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Diagnosis of *Fusarium graminearum* in Soil and Plant Samples of Wheat by Real-Time PCR

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Abstract

Early detection of Fusarium graminearum in wheat is important for the effective control of ear blight or scab. In this study, quantitative real-time PCR (qPCR) technique was used to check the susceptibility of wheat cultivar (CS-12) and proficient diagnosis of F. graminearum. For this purpose, wheat glumes were collected at 0 and 10 days post-anthesis (dpa) from three different areas and used *for the detection of F. graminearum. Conventional PCR was performed to detect F. graminearum in the glumes of all three fields while fungal DNA was quantified by qPCR analysis. Quantification of F. graminearum DNA in wheat glumes at 0 and 10 dpa revealed its highest amount in plant samples of crop area A2 while other two areas showed less contamination of F. graminearum. The quantity of fungal DNA showed synchronization at 0 and 10 days post-anthesis (dpa) in each field which indicates that the epidemic conditions of F. graminearum could be assessed at 0 dpa and can be controlled, timely. Real-time PCR also successfully detected F. graminearum in soil residues of all three areas. These results provide an easy and comprehensive approach for reliable detection of F. graminearum, even before crop cultivation and at early infection stages.*

Keywords: CS-12, fungal DNA, qPCR, Soil, Wheat

1. Introduction

Fusarium head blight (FHB), also called ear blight or scab, is a major fungal disease of important *gramineous* hosts including wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), in the temperate and warmer regions of the United States of America, China, and the southern hemisphere. The disease occurs throughout much of the world and is associated with several *Fusarium* species including *Fusarium graminearum* Schwabe (teleomorph: *Gibberellazeae* (Schwein.) Petch), and *F. culmorum* (W.G. Smith) Sacc. (1). The most common species causing FHB in many of the affected regions is *F. graminearum* (sexual stage – *Gibberella zeae*) (2). Both *F. graminearum* and *F. culmorum* may also cause root rot of small grains crops (3). In wheat, because of FHB, any part or all of the head may appear bleached. These white heads are very conspicuous in a susceptible variety. The partly white and partly green heads are indicative of the disease in wheat. The fungus may infect the stem (peduncle) immediately below the head, causing a brown/purplish discoloration of the stem tissue. Additional indications of FHB infection are pink to salmon-orange spore masses of the

fungus, often seen on the infected spikelets and glumes during prolonged wet weather. Yield reductions result from the decrease in the kernel number and the presence of dry, shriveled kernels, often referred to as "tomb-stones" because of their chalky, lifeless appearance. Infected kernels of durum often lose their amber translucence and appear chalky or opaque (4). FHB fungi produce different trichothecene mycotoxins which are toxic to animals (5). These mycotoxins including deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA) and moniliformin (MON) have been reported earlier (6). Deoxynivalenol or DON (vomitoxin) may cause vomiting and feed refusal in non-ruminant animals. The occurrence, amount, and kind of mycotoxins may depend on several factors, including environment, species of fungus, the severity of infection and the variety or kind of crop (7). Generally, different fungi follow a variety of inoculation patterns (8). The conidia of *F. graminearum* reside and multiply on infected crop residues, small grains and in the field of debris (9). Moist weather is necessary for FHB and spores of the fungi are dispersed by air movement onto the heads of cereal crops. Ascospores can come from within a crop or can be blown from surrounding crops, sometimes from long distances away (10). Rain-splash (11, 12), wind and insects have been reported as other ways for the dispersal of conidial inoculum (13, 14). Rain-splash dispersal is important for dispersal of FHB inoculum in regions with less air movement (11, 12). In this study, we used glumes for the extraction of DNA because spores of the causal fungus land on the exposed anthers at flowering time and then grow into the kernels, glumes or other parts of the head (15). Previous studies reveal that the anthers provide nutrients and possibly stimulate fungal growth through other mechanisms (16). Due to its pathogenicity, many genetic engineering approaches have been successfully used to create resistance against different *Fusarium* species (17). DNA-based techniques have been successfully used for understanding the genetic makeup, genetic diversity and phylogeny of *F. graminearum* and other fungi (18, 19). Real-time PCR analysis is a wonderful way for the accurate, reliable and high throughput quantification of target fungal DNA in plant host tissues, soil, and water (20). In the present study, real-time PCR analysis has been optimized for the reliable detection and quantification of *F. graminearum* in wheat. By using this useful and reliable technique, the relative levels of *F. graminearum* can be quantified and compared at different plant growth stages. Moreover, this study enables us to detect the presence of this fungus in soil residues. Thus, we can assign proper fields to susceptible and resistant wheat varieties.

2. Materials and Methods

2.1. Collection of plant and soil samples. Plant samples (glumes) of wheat cultivar (CS-12) were collected randomly from three different fields (designated as A1, A2 and A3), of the same ecological zone to assess disease severity of *F. graminearum* at 0 and 10 days post-anthesis (dpa). Glumes were collected randomly from each field into three groups, and each group was treated as one replica. Each selected field was at least 2 km apart from the other to get variation in disease severity because ascospore ejection distance is up to a few mm and air can carry them to 8.5 mm (21).

Soil samples were collected from above mentioned three different fields (designated as A1, A2, and A3), before the sowing of Wheat crop for the detection of *F. graminearum,* as its ascospores arise from residues (21). The soil was immediately used for DNA extraction of *F. graminearum.*

2.2. DNA extraction. Wheat glumes were collected at 0 and 10 days post-anthesis (dpa) and frozen in liquid nitrogen. Glumes (100 mg) were taken from each sample for the extraction of DNA because of spores of the causal fungus land and get nutrition from exposed

anthers (15). DNA was extracted from samples of equal weight to compare the amount of fungal DNA from different zones at different days post-anthesis. DNA extraction was performed by the CTAB method developed by Edwards et al. (22) and modified by Wilson et al. (23). To check extraction accuracy and primer specificity, the same method was used for the extraction of DNA from *F. graminearum* grown on PDA media at room temperature for 6-7 days. Prompt culturing was performed at this stage, and clean isolates were selected for fungal DNA extraction. To extract fungal DNA from soil, 100 mg soil from each zone was ground in liquid nitrogen to disrupt organisms and DNA from disrupted cells were extracted with sodium dodecyl sulphate-phenol method (24) with some modifications (25). The yield and purity of DNA samples were estimated by measuring $OD_{260\ nm}$ and $OD_{260\ nm/280\ nm}$, respectively, with a spectrophotometer SPECTRA max PLUS384.

2.3. Primer designing and analysis of primer specificity. The primer pair for partial *F. graminearum* chs1 (chitin synthase class I) gene amplification was designed from *F. graminearum* chs1 sequence in GenBank (accession number AJ312243). Whereas, mRNA sequence of the wheat housekeeping gene, Actin (accession number AB181991), was used to design a set of primers to amplify partial Actin gene from wheat genomic DNA (Table 1).

Table 1. Primers sequences used for real-time PCR analysis.

Sequence (5′-3′)	Target	Accession number	Fragment length (bp)	Product Size (bp)
CCCAGCAATGTATGTCGCAA CAGCAAGGTCCAAACGAAGG	Actin *Triticumaestivum*	AB181991.1	1163	158
CACTTCCTCGACTTCCTTCG CCAGTCCGTCCGAGTAATGT	chs1 *Fusarium graminearum*	AJ312243.1	4600	179

The absence of introns from proposed amplified partial Actin gene was confirmed from wheat genomic sequence data available in GenBank. The primers were designed by using the Primer-3 (26). Sequences of *F. graminearum* chs1 and Actin were obtained from NCBI website (http://www.ncbi.nlm.nih.gov). Primer sequences were verified by using BLAST tool available at NCBI website, and these primer sequences did not show similarity with any other gene sequences available at GenBank database. These primers were referred to amplify wheat partial Actin gene and *F. graminearum* partial chs1 gene from glumes and soil samples by simple PCR and quantitative real-time PCR. Conventional PCR was used to check the chs1 primer pair specificity of *F. graminearum* by the amplification of fungal DNA extracted from fungus grown on PDA and in wheat samples of three different areas before quantitative real-time PCR. DNA extracted from all the samples was diluted to the final concentration of 25ng/ul to normalize the difference in DNA quantities because of variable factors during extraction procedures. Concentrations of extracted DNA from collected samples are given in Table 2. PCR amplifications were performed in a 10 µl volume containing: 25 ng of total DNA, 1.6 µl of each primer (2.5 µM), 0.2 µl dNTPs (10 mM), 0.8 µl $MgCl_2$ (25 mM), 0.5 U *Taq* DNA polymerase, 1× reaction buffer. Total DNA of the selected glumes (containing both plant and fungal DNA) was denatured at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s with a final extension of 5 min at 72°C. The PCR products were subjected to gel electrophoreses on a 1.2 % agarose gel with 5 µg/ml ethidium bromide and viewed under UV light.

Table 2. Concentrations of DNA extracted from different samples.

Samples	Area	Concentration	
Plant Samples from different areas		0DPA	10DPA
	A1	10.9ug/100mg	12.2ug/100mg
	A2	11.5ug/100mg	13.4ug/100mg
	A3	11.4ug/100mg	12.9ug/100mg
Soil Samples from different areas	A1	8.5ug/100mg	
	A2	9ug/100mg	
	A3	8.2ug/100mg	
Fungal Samples were grown on PDA		13.7ug/100mg	

2.4. Real-time PCR analysis. Real-time PCR using the same DNA was performed in three replicates in an optical 96-well plate with an ABI Prism 7500 instrument (Applied Biosystems) using 25 ng DNA from each, plant (plant and fungal DNA), soil (microbial DNA), *F. graminearum* (fungal DNA) and SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycles used were as follows: 95°C for 1 min, 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. Relative quantification of fungal DNA in plant samples was calculated by using Actin as endogenous reference gene and internal positive control to normalize errors, sample-to-sample variations and to check amplification efficiency. Actin gene is present in wheat genome instead of fungal genome, but the presence of both genomes in total DNA makes it act like endogenous reference gene. In the case of DNA collected from plants, Actin acts both as reference gene as well as internal positive control, whereas, it acted as a negative control in case of DNA extracted from soil samples. Fungal DNA extracted from *F. graminearum* grown on PDA was used as an external positive control. Similar thermal cycler conditions were used for the amplification of partial *F. graminearum* chs1 gene from fungal DNA, extracted from all the samples.

2.5. Statistical analysis. All glumes were collected in three replicates. One-way analysis of variance (ANOVA) was performed using *STATISTICA* (version 5.5 a), and means were compared by DMR test ($P< 0.05$).

3. Results

3.1. Fungus growth and primer specificity analysis. *F. graminearum* was successfully cultivated on PDA medium at room temperature, and contamination free colonies were obtained in 6-7 days. Prompt culturing enabled us to select pure colonies. By means of conventional PCR, we checked the specificity of each set of primers.

F. graminearum DNA, grown on PDA, was used as a template for the amplification by chs1 gene specific primers to check primer specificity (Figure 1A). Resulting amplicons were sequenced and aligned with the sequences available in GenBank database (nr) using TBLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These obtained sequences were identical to the chs1 gene sequences present in GenBank. Primers were found to be highly specific after PCR product analysis by gel electrophoresis (Figure 1B).

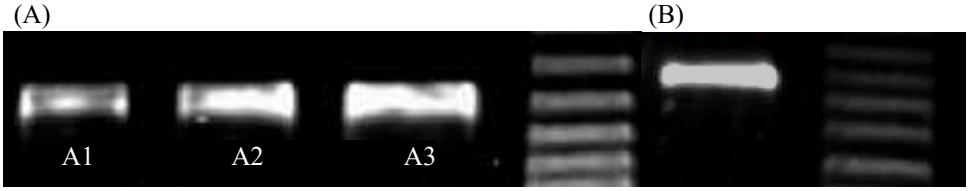

Figure 1. Conventional PCR analysis to check the specificity of each set of primers.
(A) Amplification of *Fusarium graminearum* in wheat samples of three different areas.
(B) Amplification of pure *Fusarium graminearum* DNA grown on PDA medium.

3.2. Detection and quantification of *F. graminearum* in affected glumes. Wheat glumes were collected at 0 and 10 days post-anthesis (dpa) from three different areas and analyzed for the detection of *F. graminearum*. Conventional PCR revealed the presence of *F. graminearum*in the plant samples of all three fields (Figure 1A). Using these primers, PCR product of desired fragment length was also successfully amplified from pure DNA of *F. graminearum* (Figure 1B). After the detection of *F. graminearum* by simple PCR, fungal DNA was quantified in affected glumes by quantitative real-time PCR analysis. Quantification of *F. graminearum* DNA in wheat plants at 0 and 10 dpa revealed the highest amount of *F. graminearum* DNA in plant samples (glumes) of area A2 while other two areas (A1 and A3) showed lower levels of *F. graminearum* DNA from glumes (Figure 2). Quantity of fungal DNA showed synchronization and increased with the same ratio at 0 and 10 days post-anthesis (dpa) in each area. The amount of Wheat DNA was uniform in all three areas as shown by Actin gene quantification and was not affected by the 10 days duration as opposed to fungal DNA which indicated the growth of fungus on the plant during that period.

3.3. Detection and quantification of *F. graminearum* in soil. Real-time PCR successfully detected and amplified *F. graminearum* in soil residues of all three areas (Figure 3). *F. graminearum* DNA quantities in soil samples were lesser than affected glumes. We selected 15 soil samples from each area, and *F. graminearum* DNA could not be amplified in all samples. No fluorescence signals could be detected in some samples after 40 cycles, and those amplification reactions were considered negative. The presence of *F. graminearum* was more pronounced in low tillage soil area. Wheat crops grown in these three areas had already been observed to be affected by this fungus (local observation). The absence of the Actin signal indicated the lack of wheat genomic DNA in samples. These results of the amplification of the different samples with the specific primers of *F. graminearum* provided us an easy and comprehensive approach for reliable detection of *F. graminearum* even before crop cultivation.

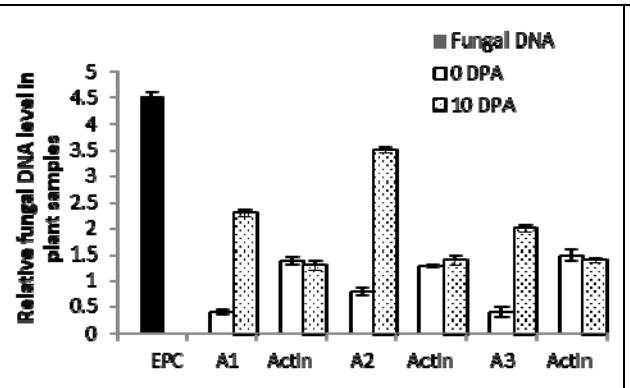

Figure 2. Relative quantification of *Fusarium graminearum* DNA by qPCR in wheat glumes of three affected areas. EPC: External positive control, A1: Area 1, A2: Area 2, A3: Area 3, dpa: days post-anthesis. Vertical bars represent standard error of means (n=3).

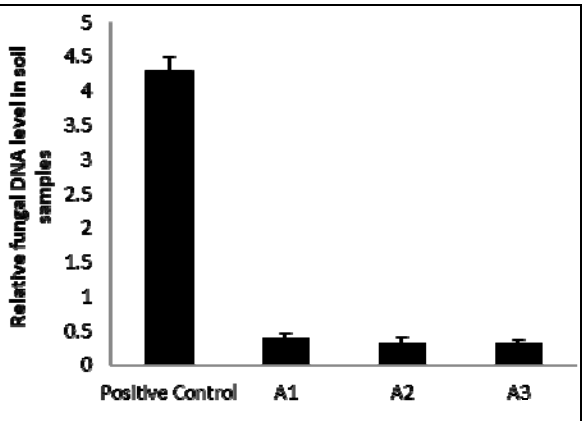

Figure 3. Relative quantification of *Fusarium graminearum* DNA from soil samples by qPCR. Relative quantification of fungal DNA in soil samples of three affected areas. A1: Area 1, A2: Area 2, A3: Area 3. Vertical bars represent standard error of means (n=3).

4. Discussion

Fusarium head blight (FHB) is caused by *F. graminearum* along with *F. culmorum, F. avenaceum* and other related fungi and its management requires an integrated approach. Early detection of this fungus helps scientists and farmers to save crop and increase yield

significantly. There are many techniques used for the detection and isolation of different fungi and qPCR is one of the most logical methods used in this modern era. Several studies have demonstrated the use of real-time PCR for the detection of different fungi in plants (27).

In this study we used wheat glumes which proved very useful for the detection of *F. graminearum* at both at 0 and 10 days post-anthesis (dpa). These results demonstrate that the glumes are the primary receptors of *F. graminearum* and fungus reside on anthers to probably get nutrition and growth stimulations (16). Hence, glumes (anthers) should regularly be observed for the earliest detection of *F. graminearum.* Up to 50-60 % reductions in FHB severity can be achieved when fungicides are applied at an early flowering stage in wheat (3). Primer specificity analysis provided us a strong base to use those primes for quantitative Real-time PCR analysis. In accordance with previous studies, use of "Actin" as an internal control proved very useful as it is one of the best housekeeping genes in wheat showing symptoms of *Fusarium* head blight (4). Simple PCR helped the detection of *F. graminearum,* but it could not be used for the quantification of fungal DNA. This detection is very important before proceeding towards the more sophisticated approach of quantitative Real-time PCR. By using this approach, the highest amount of DNA was observed in the glumes of wheat crop of Area 2 at both 0 and 10 dpa. This synchronization helped us to conclude that the epidemic conditions of *F. graminearum* could be assessed at 0 dpa under same environmental conditions. We selected all three fields from the same ecological zone to nullify environmental factors because proper environment plays key role in the spread of this fungus. Conidial production and infection rates of *F. graminearum* are favored in warmer and humid conditions (12). These results also suggest that the early inspection of glumes can assist the detection of this fungus. Successful detection of *F. graminearum* in soil by Real-time PCR analysis describes the usefulness of this technique for the selection of a suitable field for specific wheat cultivars. *F. graminearum* was subjected to detection in the soils of three regions and the presence of this fungus was detected in soil residues of all three areas which describe the presence of fungus in the soil and suggest that only fungal resistant varieties should be cultivated in these affected areas. Though none of the available commercial cultivars is an immune to *Fusarium* infection, but differences in reaction to FHB do occur and cultivars with various levels of resistance to FHB have been developed (3). This fungus survives in colonized crop residues as mycelium and may develop saprophytically on residues during the fall, winter, and spring (13). These residues can harbor perithecia and produce ascospores even after two years on the soil surface (28). Pathogen survival is enhanced within reduced tillage systems, and the fungus survives best on residue left on or above the soil surface. In wheat fields, cereal grains are prolific sources of inoculums propagules (29). For FHB, inoculum propagules include both ascospores and conidia and are found at nearly any time during the adult stages of the cereal crop unfavorable environmental conditions (30). The highest amount of fungal DNA was observed in soil samples of Area 1 while plant glumes of Area 2 showed the maximum presence of fungal DNA. This disparity suggests that the spread of fungal DNA is dependent on environmental factors. Air movement, humidity, and rain-splash may affect the dispersal of this fungus significantly. During moist weather, spores of the fungi are windblown or splashed onto the heads of cereal crops. Spores can come from within a crop or can be blown from surrounding crops sometimes long distances away (12). Epidemics of FHB are influenced by local and regional environment, host genetic make-up, and pathogen factors including adaptation and virulence (31). The most favorable conditions for infection are prolonged periods (48 to 72 h) of high humidity and warm temperatures (75 to 85 F°). However, infection occurs at cooler temperatures when high humidity persists for longer than 72 h. Early infections may produce

air-borne spores, which are responsible for the secondary spread of the disease, especially if the crop has uneven flowering due to late tillers (32).

5. Conclusion

This amplification of *F. graminearum* DNA can help scientists to successfully detect this fungus at early and later infection stages because we found a correlation between the amount of fungal DNA at 0 and 10 dpa. These primers are an useful tool for early screening of *F. graminearum* before the transport of plants or soil material.

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