Vol.66: e23210821, 2023
https://doi.org/10.1590/1678-4324-2023210821
ISSN 1678-4324 Online Edition

Article - Agriculture, Agribusiness and Biotechnology

Molecular Analysis of the Genes Responsible for Catalysing Intracellular Steps of Aurofusarin Biosynthesis in *Fusarium culmorum*

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Luiz Gustavo Lacerda

Received: 20-Dec-2021; Accepted: 12-Oct-2022.

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HIGHLIGHTS

- Molecular analysis of aurofusarin biosynthesis in *F. culmorum*.
- Mycelial pigmentation depending on gene expression and aurofusarin accumulation.
- Pigmentation variations depending on growth phase.

Abstract: *Fusarium culmorum* produces polyketide-structured aurofusarin and its precursor metabolites. Aurofusarin biosynthetic pathway is catalysed by the products of eleven genes located in gene cluster. In this study, the effects of the expression levels of *Pks12*, *Gip6*, and *Gip7*, responsible genes for the intracellular steps of the pathway, on pigmentation were investigated in an *F. culmorum* field isolate. Presence of three genes were determined via PCR and sequencing. Their expressions were investigated by qPCR at 48th, 72nd, 120th and 168th hours. Aurofusarin and rubrofusarin production were verified by HPLC at the same time periods. Mycelial pigmentation, which was initially white, turned yellow, then carmine red during the seven-day culture. *Pks12*, *Gip6*, *Gip7* genes with 1282, 449 and 1278 bps had sequence similarities (86,6%, 99,4% and 80,5% respectively) with the genes of reference (FcUK99). The ΣCp values of all genes ranged from 29th to 32nd cycles, whereas the ΣΔΔCt values were calculated between 9,59 E-02 and 1,30 E-02. Relative quantification revealed these genes were controlled by down-regulation compared to the β-tubulin expression. Aurofusarin quantities ranged from 2,355 ppm to 27,350 ppm between the 72nd and 168th hours whereas the yield of rubrofusarin decreased from 0,098 ppm to 0,063 ppm. (4) Conclusion: This study becomes first report for both investigating the expression levels of the genes responsible for the catalysis of intracellular steps of aurofusarin biosynthesis in *F. culmorum*, and for examining the relationship between gene expression and mycelium pigmentation.

Keywords: *Fusarium culmorum* 1; aurofusarin 2; mycelial pigmentation 3; qPCR 4; HPLC-DAD 5.

INTRODUCTION

Polyketide-structured aurofusarin is one of the secondary metabolites produced by *F. culmorum* [1]. Aurofusarin and its precursors participate in the formation of mycelium pigmentation [2,3]. Mycelium pigmentation may have wide range of color which differs from yellow to carmine red depending on nutrient medium [5]. The eleven genes responsible for aurofusarin biosynthesis are located as a gene cluster in the genome, and biosynthesis is carried out with multiple enzymatic steps. Nine of the eleven genes encode well described proteins while two encode hypothetical proteins. Their functions were identified through deletion mutant experiments [3, 5-6]. *Pks12*, *Gip6* and *Gip7* encode critical enzymes that catalyze the intracellular steps of aurofusarin biosynthesis. The type-I polyketide synthase, encoded by *Pks12* gene, is responsible for the catalysis of the condensation reaction between one acetyl-CoA and six manonyl-CoA units as the first step of the biosynthetic pathway. A yellow pigment, YWA1, is produced as the result of this reaction [6]. *Gip6* encodes the dehydratase required for the dehydration of YWA1 to nor-rubrofusarin, the precursor metabolite of aurofusarin [6]. The conversion of nor-rubrofusarin to rubrofusarin - another yellow colored polyketide metabolite - is carried out by the activity of O-methyltransferase, encoded by *Gip7* gene [3, 5]. Pumping of rubrofusarin across the cell membrane, dimerization of two rubrofusarin metabolites, and modification of the aurofusarin are carried out through the enzymes encoded by the remaining genes in the gene cluster. Knowledge about the relationship between expression levels of the mentioned genes and mycelial pigmentation remains limited. There are not any studies in literature for revealing the expression levels of aurofusarin genes contributing to the formation of mycelial pigmentation with in relation to the growth phases of *F. culmorum* before.

The present study investigates the effects of the alterations in the expression levels of *Pks12*, *Gip6* and *Gip7* genes - responsible for the intracellular steps of the production of aurofusarin and its precursors - on mycelial pigmentation. Each of these genes were amplified from an *F. culmorum* genome, then verified via sequencing. Their expression levels were determined by qPCR at the 48th, 72nd, 120th and 168th hours of the cultures. In vitro aurofusarin and rubrofusarin amounts were also detected with high performance liquid chromatography with diode-array detection (HPLC-DAD) at the same time marks.

MATERIAL AND METHODS

Pathogen cultivation and pigmentation monitoring

Gene expression and HPLC analyses were carried out from *F. culmorum* field isolate purified with single spore analysis. The isolate was reactivated from glycerol stock on synthetic nutrient agar (SNA) and then was transferred to potato dextrose agar (PDA). Fungal growth was carried out in controlled growth chamber at 25 ºC and 50% humidity for seven days. Radial growth rate was measured and pigmentation alterations were monitored.

Isolation of genomic DNA

Genomic DNA (gDNA) was isolated according to protocol of Aamir and coauthors (2015) with minor modifications [7]. Seven-day-old fresh mycelia were frozen with liquid nitrogen and homogenized in porcelain mortar. The homogenate was suspended in 500 µl lysis buffer (100 mM Tris HCl, 50 mM EDTA, 3% SDS, pH 8,0) and transferred into microtubes. 500 µl of LiCl solution (8 M) was added into the solution and the tube was centrifuged at 10,000 xg for 1 min at room temperature. The aqueous phase was transferred into a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added. Centrifugation was carried out at 10,000 xg for 10 min at room temperature. The supernatant was transferred into a new tube and ethanol precipitation was performed by adding a 2 × volume of absolute ethanol and a 1:10 volume of NaOAc (3 M). After incubation at -80 ºC for 30 min, gDNAs were precipitated by centrifugation at 10,000 xg for 10 min at room temperature. Quantitative and qualitative analyses of gDNAs were carried out with NanoDrop 2000 (Thermo, USA) and then the integrity of gDNAs were controlled with agarose gel electrophoresis.

PCR amplification, sequencing and alignment analysis of aurofusarin genes

The *Pks12*, *Gip6* and *Gip7* were amplified via PCR by using specific oligonucleotides. The partial sequences of the genes (FG02324, FG02325 and FG02326) belonging to *F. graminearum* PH-1 were obtained from the National Center for Biotechnology Information (NCBI). Oligonucleotide primers were

designed with the Primer3 program. Secondary structure and primer-dimer formations of the oligonucleotides were controlled with the oligoanalyzer tool of integrated DNA technologies (IDT) (Table 1). PCR components were mixed in a reaction volume of 25 µL including 1 × PCR buffer (Promega, USA), 2,5 mM $MgCl_2$, 0,08 mM dNTPs, 10 mM of each primer, 50 ng of gDNAs and 0,02 U/µL of Taq DNA polymerase (Thermo, USA). The pre-denaturation of dsDNAs was carried out at 94 ºC for 5 min. Amplification was performed in 35 cycles at 94 ºC for 1 min, at 54-55 ºC for 1 min and at 72 ºC for 2 min. The final extension step was applied at 72 ºC for 10 min. Amplification products were visualized with agarose gel electrophoresis and were bidirectionally sequenced by using the Sanger method (ABI PRISM 3100 Genetic Analyzer, USA). ABI chromatograms of gene sequences were separately exported in the FASTA format via Chromas 2.6.6, then aligned with the sequences of the FcUK99 reference genome through CLUSTALW 2.6.6.

Total RNA extraction and cDNA synthesis

Total RNAs were extracted from the 48^{th-}, 72^{nd-}, 120^{th-} and 168^{th-} hour cultures by using the Tripure RNA isolation reagent (Roche, Switzerland). 50 mg of mycelium was homogenized with liquid nitrogen in a porcelain mortar. The manufacturer's recommendations were followed as the isolation protocol. Quality and quantity of total RNAs were controlled with NanoDrop 2000 (Thermo, USA) and agarose gel electrophoresis. cDNA synthesis was carried out from total RNAs (2 µg) in a volume of 25 µl comprising of; 1 × reaction buffer, 60 µM random hexamer, 60 µM oligo-dT primer, 5 µM DTT, 1 U of protector RNase inhibitor, 1 mM dNTPs and 1 U reverse transcriptase (Roche, Switzerland). Synthesis reaction was performed in a thermal cycler (Biorad, France) in the following incubation steps; at 65 °C for 10 min, 55 °C for 30 min and 85 °C for 5 min.

qPCR analysis of *Pks12*, *Gip6* and *Gip7*

TaqMan hydrolyse probes were used in the expression analysis of the genes. Oligonucleotide primers and probes were designed for each of the genes using the IDT PrimerQuest Tool. Secondary structure and primer-dimer formations were controlled with the oligoanalyzer tool of IDT (Table 2). qPCRs were set at 20 µl final volume comprising of probe master mix, 5 pmol of each primer and cDNA derived from 2 µg of total RNA. qPCR assays were maintained using the LightCycler 480 II system (Roche, Switzerland). Each experiment was repeated at least two times. Mean crossing point (ΣCp) values were recorded. Expression levels of the genes were normalized by comparing the ΣCp values of the housekeeping gene (*β-tubulin*) and ΔCp values were calculated in accordance with the protocol proposed by Livak and Schmittgen [8]. Variance analysis was conducted on one-way ANOVA with Tukey's post-test via Graphpad Prism 5.0 software.

Aurofusarin analysis by HPLC-DAD

The HPLC-DAD quantitative analysis was performed on a Shimadzu HPLC chromatographic system (Shimadzu Corporation, Kyoto, Japan), equipped with LC-20AD pumps, a DGU-20A model degasser unit, a SIL-20 HT autosampler, a CTO-10AS column oven and an SPD-M20A diode array detector. Mediterranean Sea 18 column, 150 mm length, 4,6 mm i.d. and 5 µm particle size were used. The mobile phase was a gradient prepared from deionized water (component A) and methanol containing 0,2% o-phosphoric acid (component B). All mobile phase solutions were filtered and degassed before use. The elution program was designed as follows: 0-14 min, 50% (B) to 100% (B); 14-18 min, 100% (B) to 50% (B), the total acquisition time was 20 min. The mobile phase flow rate was 1,0 mL/min, the column temperature was set at 30°C and the sample injection volume was 20 µl. Ultraviolet absorption was fixed at 250 nm. The standard stock solution of aurofusarin (100 mg/L), rubrofusarin (100 mg/L) and quercetin (I.S.) were prepared in acetic acid and stored at 4°C. HPLC-DAD analysis was carried out from the 48th-, 72nd-, 120th- and 168th-hour cultures depending on pigmentation profile.

RESULTS

Monitoring of pigmentation and observation of radial growth rate

Mycelium pigmentation was observed as white colour in the first 48 hours of cultivation. The radial growth rate of mycelium was measured 4,37 ± 0,35 cm in diameter at this stage corresponding to the lag phase and early log phase. At the center of the culture, the mycelia acquired yellowish pigmentation between the 48th and 72nd hours, included into the log phase, and the mean colony diameter was measured as 6,9 ± 0,42 cm in this period. Maximum radial growth rate (9 cm) was reached at 96th hour and the isolate entered stationary phase from the 120th hour onwards (Table 3). Mycelium pigmentation was observed as carmine red with

yellow margins once the mycelium growth was limited. At the stationary phase (the 168th hour) all the pigmentation acquired as carmine red (Figure 1).

Amplification of Aurofusarin Genes and their Sequencing

Partial regions of *Pks12* (1118 bp), *Gip6* (593 bp) and *Gip7* (1401 bp), belonging to the open reading frames (ORFs) of the genes, were amplified and sequenced by using specific oligonucleotide primers designed for this study (Figure 2). All chromatograms of nucleotide sequences belonging to the three genes had clear peaks without baseline noise. The 950 bp length fragments of *Pks12* and *Gip7*, and 350 bp fragment of *Gip6* were all aligned with the corresponding gene regions of the reference genome (FcUK99). The highest similarity value was found as 99,4% for the *Gip6* gene, between the field isolate and the reference strain. Transversion and transmission type mutations were observed in *Gip6*. The nucleotide sequences of *Pks12* and *Gip7* genes were more polymorphic than that of the *Gip6* gene of the field isolate. Similarity coefficients were calculated as 86,6% and 80,5% for *Pks12* and *Gip7*, respectively. Sequences alignments revealed that transition and transversion type mutations are found in partial regions of both *Pks12* and *Gip7*, while in-dels and SNPs occurred only in *Gip7* gene (Figure 3).

Expressions of *Pks12*, *Gip6* and *Gip7* genes

ΣCp values revealed that the selected aurofusarin genes had already been transcribed when the mycelia had white pigmentation. Expression analysis also indicated that the expression levels of the three genes together with β-tubulin gradually decreased through lag phase to stationary phase. It was found that expression levels of *Gip6* and *Gip7* were higher than *Pks12* at the lag phase and early log phase (Table 4). At the beginning of the stationary phase (the 120th hour) the expression level of *Gip6* decreased more than *Gip7* and *Pks12*. *Pks12* and *Gip7* decrease ratio were nearly the same as *β-tubulin*. ΔCp values were calculated after normalization according to values of *β-tubulin*, the highest expression level was determined for *Gip7* (1,68E-01) at the 168th- hour. The lowest expression level was also calculated for *Gip7* (9,59E-02) at the 120th- hour. When the expressions of the aurofusarin biosynthetic genes were compared with the housekeeping gene, it was observed that fold change values of aurofusarin genes were nearly the same as that of the housekeeping gene (Table 4). Decrease in gene expression levels was observed at the 72nd hour. Expression levels of all genes increased at the 120th hour during stationary phase, and decreased again at the 168th hour (Figure 4A). Although relative quantification data indicated that the aurofusarin genes were down-regulated, all three genes were found to be transcribed even at the stationary phase (Figure 4B).

Aurofusarin Analysis by HPLC-DAD

Aurofusarin and rubrofusarin amounts were detected by HPLC-DAD at the 48th, 72nd, 120th and 168th hours corresponding to lag, log and stationary phases. The peaks were identified in comparison to the aurofusarin and rubrofusarin standards (Figure 5). HPLC-DAD analysis revealed that 4,717 ± 0,001 ppm aurofusarin and 13,443 ± 0,001 ppm rubrofusarin were found in the 48th-hour culture. The aurofusarin amount was determined as 2,355±0,001 ppm and the rubrofusarin amount was calculated as 0,098 ± 0,001 ppm at the 72nd- hour. Aurofusarin and rubrofusarin amounts were 4,742 ± 0,002 ppm and 0,068 ± 0,001 ppm respectively at the 120th- hour while they were measured as 27,350 ± 0,003 ppm and 0,063 ± 0,002 ppm respectively at the 168th- hour.

Table 1. Primers used for amplification of aurofusarin genes

Locus No	Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Product (bp)
FG02324	*Pks12*	GTGGATTGGCTTGACCAGA	CTCTACGAAGGTCGCGATG	1118
FG02325	*Gip6*	GCCATCGTTGACAAATTCCT	TGTCACCGGTCAACCTGATA	593
FG02326	*Gip7*	GCTGATGGTCAATAAGCACA	ATCATTGACCTTGCGAACG	1401

Table 2. Primers and probes used for expression analysis of aurofusarin genes

Gene	Primer	Primer 5'-3'	Probe 5'-3'	Product (bp)
Pks12	Forward	CGAGTTACAGCCACTCAAGTAT	CCGCTGCTGTGTTAGTGATATGCTCA	94
	Reverse	GCTTGTGAGACCTTGGTAAGA		
Gip6	Forward	TCAAGGACTTTCTTCGCATGA	TTTCGAGGAAACGCGGATCTGCC	80
	Reverse	GTGTCAGCAAAGTTGACGTG		
Gip7	Forward	GCCAATGTACCTGAGTCTCTAC	TTGTCATTGCCATACGCGCAACAC	76
	Reverse	TGGTTGGTTCTGCCAAGAT		

Table 3. Mean linear growth rate (LGR) values (± SE) of the field isolate during 168 hours culturing SD: standard error

Hours	LGR (cm)
24h	1,2± 0,07
48h	4,37 ± 0,35
72h	6,9 ± 0,42
96h	9,0 ± 0,00
120h	9,0 ± 0,00
144h	9,0 ± 0,00
168h	9,0 ± 0,00

Table 4. ΣC_t (± SD) and ΔC_p values of *Pks12, Gip6, Gip7 and β-tubulin* as housekeeping gene SD: standard deviation; h: hour

		48h	72h	120h	168h
ΣCt	*β -Tubulin*	25,67±0,27	25,57±0,14	27,82±0,00	27,26±0,38
	Pks12	30,28±0,17	31,04±0,06	31,89±0,05	32,01±0,05
	Gip6	29,43±0,00	30,92±0,00	32,24±0,25	33,53±0,00
	Gip7	29,11±0,07	30,74±0,55	31,20±0,00	32,57±0,00
ΔCp	*Pks12*	4,10E-02	2,24E-02	5,95E-02	3,81E-02
	Gip6	8,47E-02	2,28E-02	8,56E-02	1,30E-02
	Gip7	9,33E-02	2,83E-02	9,59E-02	1,68E-01

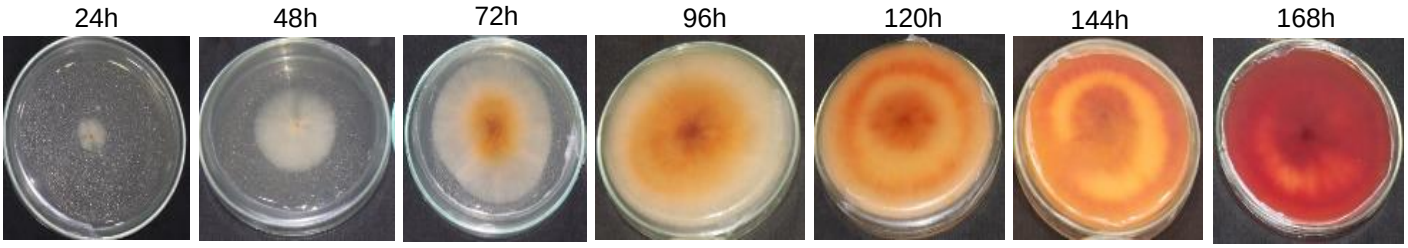

Figure 1. Mycelium pigmentations of the *F. culmorum* field isolate grown on PDA at 25 ˚C

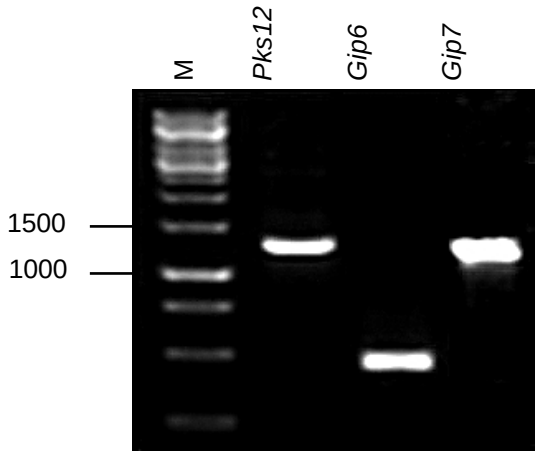

Figure 2. PCR products of *Pks12, Gip6* and *Gip7* amplified from 9F genome (M: 1 kb; Thermo, USA)

```
9F              TCAGCTTCTGG-TGCTTTTTCG----TGACCACGGTGTCAAG--TTTGTACCTCGA-GCG
FcUK99          ATGCTCCTTGGCTGCTTTTTCTCCGTTGACCACGGTGTCAAGGTCTTGTACCTCGAAGCG
                        *** *********     ****************   *********** ***

9F              GAGGTGTGGATA-GCAGCGGCTAGAGCATAGCTTGCATGACC-GCAGAACCTCCA-TCTG
FcUK99          GAGGTGTGGATAAGCAGCGGCTAGAGCATAGCTTGCATGACCCGCAGAACCTCCAATCTG
                ************ ***************************** ************ ****

9F              CAA-GAGTTAGTAAATTATTCTGAGGAGGAATGAGCCG--TGATATCACTTACATCAACA
FcUK99          CAAAGAGTTAGTAAATTATTCTGAGGAGGAATGAGGCAGTTGATATCACTTACATCAACA
                *** ******************************** *   ********************

9F              ATGAGGGATT-GTCCGGCAGGCTGGCCCAGTCAAA-CCGTTGACGAGATGAGAGAGATCC
FcUK99          ATGAGGGATTTGTCCGGCAGGCTGGCCCAGTCAAAACCGTTGACGAGATGAGAGAGATCC
                ********** ************************ ************************

9F              ATGCCTTGGCCGTCAGTCACACTTCTCATGTATCCAGAGAACTGCTTTGTCATGACAGGA
FcUK99          ATGCCTTGGCCGTCAGTCACACTTCTCATGTATCCAGAGAACTGCTTTGTCATGACAGGA
                ************************************************************

9F              CTCTGGGACAAATGATCAAAGAAGGGCAAATCATGATTGAATGCGATGTTGTAAGCTGTC
FcUK99          CTCTGGGACAAATGATCAAAGAAGGGCAAATCATGATTGAATGCGATGTTGTAAGCTGTC
                ************************************************************

9F              TCTGTCTTCTTTACGCTTCCTGGCCACTTTTCTGTAGCTTGGACCATGGCAGCAGCTGTC
FcUK99          TCTGTCTTCTTTACGCTTCCTGGCCACTTTTCTGTAGCTTGGACCATGGCAGAAGCTGTC
                ************************************************** *******
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Figure 3. Alignment data of partial fragment of *Gip7* amplified from the field isolate with reference genome. * symbolizes homologue nucleotides – symbolizes *in-dels*

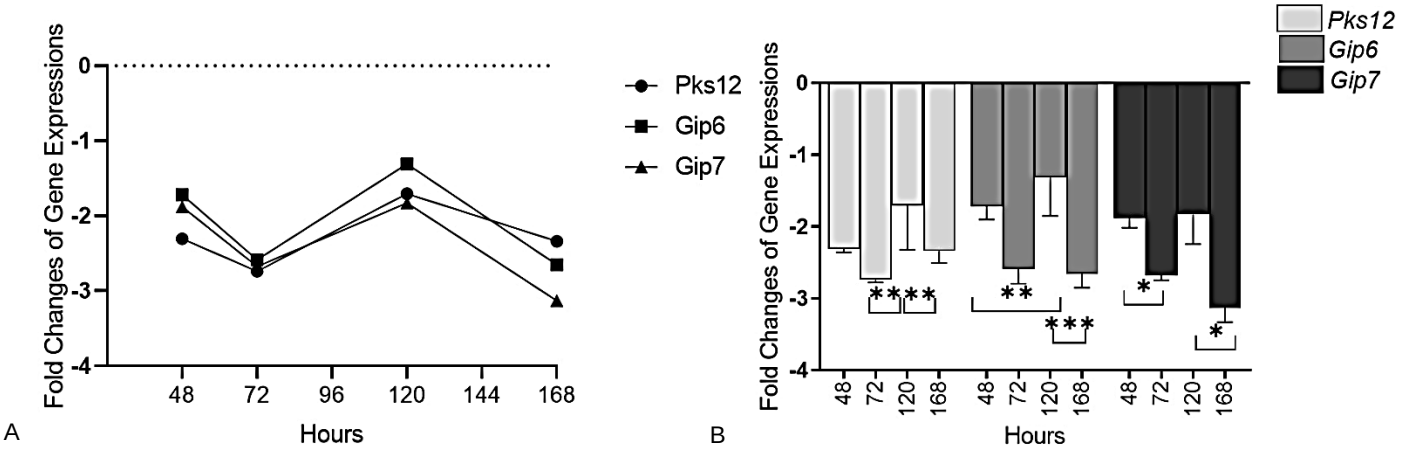

Figure 4. Relative expression patterns of the genes (A) and one-way ANOVA analysis of gene expressions of the *Pks12*, *Gip6* and *Gip7* genes, Bars represent standard errors; * p<0,5; **p<0,01; ***p<0,001 (B)

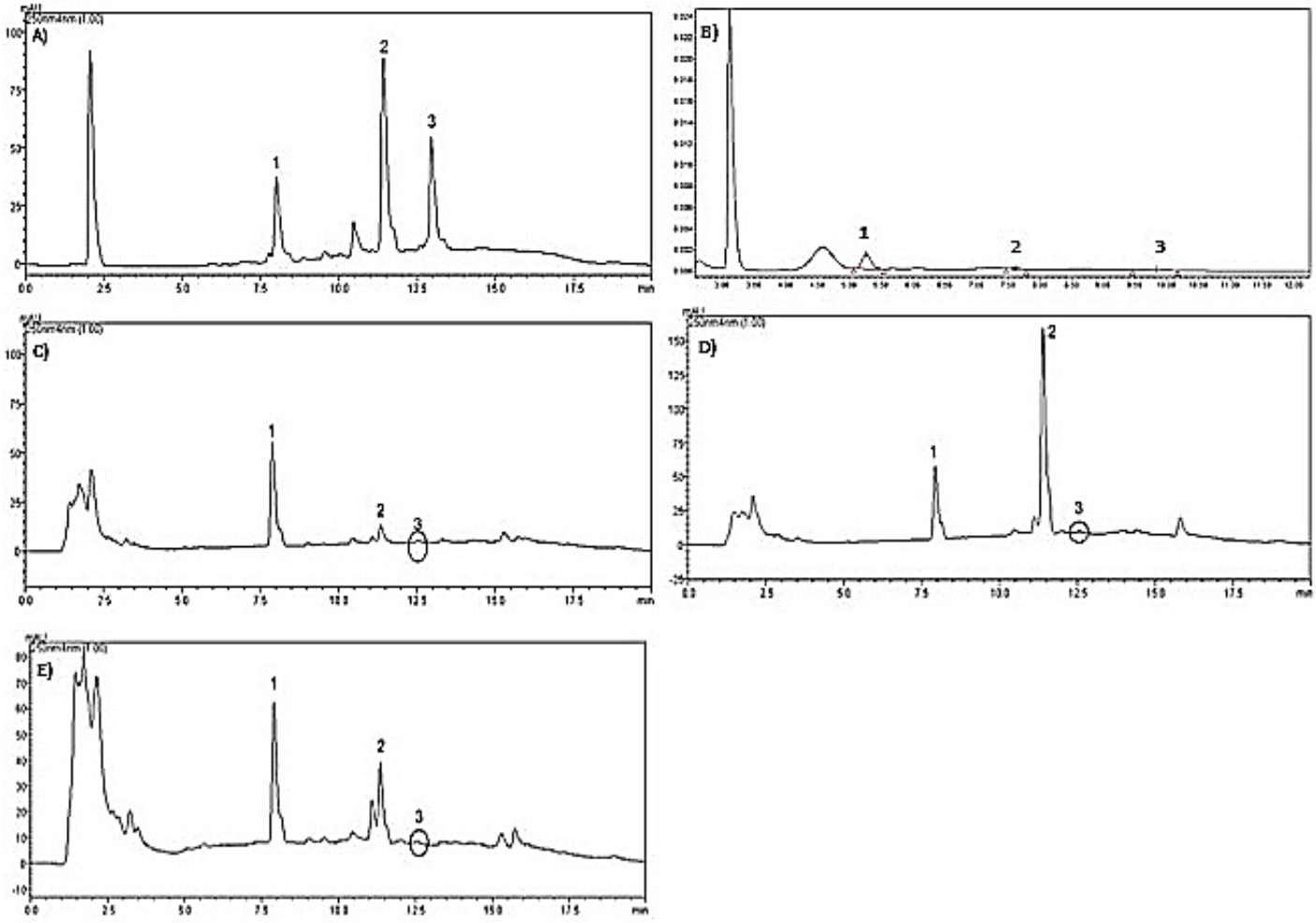

Figure 5. Chromatograms of standard mixture (A), 48th –hour culture (B), 72nd – hour culture (C), 120th –hour culture (D) and 168th –hour culture (E). Peaks were marked as 1. Quercetin I.S. (5 mg/L); 2.Aurofusarin (50 mg/L); 3.Rubrofusarin (5 mg/L)

DISCUSSION

Fusarium species synthesize aurofusarin and its precursor metabolites, which are responsible for mycelial pigmentation (yellow, tan, carmine red etc.) [4]. Their biosynthesis is carried out in intracellular and extracellular enzymatic steps. These enzymes are coded by eleven genes, which were analysed functionally via investigation of *F. graminearum* deletion mutants [3-6]. *Pks12*, *Gip6* and *Gip7*, encode enzymes which participate intracellular steps of aurofusarin production, was revealed as responsible genes for mycelium pigmentation via presented alterations in previous quelling studies [3-5, 9]. Although it was initially reported that *ΔPks12* strains were incapable of synthesizing aurofusarin and exhibited milky white pigmentation [4, 10], Jin and coauthors (2009) showed that *ΔPks12* were orange pigmented while the *ΔgzcaRA*/*Pks12* and *ΔgzcarB*/*Pks12* double mutants produced white pigments [9]. Frandsen and coauthors (2006) detected yellow/green pigmented mycelia in *ΔGip7* due to the accumulation of nor-rubrofusarin, a precursor of aurofusarin [3]. In another study, Frandsen and coauthors (2011) observed that the accumulation of another precursor, YWA1, resulted in brown/green pigmentation in *ΔGip6* mutant strains [6]. Interestingly, Cambaza and coauthors (2018) found that the mycelium of the same wild type isolate can exhibit different pigmentation profiles in the same culture conditions, changing from milky white to yellow to carmine red depending on growth phases during the cultivation [11]. Despite all these findings, our understanding of *Fusarium* pigmentation mechanisms remains limited. Up until this study, the relationship between mycelium pigmentation and expression profiles of aurofusarin biosynthetic genes had not been clearly established. In this current study, in order to understand the role of *Pks12*, *Gip6* and *Gip7* on the alteration of pigmentation patterns in *F. graminearum* and for the first time in *F. culmorum* reference strains, expression levels of these genes were investigated at different growth phases. Despite *F. culmorum* genome not having been annotated yet, based on the alignment analysis conducted with the *F. graminearum* PH-1 genome, it is proposed that

the aurofusarin gene cluster might also be responsible for pigmentation in *F. culmorum*. Oligonucleotide primers were designed according to the PH-1 aurofusarin gene cluster. These primers effectively amplified partial regions of the *Pks12*, *Gip6*, *Gip7* (1458, 593, 1401 bps long, respectively). Through the sequencing and alignment analysis of all these amplicons, the presence of SNPs and *in-del* type mutations in *F. culmorum* was revealed (Figure 3). In spite of such variations, high sequence similarities (Table 4) were determined between *F. culmorum* and *F. graminearum*, suggesting that the *Pks12*, *Gip6*, *Gip7* might be responsible for catalysing the intracellular reactions during aurofusarin biosynthesis in *F. culmorum* as well. Lind and coauthors (2017) reported that the synthesis of secondary metabolites in filamentous fungi may be affected by variations like SNPs [12]. However, this current study shows that because SNPs and in-del type mutations in *Pks12*, *Gip6* and *Gip7* do not cause frameshift or alter ORFs, such variations do not hinder the production of aurofusarin.

Through qPCR and HPLC analyses, the production of aurofusarin and rubrofusarin was verified at the 48th, 72nd, 120th and 168th hours. In milky white pigmented mycelium, gene expression analyses showed all genes to have been transcribed by the 48th hour mark, and the highest rubrofusarin amount was calculated at that time point as 13,443 ± 0,001 ppm while aurofusarin was calculated as 4,717 ± 0,001 ppm. At log phase, during which growth rates reached their highest at around the 72nd hours, the mycelium pigmentation turned yellow as rubrofusarin amounts reached 0,098 ± 0,001 ppm, and aurofusarin amounts 2,355 ± 0,001 ppm. By the 120th hour, the beginning of the stationary phase during which the mycelium pigmentation was observed to be carmine red, aurofusarin amounts had already increased to 4,742 ± 0,002 ppm and continued to gradually increase up to 27,350 ± 0,003 ppm.

Vujakovic and coauthors (2017) determined that in vitro cultures of different *Fusarium* species able to be carmine red pigmented as a result of *Pks12* expression, and a relationship between gene expression patterns and aurofusarin biosynthesis was suggested [13]. The present study revealed that in addition to *Pks12* gene expression, the *Gip6* and *Gip7* expression patterns effectively contributed to aurofusarin biosynthesis, and that the accumulation of aurofusarin was responsible for the carmine red pigmentation. The relatively decreased expression of the *Gip6* compared to the *Pks12* and *Gip7* at stationary phase explained why the concentration of nor-rubrofusarin in the cell was lower than that of rubrofusarin. It has been generally accepted that secondary metabolites are synthesized more at the stationary phase than at the log phase [14]. However, the qPCR results of this study showed the aurofusarin biosynthetic genes to have reached the highest expression levels in the log phase. Therefore, it was concluded that aurofusarin and rubrofusarin are also synthesized during the growth phase of *F. culmorum*, confirmed by HPLC data. As a precursor metabolite, rubrofusarin was detected more than aurofusarin in log phase. Throughout the stationary phase, more aurofusarin can be detected as rubrofusarin metabolites are dimerised.

In stationary phase, radial growth rate of the carmine red culture reached maximum value (9 cm). Cambaza and coauthors (2018) had previously reported that when the in vitro culture of *F. graminearum* reached its maximum diameter, mycelium turned carmine red and became darker [11]. It was showed with the current study that *F. culmorum* reached maximum growth rate faster than and went down to stationary phase earlier than *F. graminearum*. Growth dynamics in fungal cultures depend on many environmental factors such as time of nutrient exhaustion, reutilization of excreted metabolites and previous cultural conditions of inoculum [14]. Therefore, different *Fusarium* species can exhibit different in vitro growth rates, and in vitro growth might correlate with in vivo pathogenicity of *Fusarium* isolates [15]. The isolate used in this current study was purified from corn, and may have different virulence and aggressiveness compared to laboratory strains used in previous studies. This may lead to differences among the growth rates of the isolates.

Secondary metabolite biosynthesis and the gene expressions coding the enzymes responsible for the catalysis of biosynthetic steps can be affected by cellular and environmental factors, and can vary even at different growth phases in culture. Medentsev and coauthors (1993) associated the increasement of aurofusarin biosynthesis in the stationary phase of in vitro culture with excess oxidative stress and the inhibition of respiration caused by decreased nitrogen and phosphorus sources [2]. Gmoser and coauthors (2017) mention that pigment production in filamentous fungi increases in response to oxidative stress. Therefore, it should be considered that the increasement of *Pks12*, *Gip6* and *Gip7* expressions at the 120th hour and the increasement of aurofusarin levels towards the 168th hour might have been triggered by increasing oxidative stress due to the ageing of the culture [16].

Jung and coauthors (2006) noted an increase in the expression of *Pks4* and *Pks13* responsible for the synthesis of zearalenon (ZEA), a polyketide-structured mycotoxin. ZEA was synthesized in *ΔPks12* mutants, whereas no ZEA production occurred in the overexpression mutants of *Pks12,* which cause large quantities of aurofusarin synthesis. It was also reported that ZEA is synthesized at the log phase as aurofusarin is synthesized at the stationary phase antagonistically [17]. Jung and coauthors [17] (2006)'s data line up with

this study's findings that the expressions of *Pks12*, *Gip6* and *Gip7* decrease at the 72nd hour when rapid mycelium growth occurs, and that higher aurofusarin levels are detected at the 120th hour when the gene expression levels increase.

Because *Pks12*, *Gip6* and *Gip7* are regulated by the transcription factor encoded by *Gip2* of the same gene cluster [5], the expression levels of all three genes fall at the 72nd hour, are upregulated at the 120th hour, and fall back again at the 168th hour.

CONCLUSION

In conclusion, a positive correlation among gene expression levels, growth phases, and mycelium pigmentation profiles was thus established. The present study revealed for the first time that *F. culmorum* went into stationary phase earlier than *F. graminearum* upon reaching the maximum radial growth rate faster, and that *Pks12*, *Gip6* and *Gip7* are also responsible for the catalysis of intracellular reactions of the aurofusarin biosynthetic pathway in *F. culmorum*. Effects of the expressions of other genes found in this cluster on pigmentation should be investigated alongside other factors impacting pigmentation. Determination of aurofusarin and rubrofusarin concentration accumulated in mycelia during the growth phases provide useful knowledge for proper timing and environmental conditions of large-scale production of these pigment molecules.

Funding: This research was funded by THE SCIENTIFIC AND TECHNOLOGICAL RESEARCH COUNCIL OF TURKEY, grant number 216Z007.
Acknowledgments: The authors are grateful to Prof. Dr. Berna Tunali from the Department of Plant Protection, Agricultural Faculty, Ondokuz Mayis University, Samsun/Turkey, who purified the isolate previously.
Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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