Sigma J Eng Nat Sci, Vol. 42, No. 2, pp. 450–458, April, 2024

Sigma Journal of Engineering and Natural Sciences
Web page info: https://sigma.yildiz.edu.tr
DOI: 10.14744/sigma.2022.00047

Research Article

Physiological, genetic and transcriptional characterization of *Fusarium graminearum* isolates

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ARTICLE INFO

Article history
Received: 01 April 2022
Revised: 27 April 2022
Accepted: 28 May 2022

Keywords:
Fusarium Graminearum; Linear Growth Rate; Gene Expression; PCR-ITS-RFLP; qRT-PCR

ABSTRACT

Fusarium graminearum is the primary cause of Fusarium head blight (FHB) epidemics worldwide. The characterization of *F. graminearum* isolates via physiological, genetic, and transcriptional analysis was aimed in the study. A total of 31 isolates and a reference strain were grown on potato dextrose agar (PDA) for 7 days. According to measurements on the 4th and 7th day of cultivation, their minimum and maximum mean linear growth rates (LGRs) were calculated as 9.62±0.44 to 13.32±0.69 mm/day, respectively. Isolates were grouped as x<10 mm/day and 10<x<20 mm/day. Amplification products of the internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) coding sequences with 1147 bp were digested with three restriction endonucleases in all isolates. Two different restriction profiles were obtained with *Pst*I and *Eco*31I digestions whereas *Eco*RI yielded single banding profile. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping was able to distinguish isolates within the same species under four groups. *FgMgv1*, *tri4* and *MAT1-1-2* gene expression levels of selected one each isolate (FgM1, Fg174 and Fgsh4) with the highest, moderate and the lowest LGRs (13.32±0.69, 11.32±0.15 and 9.62±0.44 mm/day, respectively) were examined by qRT-PCR. Relative mRNA abundance values of *FgMgv1* were 1.715±0.199, 0.089±3.166e-004 and 0.020±1.408e-004 for FgM1, Fg174 and Fgsh4, respectively. Similarly, these values for *tri4* were calculated as 0.081±0.009, 0.016±0.004 and 0.002±4.338e-005 and for *MAT1-2* as 2.097±0.484, 1.901±0.195, 1.047±0.136. Expression levels of these genes were significantly higher in FgM1 with the highest LGR values. Outcomes showed that PCR-RFLP method may become possible to distinguish the members of *Fusarium* species complex more clearly. Moreover, determined correlation among the LGRs of isolates, their aggressiveness and mycotoxin production capacities provided basic knowledge for supporting studies intended to control FHB infections.

Cite this article as: Teker T, Khalid SA, Yörük E, Albayrak G. Physiological, genetic and transcriptional characterization of *Fusarium graminearum* isolates. Sigma J Eng Nat Sci 2024;42(2):450–458.

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This paper was recommended for publication in revised form by Regional Editor Banu Mansuroğlu

INTRODUCTION

Fusarium graminearum is among the most encountered pathogens and the most virulent species in the world as the primary cause of Fusarium head blight (FHB). Small grains, wheat and barley are primarily affected by this disease which is prominent in regions with more humid environmental conditions during the flowering season [1, 2]. FHB affects the harvest due to floret sterility and reduction in kernel weight and size [3]. The fungus also produces significant numbers of mycotoxins as secondary metabolites [3-5] in the grains, reducing their quality and making them unfit for consumption [6]. These mycotoxins mainly include trichothecene and the estrogenic mycotoxin zearalenone (ZEN). Upon consumption, they inhibit the synthesis of eukaryotic proteins and modify the immune functions in humans and animals, posing substantial health risks [7-10]. In humans, the mortality rate of systemic *Fusarium* infections is approximately 70% [11-14]. The trichothecene mycotoxins produced by *F. graminearum* include nivalenol (NIV) and deoxynivalenol (DON). Genetic profiles have been identified among the trichothecene compound, called chemotypes. The acetylated derivative of NIV, 4-acetyl nivalenol (4-ANIV), are converted from the NIV chemotype. DON chemotypes and sub chemotypes, 3-ADON (3-acetyl deoxynivalenol) and 15-ADON (15-acetyl deoxynivalenol), can be found in different geographical regions. In America, Asia, Africa and Europe where the DON and NIV chemotypes can be observed, one has been found to be dominant, suggesting a relationship between chemotypes and geographical distribution [15-17]. DON associated with FHB was found to be more dominant than NIV in Europe and USA, whereas the NIV chemotype was observed to be more dominant in the Korean population of *F. graminearum* [6].

The heterogeneity of the *Fusarium* genus makes it difficult to classify at a species level, and such a classification is of utmost importance to the identification and management of pathogens, causing yield losses across agricultural spaces [18, 19]. Traditionally, morphological characters –such as the structures and sizes of the macroconidia and microconidia, or the presence or absence of chlamydospores– have traditionally been utilized in the identification of this species [20]. The *Fusarium* species classification has also been made based on vegetative compatibility groups (VCGs) and host specificity and/or host-pathogen interaction [21]. The traditional morphological, bio-chemical, and cytological methods can sometimes be insufficient for identifying species correctly [22]. DNA based molecular approaches provide faster and more reliable detection [23, 24].

The internal transcribed spacer (ITS) sequences of fungal ribosomal RNA (rRNA) genes have been extensively used in the molecular and ecological studies [22, 25-27]. Since ITS is the most evolved region among species within a genus, this region can discriminate at species level in *Fusarium* [22, 26, 28]. Polymerase chain reaction-ITS-restriction fragment length polymorphism (PCR-ITS-RFLP) genotyping makes it possible to obtain banding patterns by characterizing isolates through the amplification and restriction enzyme digestion of this specific genomic regions of rDNA [29, 30].

Certain phenotypic traits such as linear mycelium growth rate and capacity of mycotoxin production are associated the occurrence of molecular markers in *Fusarium* species. Variations in *FgMgv1* gene that codes mitogen-activated protein kinases which are required for the cell wall integrity signaling pathways and trichodiene synthase genes can lead to significant differences in phenotypic characteristics of these species. The relationship between the mating-type and the radial growth capacity has been previously reported [31-35].

The main aim of this study is to utilize the PCR-ITS-RFLP method to detect polymorphisms among the 31 *F. graminearum* isolates obtained from different regions of Iran. Moreover, radial growth rates of these isolates were compared to their aggressiveness and mycotoxin production capacity by analyzing the expression levels of *FgMgv1, MAT1-2*, *tri4* transcripts, which effects on phenotypic characteristics. This study provides further insights into our knowledge of Iranian *Fusarium* isolates both at genotypical and phenotypical levels.

MATERIALS AND METHODS

Fungal Isolates, Linear Growth Rate Analysis

A total of 31 monosporic *F. graminearum* isolates were used in this study (Table 1). Samples obtained from diseased wheat kernels were collected from different regions of Iran [36]. H-11 *F. graminearum* reference strain was provided by Dr. Therese Lee (School of Agricultural Biotechnology, Seoul National University). Fungal isolates were maintained on a synthetic low nutrient agar SNA and stored in 20% glycerol at -80 °C. The fungus was grown on potato dextrose agar (PDA) media for a period of 7 days at 25 °C for the testing of mycelial growth, the extraction of genomic DNA and total RNA. Growth measurements were taken on the 4th and 7th days of incubation to calculate values of linear growth rates (LGR) according to protocol described by Popiel et al. [37]. Mean LGR and standard errors were calculated using GraphPad Prism 5.0 software, USA.

Genomic DNA Isolation and PCR-ITS-RFLP Analysis

The genomic DNAs of the isolates were extracted by utilizing a kit for genomic DNA isolation (Roche, Switzerland). DNA quantification was done using a spectrophotometer (Thermo, USA). Specific oligonucleotide primers, ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used for amplification [28, 38].

PCR was performed in a final volume of 25 µL by using 50 ng of genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 10 pmol of each primer and 1 U (0.04 U/µL) *Taq* DNA polymerase (Thermo Scientific, USA). PCR was carried out on thermal cycler (BioRad, France). The conditions for

Table 1. *F. graminearum* isolates and their geographic origins, chemotypes, mean linear growth rates (LGRs) and banding patterns. SE: Standard error

Code	Region	Chemotype	Mean LGR±SE (mm/day)	Banding Pattern	Code	Region	Chemotype	Mean LGR±SE (mm/day)	Banding Pattern
FgM1	Neka	NIV	13.32±0.69	B	Fgsh4	Neka	NIV	9.62±0.44	B
FgM3	Neka	NIV	10.27±0.01	C	Fgsh5	Neka	NIV	11.71±0.44	A
FgM5	Neka	NIV	11.52±0.15	D	Fgsh7	Neka	3-ADON	11.90±0.28	D
FgM6	Neka	NIV	10.75±0.73	D	Fgsh10	Neka	NIV	13.38±0.73	D
FgM7	Neka	NIV	12.32±0.10	C	Fgsh13	Unknown	15-ADON	10.64±1.38	A
FgM9	Neka	NIV	12.20±0.03	A	Fgsh14	Unknown	3-ADON	12.01±0.09	B
FgM10	Neka	NIV	9.82±0.98	D	Fgsh15	Unknown	3-ADON	12.17±0.57	D
Fg4	Mazandaran	NIV	12.95±0.46	D	FgT2	Neka	NIV	10.94±0.54	D
Fg5	Sari	NIV	13.07±0.54	B	FgT3	Neka	NIV	12.38±0.15	D
Fg18	Moghon	NIV	12.95±0.48	D	FgT7	Neka	NIV	12.95±0.48	D
Fg49	Moghon	15-ADON	13.07±0.54	D	FgT9	Neka	NIV	12.51±0.22	D
Fg56	Gorgan	NIV	11.52±0.15	A	FgT10	Neka	NIV	12.32±0.15	D
Fg165	Kordkooy	NIV	10.29±0.71	D	FgT11	Neka	NIV	13.26±0.66	D
Fg170	Gorgan	NIV	10.52±0.16	D	FgT12	Neka	NIV	12.57±0.25	D
Fg174	Gorgan	NIV	11.32±0.15	B	FgT16	Neka	NIV	12.70±0.38	A
Fgsh1	Neka	15-ADON	11.37±0.34	D	H-11	Korea	15-ADON	13.01±0.50	A

PCR amplification include pre-incubation at 94 °C for 5 mins, denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension for 1 min at 72 °C by 35 cycles. The final extension was at 72 °C for 3 mins. The PCR products were run on 1% agarose gel in TAE buffer at 70 V, 110 mA for 40 mins and stained with ethidium bromide. The images were visualized by using a gel visualization system (Avegene, Taiwan).

The restriction patterns of the ITS regions were predicted for each of the known restriction enzymes using the Restriction Mapper tool (http://www.restrictionmapper.org/). The most appropriate enzymes were selected. Three restriction enzymes, *Eco*RI, *Pst*I and *Eco*31I, were used in PCR-ITS-RFLP analysis. These enzymes had restriction site(s) in the ITS regions of the rRNA gene. Aliquots of PCR products (500 ng) were digested with 10 units of restriction enzymes *Eco*RI, *Pst*I and *Eco*31I (Thermo Scientific, USA) according to the manufacturer's instructions. The digested PCR products were electrophoresed in 1.5% agarose gel at 70 V, 110 mA in TAE buffer and stained with ethidium bromide. The restriction fragments were visualized under UV light and photographed. To verify the obtained patterns, all restriction analyses were performed thrice.

Total RNA Isolation and cDNA Synthesis

Total RNAs from 7-day-old *F. graminearum* isolates (FgM1, Fg174 and Fgsh4) were extracted using TriPure Reagent (Roche, Switzerland). 100 mg of fresh mycelia were collected from petri dishes and homogenized with liquid nitrogen and 1 mL TriPure reagent. The homogenized sample was transferred to the microtube, and the procedure recommended by the manufacturer was followed to isolate the total RNA. The isolated RNAs were checked with spectrophotometer (Thermo, USA) and agarose gel (1%) electrophoresis.

2 µg of total RNAs were used in synthesizing the cDNAs. The cDNA conversion was carried out using a commercial kit (Takara, Japan). 1 × PrimeScript master mix, 2 µg of total RNA and distilled water were mixed in a total of 20 µL of reaction volume. The mixture was incubated at 37 °C for 20 min and at 85 °C for 5 min. 1:4 diluted cDNAs were used as the qRT-PCR templates.

Gene Expression Analysis

mRNA transcript abundance of *FgMgv1, tri4, MAT1-2* and *β-tubulin* was investigated by qRT-PCR analysis in FgM1, Fg174 and Fgsh4 isolates showing relative differences in LGR. *β-tubulin* was used as the endogenous control. Sybr Green I-based fluorescence assay and QuantStudio 5.0 (Thermo-Applied Biosystems, USA) system were used in gene expression analysis. qRT-PCR mixture was prepared in a reaction volume of 20 µL containing 1 × Sybr Green mix (Takara, Japan), 20 pmol forward primer, 20 pmol reverse primer (Table 2) and a 2 µL volume of cDNA equivalent to 1 µg of RNA. Cycling conditions were as follows: 95°C for 2 min (pre-denaturation), 95 °C for 15 s, 57 °C for 15 s, 72 °C for 20 s with 45 cycles and melting curve step (95 °C for 15 s with 4.8 °C/s ramp rate and 65 °C for 1 min with 2.5 °C/s ramp rate) with a common temperature screening. 5-time log series were used in PCR efficiency determination. Two independent biological and three technical replicates were used for each experiment.

Table 2. Oligonucleotide primers used in gene expression analysis

Target Gene	Primer	Primer Sequence (F/R; 5'-3')	Size (bp)
β-tubulin	beta	AGGGTCATTACACCGAGGGT/GTACCACCACCAAGAGAGTGG	121
FgMgv1	mgv	AGGTTCAACGATTCCGACAG/GACCATTACCCTGAGGCAGA	100
tri4	tri4	ATGGATGAAAGGCTCGAGGT/ACTGTCGGTGCTTTTGACG	139
MAT1-2	mat	CGACCTCCCAAYGCYTACAT/TGGGCGGTACTGGTARTCRGG	260

Statistical Analysis

The products of RFLP were visually scored as the presence (1) or absence (0) of a band. A cluster analysis was performed to estimate the levels of intraspecific variability, using data the rDNA haplotypes defined in Table 3 by the PAST ver. 4.07 software. The dendrogram was generated based on Jaccard's coefficient according to the unweighted pair-group method with arithmetic average (UPGMA) algorithm. 10000 iterations were performed to obtain impression of the robustness of the clusters. Column statistics and one-way ANOVA tests of gene expression analysis were carried out using the GraphPad Prism 5.0 software.

RESULTS

***In vitro* Growth Capacity of Isolates**

A total of 31 *F. graminearum* isolates and a reference strain were grown on PDA for 7 days. Their radial growth capacities were tested on the 4th and 7th days of incubation and LGR values were calculated as mm/day. The minimum and maximum mean LGR values were 9.62±0.44 and 13.38±0.73 mm/day, respectively (Table 1). *F. graminearum* isolates were divided into two groups as x<10 mm/day (Group I) and as 10<x<20 mm/day (Group II) according to LGR values. Only two isolates were clustered in Group I, the remaining isolates were clustered in Group II.

Table 3. Restriction patterns of *Fusarium* isolates and estimated fragment sizes (bp) of ITS regions of rDNA digested with *Pst*I, *Eco*31I and *Eco*RI

Banding Pattern	*Pst*I	*Eco*31I	*Eco*RI
A	386, 761	310, 849	271, 876
B	386, 761	310, 849 and 404, 720	271, 876
C	420, 727	310, 849	271, 876
D	420, 727	310, 849 and 404, 720	271, 876

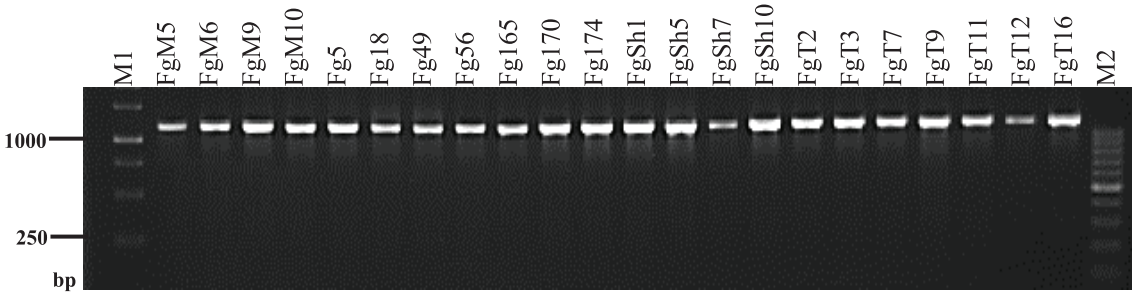

Figure 1. Amplification products with 1147 bp using ITS5/NL4 primer pair. M1: 1 kb DNA ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder); M2: 100 bp DNA ladder (Thermo Scientific GeneRuler 100 bp DNA Ladder).

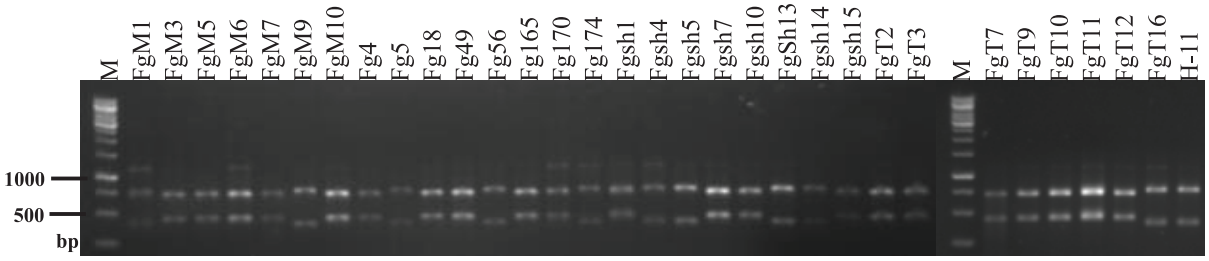

Figure 2. Digestion products of 31 samples with *Pst*I restriction enzyme. Two different profiles were observed in agarose gel electrophoresis: (1) 386 and 761 bp lengths, (2) 420 and 727 bp lengths. M: 1 kb DNA ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder).

PCR-ITS-RFLP Analysis

In the PCR amplification using ITS5/NL4 primers, a 1147 bp band was obtained in all the samples (Figure 1). Despite *Pst*I having one cutting site at 386 bp position, the digestion of PCR products with *Pst*I generated two profiles: one with 386 and 761 bp in lengths, the other with 420 and 727 bp in lengths (Table 1 and 3, Figure 2).

*Eco*31I digestion resulted in two different profiles. Of the two profiles in agarose gel electrophoresis, one contained two fragments of 310 and 849 bp in lengths, and the other, four fragments of 310, 849, 404 and 720 bp in lengths (Table 1 and 3, Figure 3).

Two bands with a length of 271 bp and 876 bp were observed in the gel via the *Eco*RI enzyme. *Eco*RI digestion resulted in an idiomorphic banding pattern in all isolates

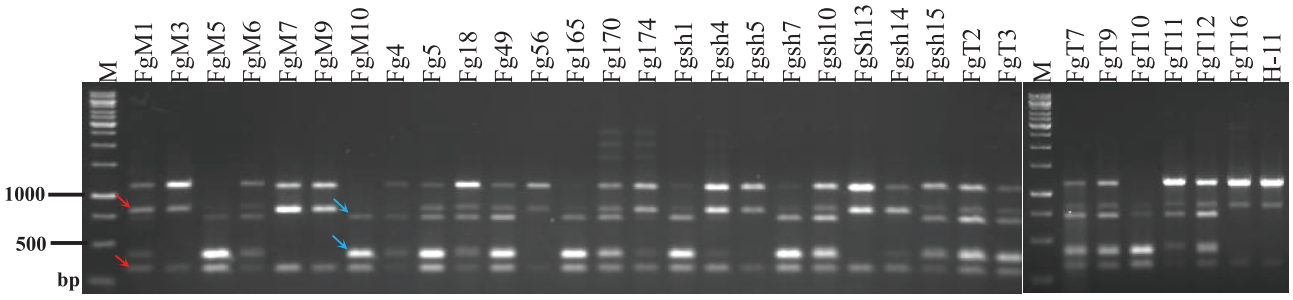

Figure 3. Restriction digestion of ITS regions with *Eco*31I enzyme resulting in two different banding profiles with 310, 849 bps (represented by red arrows); and 310, 849 and 404, 720 bps (represented by turquoise arrows). M: 1 kb DNA ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder).

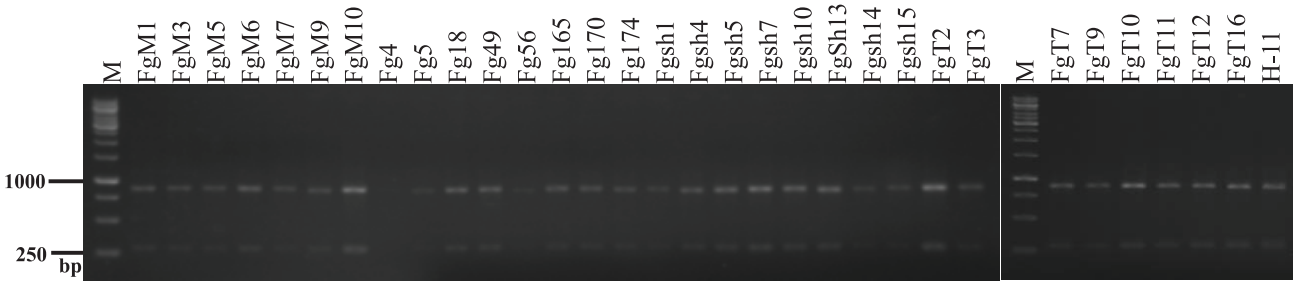

Figure 4. Gel picture of digestion products with *Eco*RI restriction enzyme. Expected banding patterns with 271 and 876 bps were observed in all isolates. M: 1 kb DNA ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder).

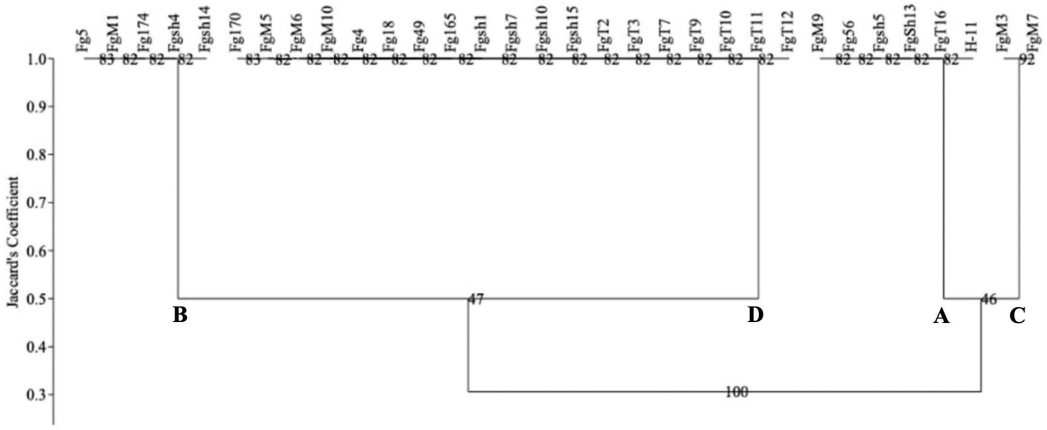

Figure 5. ITS regions phylogenies of *Fusarium* isolates listed in Table 1. Dendrogram was constructed according to UPGMA of restriction digestion patterns of ITS with three restriction endonucleases (*Pst*I, *Eco*31I and *Eco*RI). A, B, C, D represents banding patterns and the numbers at the nodes depict bootstrap values with 10000 iterations.

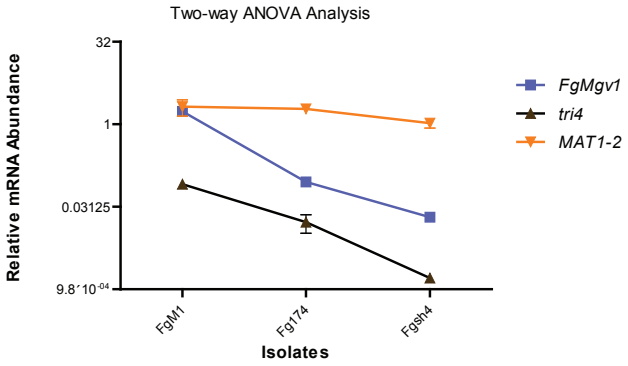

Figure 6. Relative mRNA abundance of *FgMgv1*, *tri4* and *MAT1-2* genes in three selected isolates. Alterations in relative mRNA abundance were statistically significant for each gene ($p<0.05$).

of phenotypical and genotypical diversity can be observed among *Fusarium* species [39, 40]. Popiel et al. [37] showed *Fusarium* isolates could be divided into three groups according to their radial growth rate. However, changes in morphological characteristics alone are not enough to reveal detailed data on *Fusarium* characterization. Additional methods such as molecular marker techniques can be carried out for a comprehensive analysis of *Fusarium* isolates. Like the IGS-RFLP markers, which has been reported as a suitable and rapid technique to determine the intraspecific and phylogenetic relationship of *Fusarium* strains [41, 42], ITS regions also have the potential to be a target for the investigation of intraspecies discriminations due to greater mutation frequency within these regions rather than the rDNA sequences. Minor differences in nucleotide composition within the ITS regions can lead to the different restriction patterns reflecting intraspecific variations [43-48]. In the present study, the identification of 31 *F. graminearum* isolates originating from Iran and a reference isolate was performed targeting the ITS regions with RFLP-PCR. The results showed two different band profiles obtained from gel electrophoresis by using the restriction enzyme *Pst*I. As expected, 11 *F. graminearum* isolates showed banding profiles with 386 and 761 bp fragments when restricted with *Pst*I enzymes. The other 21 samples produced a second profile with 420 and 727 bp in lengths. In digestion with *Eco*31I, the first profile was observed on 8 isolates by fragments of 310 and 849 bp lengths. Remaining 23 isolates had the second profile consisted of four bands in the gel, with band lengths of 310, 849, 404 and 720 bp, respectively. The co-occurrence of haplotypes with 310, 849 and 404, 720 bp restriction fragment sizes could be caused by heterozygous somatic individuals (dikaryons).

Table 4. Relative mRNA abundance of three selected isolates. The alteration in the expression level of each gene was found statistically significant ($p<0.05$). SE: Standard error

	FgM1	Fg174	Fgsh4
Gene	**Mean±SE**	**Mean±SE**	**Mean±SE**
FgMgv1	1.715±0.199	0.089±3.166e-004	0.020±1.408e-004
tri4	0.081±0.009	0.016±0.004	0.002±4.338e-005
MAT1-2	2.097±0.484	1.901±0.195	1.047±0.136

(Figure 4). Overall, a total of four banding patterns, A-, B-, C-, D-pattern, were obtained via PCR-RFLP analysis (Table 3). Also, the dendrogram constructed from the data of rDNA haplotypes is shown in Figure 5. The obtained restriction patterns of the ITS regions did not show any correlation with the LGR values, geographic origin and chemotypes of the isolates.

Gene Expression Analysis

0.5-1 µg/µL of total RNAs (with $\Delta_{260/280}$ = 1.9-2.0 absorbance values) were converted to cDNAs. E values for all genes were found within acceptable ranges (1.9-2.1). Relative transcript abundance values for *FgMgv1, tri4* and *MAT1-2* genes were calculated in three isolates and all values were compared to each other. Isolates with relatively higher LGR values showed significantly higher levels of *FgMgv1, tri4* and *MAT1-2* expression (Figure 6, Table 4). The alterations for each gene were found statistically significant ($p<0.05$).

DISCUSSION

F. graminearum is the most prominent cause of FHB in wheat worldwide. However, the dominant species in specific locations can vary according to their hosts, climate conditions and environmental factors. High levels

The rDNA region that was analyzed showed enough polymorphism for the intraspecific *Fusarium* identification according to specific rDNA haplotypes. As a result, it was revealed the PCR-ITS-RFLP approach appears to be a convenient tool to obtain intraspecific variability within *F. graminearum*. This method has the potential to evaluate groups of closely related species and assign new strains rapidly.

The genotyping of species complex by targeting distinct genomic regions like *FgMgv1* is a powerful approach to differentiating species and/or isolates of filamentous fungi [35]. *FgMgv1* plays crucial roles in the regulation of vegetative differentiation, the generation of multiple responses during stress conditions and the generation of different signaling pathways that are involved in the production of mycotoxins. Also, the signaling pathways dealing with the high osmolarity glycerol and cell wall integrity was provided by constituents of *FgMgv1* [49]. Hou et al. [31] reported that reductions in hyphal growth and DON production occur as consequences of the deletion of *MGV1* in *F. graminearum*. The cell walls of *mgv1* deletion mutants become hypersensitive to cell wall stressors like lytic enzymes. Their hyphal fusion blocked especially on solid media [31]. This study recorded a higher *FgMgv1* expression level in isolates with relatively higher LGR values.

Potential associations between hyphal growth and toxin production were examined by evaluating the gene expression of the *tri4* gene, required for trichothecene biosynthesis. This gene regulates several oxygenation reactions by coding a multifunctional oxygenase and is one of the commonly targeted genes for mycotoxin biosynthesis studies. Its expression level enables the selection of high or low DON producers and non-producer, which is an important criterion in plant pathology [50-52]. The expression level of the *tri4* gene displays a major up-regulation in samples with 10<x<20 mm/day LGR value.

The correlation between mycelial growth rates and expression patterns of *MAT* genes was also determined in the current study. *F. graminearum* is a homothallic (self-fertile) ascomycetous species, the genome of this species harbors two mating-type (*MAT*) genes, *MAT1-1* and *MAT1-2*. In addition to the *MGV1* gene, *MAT* idiomorphs that control two sex pheromone-receptors are required for the sexual reproduction in *F. graminearum*. In addition to primary roles of *MAT* genes in mate recognition, they regulate several genes, involved in the stages of sexual development [53, 54].

CONCLUSION

The results of this study support the association between genotypic and phenotypic traits by indicating a correlation between radial growth rate values and the expressions of genes related to selected traits of pathogenicity. Current data reflects that radial growth rate values could be associated with aggressiveness and mycotoxin production. Also, it was shown that the PCR-RFLP method may become possible to distinguish the members of *Fusarium* species complex more clearly by improving or refining a marker or genotyping profile. The outcome of the current study reveals the detailed phenotypic and genotypic characterization of *Fusarium* isolates from Iran and provide insights into better understand the genetic diversity of *Fusarium* species.

FUNDING

This study was partially funded by Scientific Research Projects Coordination Unit of Istanbul University. Project number: 26895.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Bahram Sharifnabi (Isfahan University of Technology, Iran) and Dr. Therese Lee (Seoul National University, South Korea) for providing the fungal isolates.

AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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