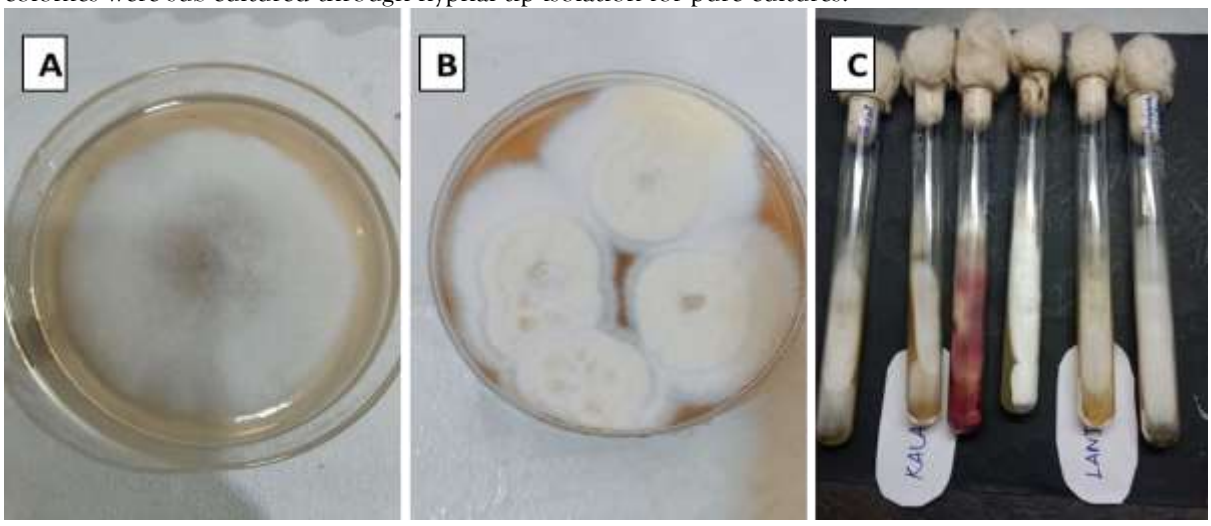


**Figure 1:** Isolation of *Fusarium spp.* from wilted black gram tissues. Surface-sterilized plant segments were plated on PDA with streptomycin sulfate (100 mg/L) to prevent bacterial growth. Plates were incubated at 28 ± 2 °C for 5–7 days. Fungal colonies were purified by hyphal tip isolation for morphological and molecular characterization.

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**Cultural and Morphological Characterization**

Colony morphology on PDA was recorded after 7 days at 28 ± 2 °C, including diameter, growth rate, pigmentation, margin, and aerial mycelial texture. For microscopic analysis, mycelial discs were mounted with lactophenol cotton blue stain. Shape, septation, and size of macroconidia, microconidia, and chlamydospores were examined under 400× magnification and compared with the Fusarium Laboratory Manual (Leslie & Summerell, 2006) for genus identification.

**Selective Isolation Using Malachite Green Agar**

To enhance selective recovery of *Fusarium spp.* from contaminated samples, Malachite Green Agar at 2.5 ppm was employed following Komada's (1975) modified method. Plant tissue segments were plated on MGA and incubated under PDA conditions. Colonies with *Fusarium*-like morphology were enumerated and re-isolated. Pathogenicity confirmation involved inoculating healthy black gram seedlings in sterile soil: seedlings were root-dipped in spore suspensions ($1 \times 10^6$ conidia/mL) for 10 minutes and transplanted into pots (Figure-2). Inoculated seedlings were monitored for 10 days, with mortality within 5 days indicating pathogenic infection.


**Figure 2:** Selective isolation and pathogenicity testing of Fusarium spp. Recovery on Malachite Green Agar showed suppression of contaminants and characteristic colony growth. Pathogenicity confirmed by root-dipping black gram seedlings in conidial suspensions ($1 \times 10^6$ conidia/mL) and transplanting to sterile soil. Mortality within 5 days confirmed virulence.

**Genomic DNA Extraction**

For molecular characterization, mycelia from each isolate were cultured in Potato Dextrose Broth (PDB) at 28 °C for 5 days, shaking at 120 rpm. Fungal biomass was filtered through sterile Whatman paper, washed with distilled water, and frozen at –20 °C. Genomic DNA was isolated using a Norgen Biotek Kit. Wash Solution was prepared by adding ethanol to reach 60 mL. A water bath was pre-heated to 65 °C, and Collection Solution was prepared for fungi other than yeast. Input was limited to $1 \times 10^8$ cfu. For plate-grown fungi, ~5 mL Collection Solution was added, and spores with mycelial fragments were harvested. Up to 1 mL biomass was pelleted at 14,000 × g for 1 min and resuspended in 500 µL Lysis Solution, with optional RNase A. The suspension underwent bead-beating for 5 min and 65 °C incubation for 10 min. Lysate was centrifuged at 14,000 × g for 2 min, mixed with equal volume ethanol and 300 µL Binding Solution, applied to a silica column, centrifuged, and washed twice with Wash Solution. DNA was eluted with 100 µL Elution Buffer, with optional second elution for improved yield. Purified DNA was stored at −20 °C or −70 °C and was suitable for PCR and sequencing. DNA quality was evaluated using a NanoDrop spectrophotometer at 260/280 nm absorbance ratio, and integrity was confirmed by 1% agarose gel electrophoresis.

**PCR Amplification of the ITS Region**

The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR reactions were performed in 25 µL containing 2 µL genomic DNA template (~50 ng), 12.5 µL PCR Master Mix (Thermo Fisher Scientific), 1 µL of each primer (10 µM), and 8.5 µL nuclease-free water. The cycling protocol was: initial denaturation at 95 °C for 5 minutes; 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 1 minute; followed by final extension at 72 °C for 10 minutes. Amplification was confirmed by electrophoresis on 1.2%

agarose gel stained with ethidium bromide, using 100 bp DNA ladder (Thermo Scientific) as size marker. Expected amplicon sizes of 600 bp indicated successful ITS amplification.

**Sequencing and Bioinformatics Analysis**

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) following manufacturer's protocol. Purified amplicons underwent bidirectional Sanger sequencing at Heredity Biosciences LLP (Bhubaneswar, India). Sequences were visualized and edited using Chromas software (Technelysium Pty Ltd., Australia), and consensus sequences were generated by aligning reads in BioEdit. For identification, sequences were compared against NCBI GenBank using BLASTn. Isolates with ≥99% similarity to reference sequences were assigned to species level. Multiple sequence alignments were performed using ClustalW in MEGA X software (Kumar et al., 2018). Phylogenetic analysis used neighbor-joining with 1000 bootstrap replicates. Sequences were deposited in GenBank and the accession numbers mention in the table-2.

**Statistical Analysis**

Each experiment was conducted three times, and the findings are expressed as the mean ± standard error. To assess significant differences in colony growth, conidial size, and pathogenicity ratings among the isolates, an analysis of variance (ANOVA) was performed using SPSS software (version 25.0, IBM Corp.). Tukey's HSD test was utilized to compare means at a significance level of $p < 0.05$.

## RESULTS

**Cultural and Morphological Characteristics of *Fusarium* Isolates**

Thirty Fusarium isolates were effectively obtained from black gram plants suffering from wilt, which were gathered from five different agroclimatic regions. On Potato Dextrose Agar (PDA), the colonies exhibited notable differences in their growth speed, color, and form. The colony colors varied from white, cotton-like mycelia to shades of pink, violet, or purple, with edges that ranged from smooth to uneven. The growth rates spanned from 4.8 ± 0.2 to 7.6 ± 0.4 cm over a period of 7 days, with *F. proliferatum* isolates demonstrating a faster growth rate compared to *F. oxysporum* and *F. solani*.

Upon microscopic analysis, three distinct morphological categories were identified, corresponding to *F. oxysporum*, *F. solani*, and *F. proliferatum*. The macroconidia of *F. oxysporum* were generally sickle-shaped with 3–5 septa, measuring on average 28–35 µm in length. In contrast, *F. solani* produced shorter, slightly curved macroconidia with 3 septa, ranging from 22–30 µm (Table-1). Meanwhile, *F. proliferatum* macroconidia were slender and had multiple septa, with lengths surpassing 35 µm. The microconidia of *F. oxysporum* were mostly oval and lacked septa, whereas those of *F. solani* were ellipsoidal and occasionally septate. Chlamydospores were found in abundance in *F. oxysporum*, were sparse in *F. solani*, and were rarely observed in *F. proliferatum*.

**Table 1. Cultural and Morphological Characteristics of *Fusarium* Isolates**

| Species | Colony Color (PDA) | Growth Rate (cm/7d) | Macroconidia (µm) | Microconidia Features | Chlamydospores |
|---|---|---|---|---|---|
| *F. oxysporum* | White to pink | 5.2 ± 0.3 | 28–35, 3–5 septa | Oval, aseptate | Abundant |
| *F. solani* | White to cream | 4.8 ± 0.2 | 22–30, 3 septa | Ellipsoidal, few septa | Moderate |
| *F. proliferatum* | Violet to purple | 7.6 ± 0.4 | >35, 5–7 septa | Slender, non-septate | Rare |

**Selective Isolation Using Malachite Green Agar**

Malachite Green Agar effectively suppressed non-target fungal contaminants, allowing for the selective isolation of *Fusarium spp.* from mixed samples. The colonies retrieved from MGA were morphologically identical to those grown on PDA, confirming its effectiveness for diagnostic use. Seedling pathogenicity tests showed that all 30 isolates caused mortality within 5 days of inoculation, demonstrating their virulent nature (Figure 2).

**Molecular Identification of Fusarium Isolates**

PCR amplification using ITS1 and ITS4 primers successfully produced amplicons of about 600 bp for all 30 isolates. The presence of strong, distinct bands was confirmed through agarose gel electrophoresis,

indicating the absence of non-specific amplification. Subsequent sequencing and BLASTn analysis against the NCBI GenBank database revealed a high similarity (≥99%) with reference *Fusarium* species, enabling precise species-level identification. Among the 30 isolates, 14 were identified as *F. oxysporum*, 10 as *F. solani*, and 6 as *F. proliferatum*. Phylogenetic analysis using the neighbor-joining method grouped the isolates into three primary clades, each corresponding to one of the identified species, with bootstrap support values exceeding 90% (Figure-3).

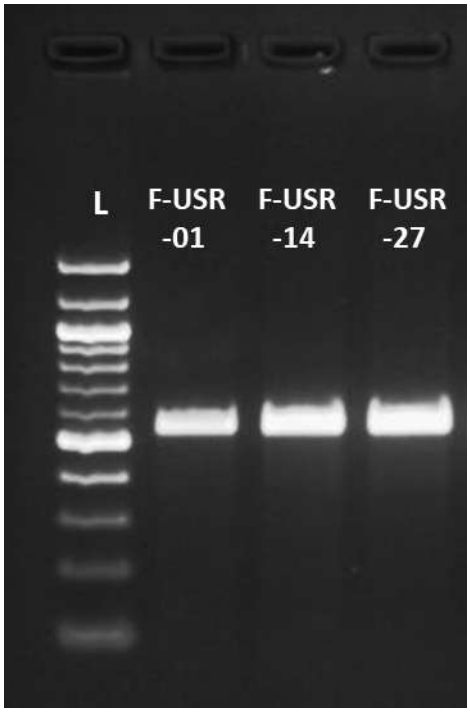


**Figure 3:** PCR amplification of ITS region using ITS1 and ITS4 primers generated ~600 bp products in all 30 fungal isolates. Gel electrophoresis showed distinct single bands, confirming successful amplification without non-specific products.

**Table 2. Molecular Identification of *Fusarium* Isolates**

| Isolate Code | BLASTn Identity | Closest Match in GenBank | Similarity (%) |
|---|---|---|---|
| F-USR-01 | *F. oxysporum* | PX313557 | 99.8 |
| F-USR-14 | *F. solani* | PX313668 | 99.6 |
| F-USR-27 | *F. proliferatum* | PX313677 | 99.4 |

**Phylogenetic Analysis**

Phylogenetic analysis utilizing ITS sequences divided all isolates into three distinct clades, corresponding to *F. oxysporum*, *F. solani*, and *F. proliferatum*. The robustness of these clades was validated by bootstrap values exceeding 90%. This molecular evidence strongly corroborated the morphological classification and confirmed the thorough methodology applied in this study.

## DISCUSSION

The current research examined *Fusarium* isolates from wilted black gram (*Vigna mungo*) plants through a combination of morphological, cultural, and molecular techniques, followed by tests for pathogenicity. A total of thirty isolates were gathered from five different agroclimatic zones, and their analysis confirmed the dominance of three primary species: *F. oxysporum*, *F. solani*, and *F. proliferatum*. This integrative method not only confirmed morphological characteristics but also offered molecular clarity, thereby enhancing confidence in the identification process. The results provide essential baseline data for understanding the distribution and pathogenic diversity of *Fusarium spp.* in black gram farming systems and contribute to the larger framework of sustainable crop protection.

Black gram is an essential pulse crop extensively consumed throughout Asia as a major source of protein. Nevertheless, its cultivation faces significant challenges due to soil-borne pathogens, with *Fusarium* wilt being the most detrimental. Previous studies have shown that *F. oxysporum* can lead to yield reductions of

over 50% when conditions are favorable (Nirmaladevi et al., 2016). Managing this disease is particularly challenging because the pathogen can persist in the soil as chlamydospores for long periods, often surviving for more than ten years (Leslie & Summerell, 2006). In the current research, isolates demonstrated high virulence, with seedling death occurring within 5–10 days after inoculation, highlighting the severe threat these pathogens pose to black gram cultivation.

Morphological features, including colony pigmentation, growth rate, and conidial structures, showed variability among isolates. *F. oxysporum* isolates had pinkish-white colonies and abundant chlamydospores, as described by Nelson et al. (1983). *F. solani* produced cream-colored colonies with shorter macroconidia, while *F. proliferatum* showed violet to purple pigmentation and slender multi-septate macroconidia. Though morphological features aid preliminary identification, environmental factors and growth conditions can influence colony appearance, leading to misclassification (Summerell et al., 2010). Thus, molecular validation was essential for accurate species identification.

Malachite Green Agar effectively suppressed background contamination and selectively isolated *Fusarium spp.* Komada's medium is reliable for isolating *F. oxysporum* from field samples (Komada, 1975). The results confirm MGA's value in diagnostic pipelines, particularly where molecular facilities are limited. Combining MGA with morphological and molecular tools enabled robust identification. Molecular characterization using ITS sequencing provided species-level identification, confirming *F. oxysporum*, *F. solani*, and *F. proliferatum* predominance. While ITS serves as the universal fungal barcode (White et al., 1990) and sometimes lacks resolution in cryptic complexes like *F. solani* (O'Donnell et al., 2009), it effectively distinguishes major pathogenic lineages. ITS-based phylogenetic analysis produced three distinct clades with strong bootstrap support (>90%), validating morphological data.

Future studies may integrate loci like translation elongation factor 1-alpha (TEF1-α) and RNA polymerase II genes (RPB1 and RPB2) to resolve intra-species variation. Such approaches have delineated cryptic species within the *F. oxysporum* complex (Laurence et al., 2014). However, ITS provided sufficient species-level resolution for this study's objectives. The variability in pathogenicity necessitates continuous monitoring of *Fusarium spp.* across regions. Differences in aggressiveness may reflect genetic diversity, indicating that breeding for resistance must consider multiple *Fusarium* species rather than just *F. oxysporum*. The findings align with reports on *F. oxysporum* and *F. solani* predominance in pulse crops (Ramesh et al., 2017). However, the high frequency of *F. proliferatum* indicates a shift in pathogen dynamics, possibly due to ecological changes or climate variability. The aggressive nature of *F. proliferatum* isolates mirrors findings in cereals, where it has emerged as dominant in regions with rising temperatures (Aloi et al., 2020).

Biological control agents like Trichoderma spp. have shown promise in suppressing Fusarium wilt (Sharma & Sharma, 2018), but their effectiveness varies in the field. Integrated disease management strategies combining resistant varieties, biocontrol, and cultural practices are likely most effective in mitigating Fusarium-induced losses. While this study identified and characterized *Fusarium* isolates, several limitations exist. ITS sequencing alone may not fully resolve species complexes, and multilocus sequence typing should be used in future work. Further investigations across additional legumes could provide insights into host-specificity, and functional genomics approaches may help unravel virulence mechanisms for developing targeted resistance strategies.

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