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Fusarium diversity affecting Soybean (*Glycine max* L.) Wilt from Marathwada region (M.S)

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Abstract

Soybean (*Glycine max* L.) is one of the most important oilseed and protein-rich crops cultivated in the Marathwada region; however, its productivity is severely affected by wilt disease caused by diverse *Fusarium* species. The present study focuses on the molecular detection and characterization of wilt-inducing *Fusarium* isolates collected from major soybean-growing districts of Marathwada such as Beed, Chhatrapati Sambhajinagar, Dharashiv, Jalna and Parbhani. A total 50 diseased Plant part and soil samples were collected, among them 28 were purified using standard mycological techniques, which having Five different *Fusarium* species were diagnosed *(F. avencanum, F. equiseti, F. hainanense, F. oxysporium and F. pernambucanum*) and Comparative study of Growth and Morphological traits on PDA and GN medium. By Molecular Techniques and Phylogenetic analysis confirm the evolutionary relationships and genetic diversity.

Keywords: Soyabean (*Glycine max* L.); *Fusarium*; Wilt; Genetic Diversity; Marathwada

1. Introduction

Soybean (*Glycine max* L.) is one of the most important leguminous crops cultivated worldwide and plays a vital role in agricultural and industrial economies. It is considered a major source of high-quality protein (40%) and oil (20%), making it indispensable for food, feed, and various industrial applications [1]. India is a significant producer of soybean, and the Marathwada region of Maharashtra has emerged as a major soybean-growing belt due to its favorable agro-climatic conditions and extensive cultivation practices. Despite its economic importance, soybean productivity is severely hampered by biotic and abiotic stresses, among which fungal diseases constitute a major constraint. Wilt disease caused by *Fusarium* species is increasingly recognized as one of the most destructive soil-borne diseases affecting soybean in the region. *Fusarium* species are ubiquitous soil inhabitants capable of causing vascular wilt, root rot, and seedling blight in a wide range of crops. In soybean, *Fusarium oxysporum, F. solani, F. equiseti, F. proliferatum,* and several other species have been implicated in wilt and root rot syndromes [2]. These pathogens invade through roots, colonize the vascular tissues, disrupt water transport, and ultimately lead to plant wilting, stunting, chlorosis, and significant yield losses. The severity of *Fusarium* infections is strongly influenced by soil type, humidity, temperature, cropping pattern, and the presence of stress factors such as drought and nutrient deficiency [3]. The disease often remains unnoticed until significant damage has occurred, making early detection and accurate identification crucial for disease management. Traditional methods for identifying *Fusarium* species rely on morphological and cultural characteristics, including colony pigmentation, conidial size, and shape. However, morphological identification can be highly subjective, labor-intensive, and unreliable due to significant phenotypic variation among isolates influenced by environmental factors [4].

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In recent years, molecular techniques have revolutionized fungal taxonomy and pathogen identification. Internal Transcribed Spacer (ITS) region sequencing, species-specific PCR primers, and phylogenetic analysis have become powerful tools for accurate and rapid detection of *Fusarium* species [5]. Molecular characterization allows differentiation of closely related species, detection of cryptic species, and understanding of pathogen diversity at the genetic level. The application of ITS-based molecular markers has proven especially effective for resolving species complexes within *Fusarium*. These markers provide high amplification success, sufficient genetic variability, and wide availability of reference sequences in public databases. Studies employing ITS, TEF-1α (Translation Elongation Factor), and β-tubulin markers have highlighted significant genetic diversity among *Fusarium* isolates infecting soybean across different geographical regions [6]. Understanding this diversity is imperative for predicting pathogenic behavior, virulence patterns, host specificity, and potential emergence of new pathogenic strains.

Marathwada is characterized by semi-arid climatic conditions, irregular rainfall patterns, and predominantly black cotton soils that favor the proliferation of soil-borne pathogens, including *Fusarium*. With soybean cultivation expanding rapidly in the region, reports of wilt symptoms and root infections have increased significantly in recent years. However, scientific information on the identity, distribution, and molecular diversity of *Fusarium* species causing soybean wilt in Marathwada remains limited. Most existing studies have focused on morphological identification or general pathogen surveys, with limited emphasis on molecular detection. Given the economic significance of soybean and the threat posed by *Fusarium* wilt, a systematic investigation employing modern molecular tools is urgently needed. Accurate identification of the causal species will support the development of region-specific disease management strategies, such as resistant cultivars, crop rotation schedules, soil health monitoring, and biological control approaches. Moreover, understanding genetic variation among isolates may help predict disease outbreaks and guide policy-making in plant health and crop protection. The present study aims to detect and characterize the *Fusarium* species associated with wilt disease in soybean (*Glycine max* L.) in the Marathwada region using molecular markers. This study is to provide detailed molecular insights that will enhance understanding of pathogen diversity and contribute to developing effective management strategies to reduce yield losses and ensure sustainable soybean production in Marathwada.

2. Materials and methods

2.1. Field Collection of Wilted Plant Samples

The Wilt-affected soybean crop samples were collected from different localities of the Marathwada region, including Beed, Chhatrapati Sambhajinagar, Dharashiv, Jalna and Parbhani. Samples showing typical wilt symptoms such as yellowing, drooping, vascular browning, and root deterioration were carefully uprooted for examination (Figure 01 and 02). Collection was carried out during early morning hours to minimize desiccation and maintain sample integrity. Each infected plant sample was placed in a sterile paper bag or clean zip-lock polythene bag to prevent contamination. At every sampling site, GPS-enabled photographs were taken to record the exact geographical location. All sample bags were properly labeled with details such as locality name, GPS coordinates, date, and time of collection. After field collection, the samples were transported immediately to the laboratory under cool conditions. In the laboratory, the wilted samples were stored at 4°C in a refrigerator to preserve pathogen viability and prevent excessive microbial growth. These preserved samples were then used for further isolation.

2.2. Surface Sterilization, Isolation and Purification of *Fusarium* species

The infected soybean wilted samples were initially subjected to surface sterilization to eliminate external contaminants. Small pieces of the diseased root or stem tissues were excised and treated with 0.1% mercuric chloride ($HgCl_2$) for 30 seconds to ensure effective disinfection. Following sterilization, the tissues were rinsed three to four times with sterile double-distilled water to completely remove any residual traces of $HgCl_2$, which may otherwise inhibit fungal growth. The thoroughly washed and surface-sterilized tissue pieces were then aseptically transferred onto Potato Dextrose Agar (PDA) medium supplemented with streptomycin (0.2 g/L) to suppress bacterial contamination. The inoculated Petri plates were incubated at 30°C for 5–7 days under controlled environmental conditions. During incubation, fungal growth emerging from the infected tissues was monitored, and freshly growing colonies of *Fusarium* species were subsequently subculture onto fresh media for purification and further identification.

2.3. Composition of Media

2.3.1. Potato Dextrose Agar (PDA)

Peeled Potato – 200g, Dextrose – 20g, Agar-Agar – 20g, Streptomycin – 0.2g, Distilled Water (D/W) – 1000ml, $_{p}$H -5.5 to 5.6

2.3.2. Glucose Nitrate (GN) Liquid Media

Glucose – 10g, Potassium Nitrate (KNO_3) – 2.5g, Potassium Dihydrogen Phosphate (KH_2PO_4) – 1.0g, Magnesium Sulphate Heptahydrate (MgSO4.7H2O) - 0.5g and Distilled Water (D/W) – 1000ml, pH – 5.5 to 5.6

2.4. Taxonomic Identification of Different *Fusarium* species

After the growth of *Fusarium* isolates on potato dextrose agar (PDA) petri plates, detailed macroscopic observations were carried out. These included the examination of external colony features such as texture, pigmentation on the surface and reverse side, colony colour variations, and the overall growth rate. Microscopic characteristics were also studied, with particular emphasis on the arrangement, shape, and size of conidia, the nature of conidiophores, and other distinguishing reproductive structures. Both macroscopic and microscopic features play a crucial role in the accurate identification of *Fusarium* species, as the genus exhibits considerable morphological diversity. For precise identification, the observations were compared with descriptions available in standard taxonomic resources, including various research publications, monographs, and authoritative literature such as *Fusarium* Species: An Illustrated Manual for Identification [7], The *Fusarium* Laboratory Manual [8] and The Fuskey: *Fusarium* Interactive Key [9]. These references helped confirm the identity of the isolated *Fusarium* species with greater accuracy and reliability.

2.5. Comparative Study of Growth and Morphological Traits of Different *Fusarium* Species on Potato Dextrose Agar (PDA) and Glucose Nitrate (GN) Liquid Medium.

To study the growth pattern and morphological characteristics of *Fusarium* species obtained from wilted soybean crops, the *Fusarium* species were cultured on solid Potato Dextrose Agar (PDA) medium. The cultures were incubated at room temperature (approximately 30 °C) for seven days. After incubation, macroscopic characteristics such as colony colour, colony pattern, surface texture, and colony diameter were recorded. The microscopic examination, the morphology of macroconidia, microconidia, and chlamydospores was studied using seven-day-old cultures grown on PDA. A small portion of mycelial growth was stained with 0.1% lactophenol cotton blue, mounted on a glass slide, and observed under a compound microscope to assess the arrangement, shape, and size of the conidia. To compare the growth behavior and sporulation capacity among different *Fusarium* species isolated from wilted Soybean crop, the isolates were further cultured in Glucose Nitrate (GN) liquid medium. The flasks were incubated at room temperature (30 °C) for seven days. Observations were recorded in terms of mycelial colour, changes in media colour, mycelial dry weight, and sporulation levels. This combined macroscopic and microscopic assessment provided a comprehensive understanding of the growth dynamics and morphological diversity of the *Fusarium* species.

2.6. DNA Isolation

Application of the standardized protocol with slight modifications resulted in DNA that was readily amenable to PCR amplification [10].

2.7. Preparation of Diluted DNA Samples

A portion of the extracted DNA was diluted with an appropriate volume of sterile double-distilled water to obtain a working concentration of 155 ng. This working DNA sample, along with 10pM of each primer, was used for PCR amplification and subsequently stored at 4 °C.

2.8. PCR-Based DNA Fingerprinting Profiles

The ITS-PCR protocol was used with some modification to produce DNA fingerprinting profile to produce three different fungal isolates of *Fusarium* species. The PCR amplification reaction was optimized by varying concentration of PCR component. Amplification reaction was carried out in 50 µL reaction mixtures containing 155ng of fungal genomic DNA, 10X PCR buffer, 3.2 mM MgCl2 0.5 mM dNTPs, 10pM primers and 3U of Taq DNA polymerase. PCR amplification was performed in master cycler gradient, Eppendorf PCR thermocycler, and the program consisted of an initial denaturing at 94˚C for 3 min, followed by 30 cycles comprising denaturation at 94˚C for 1 min, annealing at 50˚C and extension of 2 min. at 72˚C. The final extension was set at 72˚C for 7 min. PCR amplified product was separated by electrophoresis on 1.5% agarose gel in 10X TAE buffer, stained with Ethidium bromide and visualized under gel documentation system.

2.9. ITS Primer Profiling

The ITS primers were screened, and those showing clear sequence variation were selected for assessing the genetic diversity among the three different *Fusarium* species. The PCR amplification was carried out in a reaction volume of 50 µL.

2.10. PCR Amplification Procedure

The master mix was prepared using the afore mentioned reagents and subsequently divided into six equal aliquots of 50 µL each, dispensed into separate PCR tubes. To each tube, 1 µL of genomic DNA from the three different *Fusarium* species was added, using fresh sterile tips for each sample to prevent cross-contamination, thereby maintaining a final reaction volume of 50 µL. The PCR tubes were then placed in a thermal cycler, and amplification was carried out according to the standardized protocol, with minor modifications depending on the selected primer [11].

3. Result and Discussion

3.1. Incidence of *Fusarium* Species on Soybean Crop Field

The present investigation focused on the isolation and identification of different *Fusarium* species associated with soybean crops collected from five major regions of Marathwada, namely Beed, Chhatrapati Sambhajinagar, Dharashiv, Jalna, and Parbhani. The results revealed a considerable diversity of *Fusarium* fungi in the soybean-growing areas. A total of 28 *Fusarium* isolates were obtained, representing five distinct species: *F. avencanum, F. equiseti, F. hainanense, F. oxysporum,* and *F. pernambucanum* (Figure 03). Among the surveyed localities, Jalna exhibited the highest occurrence, with eight isolates recorded. Beed and Chhatrapati Sambhajinagar followed, each contributing six isolates. In contrast, Dharashiv and Parbhani showed the lowest diversity, with only four isolates recovered from each region (Table 1). These findings indicate notable regional variation in the distribution of *Fusarium* species across the Marathwada region.

Table 1 Incidence of Different *Fusarium* Species on Soybean Crop field

Sr. No.	Name of Fusarium species	Beed	Chhatrapati Sambhajinagar	Dharashiv	Jalna	Parbhani
1	*Fusarium avencanum*	+	-	-	+	-
2	*Fusarium equiseti*	++	+	++	++	-
3	*Fusarium hainanense*	-	+	-	+	+
4	*Fusarium oxysporum*	++	++	+	+++	++
5	*Fusarium pernambucanum*	+	++	+	+	+

-: Absent (0%), +: Minimum (20-25%), ++: Moderate (45-50%), +++: Maximum (70-75%), ++++: Abundant (>70%)

3.2. Morphological Diversity of Different *Fusarium* species

In the present investigation on *Fusarium* wilt of soybean, a total of five distinct *Fusarium* species were successfully isolated from soybean fields across various districts of the Marathwada region, including Beed, Chhatrapati Sambhajinagar, Dharashiv, Jalna and Parbhani. These isolates were subjected to detailed morphological characterization to aid in their identification and differentiation. The morphological features studied included the colour of the colony surface (front view), reverse colony colour, colony diameter, and the nature of mycelial growth on Potato Dextrose Agar (PDA) medium. The comparative morphological characteristics of all five *Fusarium* species are presented in Table 2, clearly highlighting the distinct variations observed among the isolates (Figure 03).

Table 2 Comparative Morphology of Different *Fusarium* Species Cultured on Potato Dextrose Agar (PDA) Medium

Sr. No.	Name of Fusarium Species	Front colony colour	Reverse colony colour	Colony Diameter (Cm)	Type of Mycelium
1	*Fusarium avencanum*	Whitish Pink	Purplish	3.4	Fluffy, Cottony, Floccose Aerial
2	*Fusarium equiseti*	Cream	Tan	3.3	Irregular, Cottony, Smooth Aerial
3	*Fusarium hainanense*	Peach Orange	Pale Cream	3.5	Rounded, Compact, Smooth Aerial
4	*Fusarium oxysporum*	Pinkish White	Pale Yellowish	3.1	Rounded, Cottony, Fluffy aerial
5	*Fusarium pernambucanum*	Whitish Cream	Pale Brown	3.2	Cottony, Fluffy, Floccose Aerial

Note: Diameter of colony after 7 days of growth at 30°C

3.3. Effect of Growth and Sporulation of different *Fusarium* species

During the screening of five different *Fusarium* species on Glucose Nitrate Broth (GNB) medium, considerable variation was observed in their growth behaviour, sporulation intensity, mycelial pigmentation, and mycelial dry weight. All five isolates exhibited differential responses to the nutrient medium, indicating species-specific growth patterns. Although most *Fusarium* species demonstrated high sporulation capacity, their biomass accumulation varied markedly. Among the tested species, *Fusarium hainanense* recorded the highest mycelial dry weight (0.17 g), suggesting its superior ability to utilize the GNB medium for biomass production. This was followed closely by *Fusarium pernambucanum*, which achieved a dry weight of 0.16 g. In contrast, *Fusarium avencanum* exhibited the lowest mycelial dry weight (0.09 g), indicating comparatively slower growth or reduced nutrient assimilation. These differences highlight the inherent physiological variability among *Fusarium* species when cultured under identical conditions (Table 3).

Table 3 Comparative Study on Growth and Sporulation Patterns of Different *Fusarium* Species on Glucose Nitrate (GN) Liquid Media

Sr. No.	Name of *Fusarium* Species	Sporulation	Mycelial Colour	Culture Filtrate (C.F.) Colour	Mycelial Dry Weight (g)
1	*Fusarium avencanum*	+++	Whitish Pink	Off-white	0.09
2	*Fusarium equiseti*	++	Whitish Cream	Off-white	0.11
3	*Fusarium hainanense*	++++	Light Orange	Creamish	0.17
4	*Fusarium oxysporum*	++	Whitish	Pale Yellowish	0.10
5	*Fusarium pernambucanum*	++++	Light Orange	Off-white	0.16

3.4. Genetic Diversity Analysis of *Fusarium* Species Through ITS Marker Profiling.

The analysis of the genomes of three *Fusarium* species using ITS primers i.e. *F. equiseti, F. hainanense* and *F. pernambucanum*. It provided clear evidence of significant genetic variation among these pathogenic fungi. This genetic variability is an important factor to consider when assessing their impact on soybean crops. To evaluate the extent of genetic diversity and its association with cultural and pathogenic traits, DNA fingerprints were generated using ITS primers based on the DNA sequences of the three *Fusarium* species. These species were successfully differentiated according to their ITS primer–derived sequence patterns. The results revealed substantial genetic variation among the selected *Fusarium* species, indicating a high level of genetic diversity within this economically important group of plant pathogens. Such molecular-level differentiation enhances the understanding of species-specific variability and supports improved strategies for disease diagnosis, management, and the development of resistant soybean cultivars (Table 4).

Table 4 DNA Sequencing of different *Fusarium* species used by ITS Primer

Sample Code	Name of *Fusarium* species	Primer	Sequence (5`à 3`)
F-33	*Fusarium equiseti*	ITS-1	TCCGTAGGGGGGACCTGCG
F-34	*Fusarium hainanense*	ITS-2	TCCTCCGCTTATTGATATGC
F-35	*Fusarium pernambucanum*	ITS-3	CAACTCCCAAACCCCTGTGA

3.5. ITS-Based Phylogenetic Analysis for Assessing Genetic Diversity Among Three Different *Fusarium* Species

The phylogenetic tree provides a clear representation of the evolutionary relationships among the three *Fusarium* samples F33, F34, and F35 showing how they have diverged from a common ancestral node (Node 4). The very short initial branch length (0.1) from the root to Node 4 suggests that only minimal genetic variation occurred prior to the first major evolutionary split. From this ancestral point, sample F34 separates onto its own branch with the longest evolutionary distance (1.0). This extended branch length indicates that F34 has undergone the greatest amount of genetic change, making it the most divergent and evolutionarily distinct among the three isolates. In contrast, samples F33 and F35 remain clustered together beyond Node 4, sharing a more recent common ancestor. Their respective branch lengths 0.6 for F33 and 0.8 for F35 indicate moderate genetic divergence, yet still significantly closer to each other than either is to F34. This closer relationship demonstrates that F33 and F35 likely belong to a similar genetic lineage or species complex, forming a distinct cluster within the phylogenetic framework (Figure 04).

Figure 1 Collection of Soybean Wilted crop sample

Figure 2 Vascular Browning of Fusarium Wilt in Soybean Crop

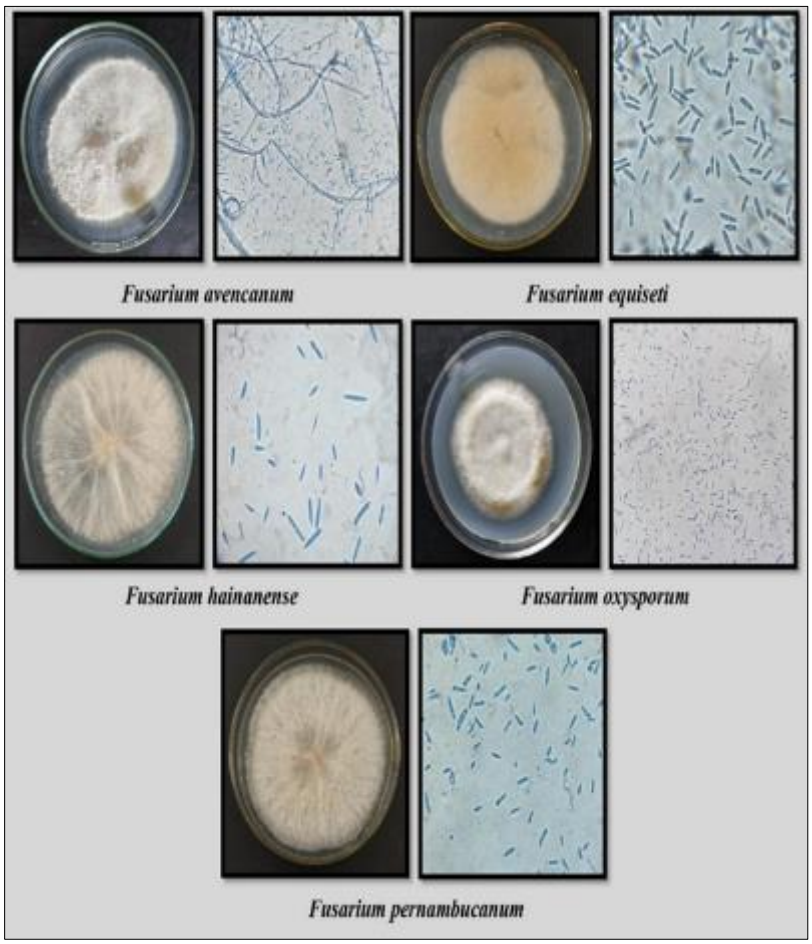

Figure 3 Pure Culture and Microscopic Photograph of Different Fusarium species

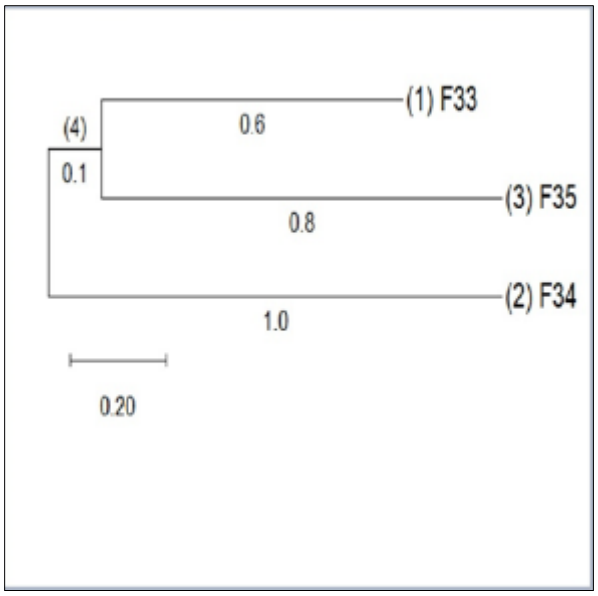

Figure 4 Graphical Representation of Phylogenetic Relationships Among Three Different Fusarium Species

4. Conclusion

The present investigation revealed a considerable level of diversity among *Fusarium* species associated with soybean crop fields. A total of five different *Fusarium* species were identified from 28 isolates collected across various localities in the Marathwada region. During the study, the pathogenic fungus *Fusarium* exhibited a high degree of genetic and morphological variability, likely influenced by differences in geographical and environmental conditions. To assess molecular diversity, ITS primers were employed, enabling the detection of genetic variation among the different *Fusarium* species. A significant level of genetic variability was recorded among the three selected species i.e. *F. equiseti, F. hainanense* and *F. pernambucanum*. This genetic variation corresponded closely with their cultural characteristics and pathogenic behaviour. Overall, the findings clearly demonstrate the extent of genetic diversity present within these *Fusarium* species and highlight their strong association with distinct morphological, cultural, and pathogenic traits.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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