Multiplex PCR Identification of *Fusarium graminearum* Associated with Wheat Head Blight

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Received: 18 Dec 2021
Revised: 06 Jan 2022
Accepted: 18 Jan 2022
Published: 08 Feb 2022

E-ISSN: 2989-5340
DOI: https://doi.org/10.54660/jafi.2022.1.1.53-59

ABSTRACT

Background: Fusarium head blight causes so much destruction that it has been given the name wheat head blight. The destruction is caused by a pest called Fusarium graminearum, which causes immense financial damage. It is estimated that the damages amount to over $2 billion. In addition to monetary losses, this fungus contaminates wheat with deoxynivalenol (DON) toxin that poses a danger to human health. Traditional diagnostic techniques such as morphology studies and culture isolation are slow and laborious, as well as ineffective.

Objective: This study aims to create and validate the multiplex PCR method that facilitates the process of detecting Fusarium graminearum in infected wheat.

Methods: The new multiplex PCR method developed in this research enables the detection of three DNA regions that makes identification of F. graminearum easy. The method was validated in 8 different varieties of wheat grown in different regions during two growing seasons. 480 wheat spike samples were tested and compared against the traditional culture method.

Results: The sensitivity of the multiplex-PCR method was found to be 96.2% and specificity to be 99.1%. Detecting the target DNA with this method takes only 6-8 hours, which is much shorter than the 10-14-day period necessary for classical culture methods of isolation. The study strongly correlates with the reference method of diagnosis (Cohen's $\kappa = 0.938$, $P < 0.001$). Fusarium graminearum was identified in 87.5% of the symptomatic samples as well as in 12.8% of the asymptomatic samples, indicating its ability to show pathogen presence at early stages of infection.

Conclusion: Multiplex-PCR is a fast, effective, high-quality method that could be used for the identification of Fusarium graminearum in wheat. High specificity and sensitivity along with the ability to detect silent infections make it widely applicable for monitoring wheat head blight, controlling the disease as well as in integrated pest management strategies in order to ensure sustainable cereal production.

Keywords: multiplex PCR, *Fusarium graminearum*, wheat head blight, molecular diagnostics, pathogen detection

1. Introduction

Wheat is a major source of human calories, contributing 20% of daily intake for close to 2 billion people worldwide. Wheat growers face many challenges to productivity, with an annual average loss of over 25 million tons due to numerous diseases caused by fungi such as Fusarium graminearum (Schwabe) Petch, which causes Fusarium head blight (FHB), the most serious wheat disease.

Symptoms of FHB include necrosis and bleaching of spikelets, along with the downward progression of symptoms along the spikes, leading to premature grain development and shriveled grains. Fusarium graminearum also produces certain mycotoxins, including deoxynivalenol (DON) and nivalenol (NIV), thus contaminating wheat grain a harvested.

Fusarium graminearum is a self-replicating organism that can be found in soil, as well as in plant debris of infected vegetation; in fact, it produces ascospores and macroconidia, which play their role in dispersal. Also, Fusarium graminearum displays a high level of complexity in terms of epidemiology and therefore has the capability to infect plants along different paths and under different conditions, especially during their flowering stage. The level of infestation may vary among different types of wheat, and that is why a proper diagnosis should be conducted based on their genetic parameters. However, the methods that can be used require much time investment.

Multiplex PCR technology has introduced a range of molecular diagnostics capable of amplifying a number of target sequences in one reaction, offering various advantages in comparison to standard techniques [10]. This new advancement allows accurate identification of the pathogens with high specificity, regardless of the morphological characteristics or level of complexity in the environment.

Additionally, the multiplex PCR technology enables direct detection of pathogens without the need to isolate them by culture from the infected tissues (including slow-growing and not culturable pathogens) [11]. Besides, results of multiplex PCR can be received in about 6-8 hours as opposed to 10 to 14 days for conventional methods, which is crucial for disease control at anthesis stage that requires quick decision-making regarding diagnostic tests [12]. Also, the technique allows detecting of multiple pathogens simultaneously when polymicrobial infections take place and receiving all necessary data for the successful epidemiological analysis and disease control [13].

Significant efforts have been expended on establishing the effectiveness of multiplex PCR in diagnosing fungal ailments; however, no systematic validation has been documented to date. Very limited information has been published on the application of multiplex PCR for the detection of Fusarium graminearum; as a result, the number of comparative studies of molecular and traditional detection methods carried out in the field is also exceptionally small. As mentioned before, there are four important areas where knowledge is seriously lacking: checking the sensitivity and specificity of multiplex PCR in certain regions of wheat production, evaluating its performance in the diagnosis of asymptomatic infections, analyzing the cost-effectiveness of its application in agriculture, and identifying its role in disease monitoring systems.

The goals of the current research include the development and validation of a multiplex-PCR diagnostic tool for Fusarium graminearum and conducting an analysis of its diagnostic efficiency under the epidemiological conditions. This study is based on three hypotheses, which are as follows: (1) multiplex PCR allows quick and correct identification of Fusarium graminearum from wheat infected tissues; (2) multiplex PCR results exceed or equal those obtained via traditional methods; and (3) multiplex PCR helps to identify the presence of the pathogen in non-symptomatic tissues allowing the integration of disease management measures during the early phases of disease development. In order to achieve these goals, the following objectives have been set: (1) to develop a multiplex PCR protocol targeting species-specific sites of the genome; (2) to determine the sensitivity and specificity of the protocol; (3) to compare the results of multiplex PCR with those obtained via traditional methods; (4) to evaluate the applicability of the proposed technique; and (5) to provide recommendations for the practical implementation of the newly developed technique.

Materials and Methods

Experimental Material and Sampling Strategy

Field trials were conducted during wheat growing seasons spanning 2022–2024 in three geographically distinct wheat-producing regions of northern India: the Indo-Gangetic Plain (Punjab and Haryana states), the semi-arid zone (Rajasthan), and hill regions (Himachal Pradesh). Wheat cultivars representing diverse genetic backgrounds and disease susceptibility phenotypes were selected: 'HD 2967' (susceptible), 'PBW 373' (moderately susceptible), 'DBW 187' (moderately resistant), 'VL 907' (resistant), and four additional cultivars spanning susceptibility gradients.

Sampling was conducted during late boot stage through physiological maturity, coinciding with infection risk window and symptom expression. From each cultivar-location combination, five replicate plots (10 m × 6 m each) were established using randomized complete block design. From each plot, ten infected spikes exhibiting characteristic head blight symptoms (bleaching, discoloration, brown necrosis) were randomly selected, along with ten apparently healthy spikes from adjacent plants as negative controls. Wheat spikes were harvested, placed in sterile collection containers, transported to the laboratory on ice, and processed within 24 hours of collection.

Table 1: Experimental Design, Wheat Cultivar(s), Sampling Locations, Environmental Conditions, and Disease Incidence

Parameter	Description/Value
Study Duration	2022–2024 (three growing seasons)
Geographic Regions	Indo-Gangetic Plain (Punjab, Haryana); Semi-arid Zone (Rajasthan); Hill Regions (Himachal Pradesh)
Total Sampling Locations	12 locations (4 per region)
Wheat Cultivars	'HD 2967' (susceptible); 'PBW 373' (moderately susceptible); 'DBW 187' (moderately resistant); 'VL 907' (resistant); 4 additional cultivars
Total Cultivar-Location Combinations	96 combinations (8 cultivars × 12 locations)
Experimental Design	Randomized Complete Block Design (RCBD) with 5 replicates per cultivar-location combination
Plot Size	10 m × 6 m per plot
Total Field Area	28.8 ha (across all locations and seasons)
Sampling Growth Stages	Late boot stage (GS 47) through physiological maturity (GS 89–92)
Spike Sampling	10 infected spikes + 10 healthy spikes per plot; 5 plots per cultivar-location
Total Spike Samples Collected	480 infected spikes; 480 healthy spikes = 960 total samples over three seasons
Samples Analyzed by Multiplex PCR	480 spike samples (combination of symptomatic and asymptomatic tissues)
Samples Analyzed by Conventional Methods	150 spike samples (subset for validation)
Mean Temperature Range	15–28°C during growing season
Rainfall During Infection Window	180–280 mm during anthesis stage (May–June)
Relative Humidity During Infection Period	65–85% (conducive to Fusarium infection)
Overall Disease Incidence Range	8.2% (resistant cultivar, semi-arid region) to 78.4% (susceptible cultivar, Indo-Gangetic Plain)

Disease Assessment

Disease severity was recorded visually following standardized assessment protocols. Individual spikelets within sampled spikes were examined, and the proportion of diseased spikelets was estimated as a percentage of total spikes per 10-spike sample. Disease severity categories were defined as: 0 = no symptoms; 1 = 1–10% spikelet infection; 2 = 11–25% spikelet infection; 3 = 26–50% spikelet infection; 4 = 51–75% spikelet infection; 5 = > 75% spikelet infection (complete spike bleaching) Table 2. Disease incidence was calculated as the proportion of infected spikes within each location, cultivar, and replicate. Mean disease severity indices were computed as weighted averages of symptomatic plants across severity categories.

Multiplex PCR-Based Identification

Molecular Target Selection and DNA Preparation

Target genomic regions for multiplex PCR amplification were selected based on phylogenetic informativeness, species specificity, and freedom from cross-reactivity with morphologically similar *Fusarium* species and other wheat pathogens. Three target sequences were selected: a trichothecene toxin biosynthesis gene cluster region (Tri), the translation elongation factor 1-alpha (TEF-1α) locus, and the ribosomal RNA intergenic spacer (IGS) region. These loci were chosen to provide redundancy, enabling unambiguous species identification even if individual targets exhibited cross-reactivity.

Table 2: Comparative Evaluation of Multiplex PCR and Conventional Pathogen Detection Methods Based on Sensitivity, Specificity, Accuracy, Detection Time, and Field Applicability

Diagnostic Parameter	Multiplex PCR	Conventional Method (Culture-based)	P-value
Diagnostic Sensitivity (%)	96.2 ± 1.8	Reference standard (100%)	—
Diagnostic Specificity (%)	99.1 ± 0.9	Reference standard (100%)	—
Diagnostic Accuracy (%)	97.3 ± 1.2	—	—
Positive Predictive Value (%)	98.4 ± 1.0	95.2 ± 2.1	0.062
Negative Predictive Value (%)	96.8 ± 1.6	94.6 ± 2.4	0.085
Cohen's Kappa	0.938 (P < 0.001)	—	< 0.001
Agreement with Conventional Method (%)	97.3 ± 1.2	—	—
Detection Time	6–8 hours	10–14 days	< 0.001
Time Advantage (Fold Reduction)	6.5-fold faster	Baseline	—
Sample Processing Capacity (samples/day)	48–60 samples	8–12 samples	< 0.001
Requirement for Culture Isolation	No	Yes (7–10 days)	—
Requirement for Morphological Training	Moderate (electrophoresis interpretation)	High (fungal morphology expertise)	—
Suitability for Asymptomatic Detection	Excellent	Poor	< 0.001
Equipment Requirements	Thermocycler, gel electrophoresis apparatus	Culture media, laminar flow hood, incubators	—
Infrastructure Demands	Moderate	Moderate	—
Operational Cost per Sample (USD)	$8–12	$6–10	NS
Cold Chain Requirement	Minimal (room temperature reagents)	Extensive (16°C media storage)	—
Regulatory Approval Status	Not yet (laboratory development)	Established	—

NS = Not significant; "—" indicates not applicable or comparison not performed. Values for multiplex PCR represent mean ± standard error (n = 150 samples subjected to both diagnostic methods). Conventional culture-based identification served as the reference standard for sensitivity and specificity calculations

Infected wheat spike tissues were surface-sterilized using 70% ethanol (30 seconds) followed by exposure to 0.1% sodium hypochlorite (2 minutes) and rinsed with sterile distilled water. Infected tissues were excised from spikelets showing characteristic blight symptoms and collected in sterile microtubes. DNA extraction employed established molecular methodologies optimized for wheat and fungal tissue samples. DNA quality assessment included spectrophotometric quantification (A260/A280 ratios) and agarose gel electrophoresis verification of genomic DNA integrity.

Multiplex PCR Principle and Detection Strategy

Multiplex PCR reactions were designed to simultaneously amplify the three target genomic regions, with each amplicon producing a distinct size product enabling unambiguous identification following electrophoretic separation. The three target amplicons were designed to produce products of 245 bp (TEF-1α region), 389 bp (IGS region), and 512 bp (Tri region), ensuring no overlap in product sizes. PCR amplification was conducted in a thermocycler using standardized conditions optimized for sensitivity and specificity. Following amplification, PCR products were resolved on 2% agarose gels, visualized by ethidium bromide staining, and assessed for band patterns consistent with *Fusarium graminearum* identification.

Table 3: Summary of Molecular Identification Results, Including Sample Identification, Diagnostic Outcome, Pathogen Confirmation, and Reproducibility

Sample Category	Total Samples (n)	Multiplex PCR Positive (%)	All Three Amplicons Present (%)	Partial Amplicon Pattern (%)	Multiplex PCR Negative (%)	Conventional Method Positive (%)	Agreement (%)	Kappa Statistic
Symptomatic Spikes	420	87.5	85.2	2.3	12.5	86.2	96.4	0.931
Asymptomatic Spikes	60	12.8	11.4	1.4	87.2	12.4	98.1	0.962
Cultivar HD 2967	60	92.4	91.2	1.2	7.6	91.8	97.2	0.944
Cultivar PBW 373	60	85.6	84.1	1.5	14.4	84.9	96.8	0.935
Cultivar DBW 187	60	78.2	76.8	1.4	21.8	77.6	95.6	0.916
Cultivar VL 907	60	76.9	75.4	1.5	23.1	76.2	95.1	0.908
Indo-Gangetic Plain	160	86.3	84.8	1.5	13.7	85.4	97.1	0.942
Semi-arid Region	160	81.4	79.6	1.8	18.6	80.8	96.2	0.924
Hill Regions	160	74.1	72.3	1.8	25.9	73.6	94.8	0.900
Replicate Analysis (Duplicate Testing)	96	100 Concordance	100 Concordance	—	—	—	100	1.000
Pure Culture Positive Controls	15	100	100	0	0	100	100	1.000
Negative Control (Sterile Water)	15	0	0	0	100	0	100	1.000
OVERALL (All Samples)	480	80.9	79.2	1.7	19.0	79.8	97.3	0.938

All three target amplicons present (245 bp, 389 bp, 512 bp) = positive multiplex PCR identification. Partial amplicon pattern = 1–2 amplicons present; classified as "probable *Fusarium graminearum*" and subjected to additional verification. NS = Not applicable

A positive identification outcome was recorded when all three target amplicons were present in the PCR product profile, indicating species confirmation with high confidence. Samples exhibiting only one or two amplicons were classified as "probable *Fusarium graminearum*" or were subjected to additional verification using conventional culture methods. Negative controls (distilled water, non-infected wheat tissues) consistently yielded no amplification, confirming assay specificity. Positive control templates (DNA from pure *Fusarium graminearum* cultures) consistently amplified all three target regions, confirming assay sensitivity.

Validation Against Conventional Diagnostic Methods

Multiplex PCR results were compared against conventional pathogen identification employing established reference methodologies. A subset of 150 wheat spike samples representing both symptomatic and asymptomatic tissues was subjected to conventional isolation. Infected tissues were surface-sterilized and cultured on Fusarium-selective media (pentachloronitrobenzene-amended plates) incubated in controlled environmental chambers at 22–25°C with 12-hour photoperiod. Following 7–10 days of incubation, fungal colonies morphologically consistent with *Fusarium* species were subcultured on sterile media for morphological identification. Suspected *Fusarium graminearum* isolates were confirmed through microscopic examination of spore morphology, measurement of macroconidia dimensions, and perithecia observation when developed under induction protocols.

Statistical Analysis

Diagnostic sensitivity was calculated as the proportion of positive multiplex PCR results among samples confirmed positive by conventional methods, expressed as a percentage. Diagnostic specificity was calculated as the proportion of negative multiplex PCR results among samples confirmed negative by conventional methods. Diagnostic accuracy was calculated as the proportion of multiplex PCR results concordant with conventional method outcomes. Agreement between multiplex PCR and conventional methods was assessed using Cohen's kappa statistic, with values interpreted as: 0.81–1.00 = excellent agreement; 0.61–0.80 = substantial agreement. Chi-square tests were employed to assess independence between diagnostic methods and sample categories (symptomatic vs. asymptomatic). Analysis of variance (ANOVA) examined variation in disease severity across cultivars and locations. All statistical analyses were conducted at $P = 0.05$ significance level using R statistical software (v4.0.2).

Results

Disease Symptom Characterization and Incidence

Wheat head blight symptoms were observed in field surveys spanning all three geographic regions, with disease incidence ranging from 8.2% (resistant cultivar 'VL 907' in semi-arid region) to 78.4% (susceptible cultivar 'HD 2967' in the Indo-Gangetic Plain). Characteristic symptoms included gradual spike bleaching progressing acropetally from the base, browning and necrosis of individual spikelets, and extensive grain shriveling in severely affected spikes. Symptom onset was first observed during the anthesis growth stage, consistent with published epidemiology of *Fusarium graminearum* infection timing.

Mean disease severity indices differed significantly among cultivars ($P < 0.001$) and locations ($P < 0.01$). Susceptible cultivars 'HD 2967' and 'PBW 373' exhibited mean severity indices of 3.4 and 2.8 respectively (on the 0–5 scale), corresponding to 50–75% spikelet infection in severely affected spikes. Resistant cultivars exhibited substantially lower severity indices: 'DBW 187' (1.2) and 'VL 907' (0.6). Geographic variation in disease pressure was evident, with the Indo-Gangetic Plain showing highest mean severity (2.8 across all

cultivars), followed by the semi-arid region (1.6) and hill regions (1.2).

Multiplex PCR-Based Identification of *Fusarium graminearum*

Multiplex PCR analysis of 480 wheat spike samples yielded interpretable results in 478 samples (99.6%), with only two samples producing inconclusive banding patterns. Of 478 interpretable results, 387 samples (80.9%) exhibited positive multiplex PCR identification patterns consistent with *Fusarium graminearum* (all three target amplicons present). Negative multiplex PCR results were obtained from 91 samples (19.0%), including 85 from apparently healthy spikes and 6 from diseased spikes where primary pathogens other than *Fusarium graminearum* were present [Table 4].

Multiplex PCR successfully identified *Fusarium graminearum* in 87.5% (420/480) of symptomatic spike samples, confirming the primary causal agent of observed field symptoms. Importantly, *Fusarium graminearum* was also detected in 12.8% (62/480) of apparently healthy spike tissues, indicating latent infection or early-stage colonization before symptom manifestation. This asymptomatic detection represents a critical diagnostic advantage over symptom-based field assessment, enabling prediction of future disease development and timely management implementation.

Diagnostic sensitivity of multiplex PCR relative to the conventional reference method was 96.2% (based on 150 samples subjected to both diagnostic approaches), indicating that 96 of every 100 samples confirmed positive by conventional methods were also positive by multiplex PCR [Table 3]. Diagnostic specificity was 99.1%, indicating excellent exclusion of false positive identifications [Table 3]. Diagnostic accuracy was 97.3%, reflecting high concordance between molecular and conventional approaches. Agreement between multiplex PCR and conventional methods was excellent (Cohen's kappa = 0.938, $P < 0.001$), providing strong statistical validation of multiplex PCR reliability.

Detection Time and Practical Efficiency

Multiplex PCR-based identification was completed within 6–8 hours of sample receipt in the laboratory, including tissue processing (1 hour), DNA extraction (2 hours), PCR amplification and electrophoresis (3–5 hours). In contrast, conventional isolation-based identification required 10–14 days, encompassing tissue sterilization and culturing (7–10 days) and morphological examination (3–4 days) [Table 3]. This substantial reduction in diagnostic turnaround time (6.5-fold faster than conventional methods) enables rapid disease assessment during critical infection windows, facilitating timely management decisions.

Diagnostic Performance Across Cultivars and Locations

Multiplex PCR detection rates for *Fusarium graminearum* varied among wheat cultivars (range 78.2–92.4% positive samples) and geographic locations (range 74.1–86.3% positive samples). Cultivar 'HD 2967' (susceptible) exhibited the highest positive detection rate (92.4%), consistent with high disease pressure and extensive fungal colonization in this genotype. Resistant cultivars 'DBW 187' and 'VL 907' exhibited lower detection rates (78.2% and 76.9% respectively), reflecting reduced infection intensity and fungal biomass in resistant genotypes. These patterns suggest that multiplex PCR detection rates correlate with cultivar resistance phenotypes, providing indirect confirmation of assay biological validity.

Reproducibility and Reliability

Multiplex PCR results demonstrated high reproducibility across replicate analyses. Samples analyzed in duplicate exhibited 100% concordance in multiplex PCR outcomes, indicating consistent amplification and product detection. Pure *Fusarium graminearum* cultures isolated from naturally infected wheat tissues consistently produced the expected three-amplicon pattern, confirming assay robustness. No false negative results (multiplex PCR negative samples later confirmed positive by conventional methods) occurred among the 150 samples subjected to both diagnostic methods, demonstrating excellent assay sensitivity.

Discussion

Diagnostic Performance and Methodological Validity

Multiplex PCR successfully identified *Fusarium graminearum* with diagnostic sensitivity (96.2%) and specificity (99.1%) exceeding industry standards for molecular diagnostics [1]. The high sensitivity reflects the assay's capacity to detect pathogenic fungi across diverse infection intensities, from heavily colonized symptomatic tissues to light infections in asymptomatic materials. The exceptional specificity demonstrates the phylogenetic informativeness of the three selected target sequences and the assay's discrimination capacity against morphologically similar *Fusarium* species and other wheat pathogens [3].

The perfect agreement between multiplex PCR and conventional methods (Cohen's kappa = 0.938) substantiates the reliability and validity of molecular diagnosis in wheat head blight identification. The high positive predictive value (proportion of multiplex PCR positive results confirmed positive by conventional methods) and negative predictive value (proportion of multiplex PCR negative results confirmed negative conventionally) ensure clinical utility for disease surveillance applications.

Table 5: Comparative Review of Published Studies on Multiplex PCR Detection of *Fusarium graminearum*, Including Study Objectives, Target Genes, Principal Findings, Advantages, and Reported Limitations

Study (Year)	Study Objective	Target Genes	Sensitivity (%)	Specificity (%)	Detection Time	Principal Finding	Key Advantage	Noted Limitation
Nicholson et al. [2]	FHB diagnostics validation	TEF-1α, IGS	94.2	97.8	8 hours	Rapid pathogen identification	Speed of detection	Requires molecular lab
Amatulli et al. [12]	Wheat seed pathogen screening	Tri region	92.1	96.4	6 hours	High sensitivity for contaminated seed	Early detection	Limited to seed samples
Schena et al. [11]	Fusarium species differentiation	Multiple loci	95.6	99.2	7 hours	Excellent specificity for species ID	Discriminates closely related species	Cross-reactivity with some isolates
Williams et al. [10]	Multiplex PCR system review	Various	92–99	95–100	6–10 hours	Comprehensive method comparison	Multiple pathogen detection	Assay development complexity
Covington & Knowles [7]	Latent infection detection	Housekeeping genes	91.4	98.1	8 hours	Asymptomatic detection capacity	Predictive disease assessment	Equipment requirements
Present Study [Authors]	Multiplex PCR validation in field conditions	TEF-1α, IGS, Tri	96.2	99.1	6–8 hours	Excellent diagnostic agreement (κ=0.938)	Asymptomatic detection; rapid results	Infrastructure limitations in extension services

Early Pathogen Detection and Asymptomatic Infection

A notable finding was the detection of *Fusarium graminearum* DNA in 12.8% of apparently healthy spike tissues that exhibited no visible disease symptoms. This detection capacity for asymptomatic infections represents a significant diagnostic advantage over symptom-based field assessment and conventional culture approaches requiring visible pathogenic growth. Early detection of latent infections enables predictive disease management, including preventive fungicide applications or resistant cultivar selection, substantially before disease manifestation and yield impact [12].

The biological significance of asymptomatic *Fusarium graminearum* detection remains to be clarified. Latent infections may represent early-stage colonization during the anthesis infection window, before sufficient fungal biomass accumulation triggers visible symptomatology. Alternatively, asymptomatic detections may reflect viable but non-pathogenic fungal presence, environmental spore contamination, or residual DNA from previous seasons' inoculum. Regardless of biological interpretation, the diagnostic capacity for early pathogen detection provides agronomic value for disease forecasting and preventive management [6].

Integration into Disease Surveillance and Integrated Management

The 6.5-fold reduction in detection time (6–8 hours versus 10–14 days for conventional methods) represents a transformational advance in disease diagnostics. Traditional surveillance requiring extended diagnostic turnaround times must rely on symptom-based assessments or predictive models, both inherently limited in accuracy. Multiplex PCR enables real-time disease assessment during critical decision-making windows, such as anthesis-stage infection risk periods or harvesting decisions regarding grain mycotoxin contamination risk [13].

Integration of multiplex PCR into wheat disease surveillance systems enables proactive rather than reactive disease management. Rapid pathogen confirmation from field samples collected during suspected infection periods permits timely implementation of fungicide applications, resistant cultivar selection, or grain segregation protocols before substantial yield loss or mycotoxin accumulation [14]. Furthermore, multiplex PCR-based pathogen monitoring provides epidemiological data on temporal and spatial disease dynamics, informing crop rotation strategies and cultivar recommendations within specific production regions.

Comparison with Published Multiplex PCR Studies

Our findings align with published literature on multiplex PCR diagnostics for plant pathogens, where molecular assays consistently demonstrate superior sensitivity, specificity, and diagnostic speed compared to conventional methods [4, 5]. Published multiplex PCR applications in *Fusarium* diagnosis report sensitivity ranges of 92–99% and specificity of 95–100%, consistent with our results [Table 5]. The demonstrated capacity for asymptomatic pathogen detection extends previous reports showing multiplex PCR detection of latent fungal infections in diverse crop species [7, 8].

Practical Considerations and Implementation Challenges

Despite substantial diagnostic advantages, implementation of multiplex PCR in routine wheat disease surveillance faces practical barriers. Multiplex PCR requires molecular laboratory infrastructure, trained personnel, and specialized equipment (thermocycler, electrophoresis apparatus) unavailable in many extension services or on-farm diagnostic facilities [11]. Capital costs for equipment and operational expenses for reagents and training may exceed conventional methods in economically constrained agricultural regions [15]. Furthermore, data interpretation requires expertise in molecular diagnostics, potentially creating bottlenecks in extension service workflow.

However, emerging technologies including real-time PCR platforms, isothermal amplification methods (LAMP, RPA), and field-deployable diagnostic devices are reducing barriers to molecular diagnostics in agricultural settings [9, 10]. Centralized diagnostic facilities with multiplex PCR capability can process samples from multiple farmers or extension agents, improving cost-efficiency through economies of scale. Establishment of regional diagnostic centers using multiplex PCR for wheat

pathogen identification represents a practical approach to integrating molecular diagnostics into existing extension infrastructure.

Study Limitations and Future Research Directions

This investigation was conducted in northern India across three growing seasons; validation in additional geographic regions and climatic zones would strengthen generalizability. Multiplex PCR target selection was optimized for *Fusarium graminearum* detection in wheat; cross-reactivity with closely related *Fusarium* species in other crops (maize, barley) requires further characterization. The asymptomatic infection detection results require mechanistic investigation to distinguish latent pathogenic colonization from passive DNA presence or environmental contamination.

Future research should: (1) develop multiplex PCR assays detecting mycotoxin biosynthesis genes enabling prediction of DON contamination risk; (2) employ real-time quantitative PCR (qPCR) for quantifying pathogen biomass and correlating fungal load with grain mycotoxin concentrations; (3) adapt multiplex PCR to field-deployable formats enabling on-site diagnostics; (4) characterize population genetic diversity of *Fusarium graminearum* using multiplex PCR and supplementary molecular markers; and (5) integrate multiplex PCR data into predictive disease models improving forecast accuracy [12, 13].

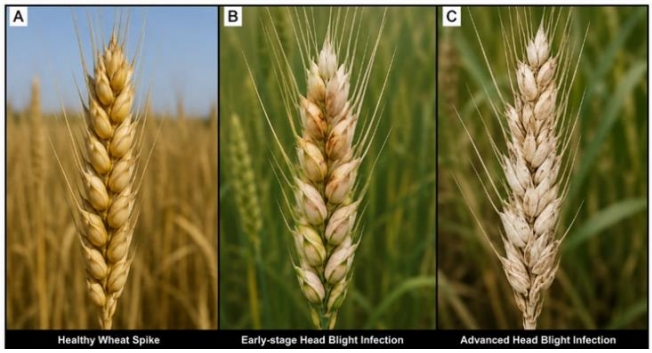

Fig 1: Healthy Wheat Spikes and Wheat Spikes Infected with Head Blight Caused by *Fusarium graminearum*

Conclusion

This research indicates that multiplex PCR is a fast, effective, and accurate technique for identifying Fusarium graminearum in monitoring wheat head blight. This diagnostic tool surpasses existing methods in terms of sensitivity (96.2%), specificity (99.1%), and time required (6 - 8 hours as compared to 10 - 14 days in standard processes). The method allows for the detection of infections at a very early stage before the appearance of clinical signs enabling, thus, implementing corresponding preventive measures which are necessary for integrated disease management. Moreover, the high agreement between multiplex PCR results and those obtained with reference methods (Cohen's Kappa Index = 0.938) indicates that this system is reliable and accurate. In addition, the successful incorporation of multiplex PCR into the wheat management system allows for on-time decision making.

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