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Morphological, Transcriptional, and Epigenetics Alterations due to L-arginine and L-methionine Treatment in *Fusarium culmorum*

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ABSTRACT

Fusarium culmorum is a worldwide phytopathogenic fungus of small-grain cereals. Struggling strategies such as fungicide treatment and biocontrol agent usage are not long-term solutions due to the potential adverse effects on ecological environment and resistance development in fungal pathogens. In this study, potential suppressive effects of amino acid supplementation on *F. culmorum* were investigated. Potato dextrose agar (PDA) medium amended with 1 mg mL^{-1} and 2 mg mL^{-1} concentrations of L-arginine and L-methionine were used as experimental sets. PDA with no supplement and PDA amended with nicotinamide of 1 mg mL^{-1} and 2 mg mL^{-1} concentrations were used as negative and positive control sets, respectively. While L-arginine treatment led to significant increase in linear growth rate (LGR) with $p<0.01$, L-methionine decreased LGR values ($p<0.001$). Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) essays yielded very similar alterations in terms of genomic template stability within the experiment groups of L-arginine and L-methionine treated sets. UPGMA-dendrogram (unweighted pair group method with arithmetic mean) revealed co-clustering of L-methionine and nicotinamide treated sets. Methylation-specific PCR (MSP) analysis showed that there was Type-II and Type-III methylation present in 2 mg mL^{-1} L-methionine treated sets. Gene expression analysis showed that L-methionine and L-arginine treatment led to contrast alteration in expressions of *tri6* and *FcStuA* genes with significant differences ($p<0.05$-$p<0.0001$). Our results showed that L-methionine treatment could suppress potential aggressiveness of *F. culmorum* at phenotypic, epigenetics, and transcriptional levels.

Keywords: *Fusarium culmorum*, Gene expression, CRED-RA, L-arginine, L-methionine.

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Introduction

Phytopathogenic fungi are responsible for various diseases of cereals, contamination of cereals by mycotoxins and associated risks to human and animal health [1,2]. Mycotoxins are natural secondary metabolites produced by various fungal species. These compounds are significantly different from most synthetic food contaminants. Their presence in food is almost inevitable and largely depends on climatic conditions that are difficult to control [2-4]. The only way to keep these substances in food at the lowest possible level is to observe good agricultural practices, ensure proper storage conditions, ensure crop diversity, use plant varieties resistant to fungal diseases, and apply fungicides [5–8]. Furthermore, fungus-infected seed grains are of lower quality and have lower yields, so the economic consequences must also be seriously considered.

Fusarium species are known worldwide as the causal agent of devastating diseases, especially in cereal crops of economic value (such as barley, wheat, maize, rice, rye, and oats), but they also infect a wide variety of plant species [9,10,8]. In Turkey and worldwide, *Fusarium* spp. infect cereal crops such as barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.), causing economic losses by causing head blight and crown rot. *F. culmorum* Schwabe is one of the most important agents of spike blight and root rot [9, 11–13]. *F. culmorum* produces different types of mycotoxins including deoxynivalenol (DON), acetylated derivatives of DON, zearalenone, and fusarins, and these phytopathogenic fungi have been reported to present moderate- and high-level genetic diversity among different populations worldwide. Since there is still failure in disease management and increased epidemics, struggling with diseases caused by *F. culmorum* (and also *F. graminearum*) has been still interest of plant pathologist for management of head blight and crown rot [13-18].

Fusarium species have been reported to cause increasing adverse effects over larger areas due to climate changes caused by global warming [19–21]. Thus, disease epidemics should be monitored every year and precautions must be taken. In this context, different disease management strategies have been carried out for head blight and crown rot diseases including fungicide management, biocontrol agent usage, and resistant plant cultivation [6,11,22,23]. However, fungicide resistance development, increased genomic plasticity, adverse effects on environment, high labor efforts, long periods, and some other reasons could yield with failure in management of head blight and crown rot in fields [7,11,24]. Considering this situation, it is almost inevitable

to use alternative chemicals or to develop new approaches to control spike blight and root rot diseases.

In recent years, researches have been increasingly focused on understanding the molecular mechanisms underlying *Fusarium* pathogenicity and toxin production to develop more targeted control strategies. *FcStuA*, an APSES-type transcription factor in the phytopathogenic fungus *F. culmorum*, plays a crucial role in regulating fungal development, virulence, and secondary metabolism. Functional analyses have demonstrated that *FcStuA* is essential for the proper formation of reproductive structures and conidia, and its deletion results in significant morphological abnormalities [25,26]. Moreover, *FcStuA* contributes to the biosynthesis of the trichothecene mycotoxin deoxynivalenol (DON) by facilitating the transcriptional activation of *tri6* via recruitment of the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex. The acetylation of *FcStuA* enhances its affinity for the *tri6* promoter, thereby promoting gene expression critical to mycotoxin production [27]. *FcStuA* regulates the expression of *tri6*, thereby indirectly controlling DON biosynthesis. This regulatory relationship is crucial for the pathogenicity of *F. culmorum* and its ability to produce trichothecene toxins *in planta*. Consequently, both *FcStuA* and *tri6* are considered key molecular targets in the development of strategies aimed at controlling Fusarium head blight (FHB) and reducing trichothecene contamination in cereal crops.

In this study, an alternative approach was investigated to combat the resistance of the *F. culmorum* pathogen to conventional agrochemicals by exploring epigenetic control strategies using adjuvant compounds on a reference strain of *F. culmorum*. Specifically, the study aimed to reveal potential suppressive effects of L-arginine and L-methionine amino acids on *F. culmorum* FcUK99 reference strain. The morphological, transcriptional, and epigenetics alterations by their treatment through various analytical approaches.

Materials and Methods

In vitro Growth Assays

Fusarium culmorum FcUK99 reference strain was kindly provided by Dr. Pierre Hellin (Walloon Agricultural Research Centre, Belgium). The fungal cultures were grown on the PDA (potato dextrose agar) media for 7 days at 28 ± 2°C. PDA medium amended with 1 mg mL^{-1} and 2 mg mL^{-1} concentrations of L-arginine and L-methionine were used as experimental sets. PDA with no supplement and PDA amended with nicotinamide of 1 mg mL^{-1} and 2 mg mL^{-1} concentrations were used as negative and positive control groups, respectively. control and experimental sets were named as follows due to PDA medium content: FCC (Control set), FCARG1(PDA-amended with 1 mg mL^{-1} L-arginine), FCARG2 (PDA-amended with 2 mg mL^{-1} L-arginine), FCMET1 (PDA-amended with 1 mg mL^{-1} L-methionine), FCMET2 (PDA-amended with 2 mg mL^{-1} L-methionine), FCNIC1 (PDA-amended with 1 mg mL^{-1} nicotinamide), and FCNIC2 (PDA-amended with 2 mg mL^{-1} nicotinamide). *In vitro* growth capacities of fungal cultures were analyzed by measuring the linear growth rate (LGR) on the 4th and 7th days. Each experiment set included at least three repeats. Statistical changes were tested using GraphPad Prism 9.0 software with confidence intervals of 0.05 and Dunnet's post-test.

Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) Assays

The genomic template stability (GTS) and genetic polymorphisms in L-arginine and L-methionine-treated *F. culmorum* were investigated via CRED-RA assay. First, genomic DNA (gDNA) was extracted from 7-day-old mycelia cultures. Then, diluted gDNA molecules were used in CRED-RA assays. A commercial kit (Anatolia Geneworks, Türkiye) was used to obtain gDNA. 50 mg fresh mycelium was homogenized using liquid nitrogen, a sterile pestle, and a sterile mortar. For binding, washing, and elution steps, the recommendations of the manufacturer were followed. 1.0% agarose gels and spectrophotometer (Thermo Fisher Scientific-Multiskan Go, U.S.A.) were used for the qualitative and quantitative gDNA analyses, respectively.

*Msp*I and *Hpa*II restriction enzymes were used to digest the gDNA of control and experimental sets. Digestion reactions were carried out in a volume of 50 µL including 10 U of digestion enzyme (Thermo Fisher Scientific, U.S.A.), 1X reaction buffer with 0.1X BSA, and 500 ng gDNA. The digestion reaction was performed by two-step incubation processes including 37C for 75 min and 80°C for 10 min. Non-digested gDNA of 50 ng μL^{-1} was used to form internal control set of CRED-RA. PCRs were carried out using common OPERON primers given in Table 1. The cycling conditions were as follows: Initial denaturation (94°C for 5 min), 45 cycles of three steps (1 min at 94°C, 1 min at 40°C, and 2 min at 72°C), and final elongation (72°C for 10 min). PCR bands were separated using 1.7% agarose gels and visualized with a U.V. transilluminator. Bands were scored as "0" or "1" as being related to their absence or presence on the experimental set. GTS, similarity matrix, and unweighted pair group method with arithmetic mean (UPGMA) dendrogram was obtained using MVSP software combined with Nei and Li's coefficient [28].

Methylation Specific PCR (MSAP)

Alteration in methylation on *FcStuA* and *tri6* genes which are related to asexual growth and trichothecene biosynthesis process were evaluated by MSA-PCR. Primers were designed in this study (Table 2). gDNA molecules which were non-digested (by any restriction enzyme), *Msp*I-digested, and *Hpa*II-digested were used in PCR assays. PCRs were conducted in a reaction volume of 25 µL containing 50 ng gDNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTP mix, 10 pmol of each primer, 0.04 U μL^{-1} *Taq* DNA polymerase (Nepenthe, Türkiye). The cycling conditions included initial denaturation at 94°C for 5 min, a loop of 35 cycles (94°C for 45 s, 61°C for 45 s, 72°C for 2

min), and final extension at 72°C for 5 min. PCR bands were separated on 1.7% agarose gels as described above.

Quantitative Real-Time PCR (qRT-PCR) Assays

tri6 (a zinc finger transcription factor) involved in DON biosynthesis and *FcStuA* (APSES protein) related to asexual growth, were used as target genes in qRT-PCR assays. *β-tubulin* was used as an endogenous gene (Table 2).

Sybr Green-I reagent, QuantStudio 5.0 (Applied BioSystems-Thermo F.S., U.S.A.), and $2^{-\Delta\Delta CT}$ formula [29] were used as dsDNA binding dye, thermal cycler, and fold change calculation formula, respectively. For this purpose, the total RNA was first extracted, and then the total RNA was converted to cDNA. cDNA was used in qRT-PCR assays.

Table 2. MSAP and qRT-PCR primers used in this study

Aim	Target gene	Primer set	5'-3 ' sequence (forward/ reverse)	Band size
MSAP	*FcStuA*	2SPANSTUA-F/R	ttccccaattgctagacctg / aaacacttcgcttgaccttga	2001 bp
MSAP	*tri6*	spantri6F/R	taccaatcgtgtcccctctc / cgccaaactcgtcatcattt	874 bp
qRT-PCR	*FcStuA*	stuartf/r	gcccctactggatacgatca / ttgccttctagggacattgg	100 bp
qRT-PCR	*tri6*	tri6qpcrf/r	acggtggattcaaccaagac / tggtactgcgtccaacagtc	117 bp

The total RNA was extracted from 7-day-old fungal mycelium of 50 mg. Mycelium was extracted by using liquid nitrogen and 0.8 mL of NucleoZOL compound (Macherey-Nagel, Germany). The recommendations of the manufacturer were followed to obtain total RNA. After the qualitative (1% agarose gel) and quantitative (spectrophotometer, Thermo Fisher Scientific Inc.) analyses of RNA, cDNA conversion (from 1000 ng total RNA) was performed by a commercial cDNA synthesis kit following the protocol of the manufacturer (Nepenthe, Türkiye). The reactions were conducted in a 12 µL volume containing cDNA amount corresponding to 50 ng total RNA, 5 pmol of each primer, and 1X SYBR Green Mix (Episozyme, Türkiye). The cycling conditions were as follows: the pre-denaturation at 95°C for 2 min, the amplification with 40 cycles of 95°C for 15 s, 57°C for 20 s, and 72°C for 20 s, and the melting curve temperature scan step. Each Cp and fold change was recorded for each gene and experimental set separately.

Results and Discussion

In vitro Growth Assays

Linear growth rate (LGR) values were recorded in *F. culmorum* FcUK99 strain treated with L-arginine, L-methionine, and nicotinamide with two concentrations sets of 1 mg mL^{-1} and 2 mg mL^{-1}. The control set grown on PDA medium yielded 11.55±0.14 mm/day LGR values. LGR values for FCARG1 and FCARG2 were recorded as 13.40±0.31 and 13.42±0.30, respectively. In contrast to increased LGR values, L-methionine treatment decreased growth rate with LGR values of 9.01±0.23 (FCMET1) and 7.04±0.23 (FCMET2). Similar results were obtained from nicotinamide treatment with 8.51±0.23 (FCNIC1) and 7.39±0.18 (FCMET2). All comparisons among control and experimental sets by Dunnett's post-hoc test yielded significant changes from $p<0.05$ to $p<0.0001$ (Figure 1). These findings led to knowledge about the contrary effects of L-arginine and L-methionine on the *in vitro* growth capacity of *Fusarium* spp. Our findings align with previous studies on fungal organisms, which indicate that L-arginine treatment may serve as a positive regulator of asexual and/or sexual growth while acting as a negative regulator of mycotoxin biosynthesis in fungi, including *Coniothyrium* spp., *Alternaria* spp., and *Fusarium* spp. [30–32]. Additionally, L-methionine's adverse effect on fungal growth, mycotoxin production, and plant-protective effect against fungal phytopathogenic species were present in previous studies.

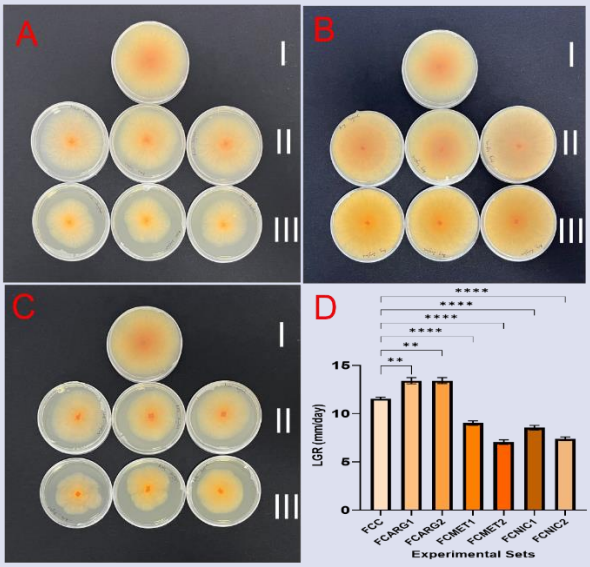

Figure 1 *in vitro* growth profile of L-methionine (A), L-arginine (B), and nicotinamide (C) treated *F. culmorum* FcUK99 strain on petri dishes (A, B, and C) and boxplot graphic (D). I, II, and III show 0 mg mL^{-1}, 1 mg mL^{-1}, and 2 mg mL^{-1} concentrations of supplements, respectively. "**" means significant changes with $p<0.01$ and "****" means $p<0.0001$.

Our study revealed similar data on decreased fungal growth [33,34]. However, further studies could include *in planta* analysis including plant protective effects of L-methionine for *Fusarium* spp. and its plant host.

CRED-RA Analysis

After checking the quality and the quantity of gDNA molecules, CRED-RA assays were carried out. In CRED-RA assays, 10 primers were used in PCRs. OPA3 and OPB9 yielded no amplicon in all experimental sets (Table 1).

Table 1. OPERON primers used in CRED-RA analysis

Primer	5'-3' sequence	GC%	Highest no of bands in any sample	Total band no	Polymorphic band no
OPM1	gttggtggct	60	21	25	1
OPM4	ggcggttgtc	60	12	14	0
OPM7	ccgtgactca	60	17	18	0
OPM8	tctgttcccc	60	6	6	1
OPB9	tgggggactc	60	0	0	0
OPB13	ttcccccgct	70	9	9	1
OPB14	tccgctctgg	70	11	11	1
OPG13	ctctccgcca	70	10	12	0
OPC5	gatgaccgcc	70	17	20	1
OPA3	agtcagccac	60	0	0	0

By excluding OPB9 from CRED-RA analysis, the amplicon numbers ranged from 6 (OPM8) to 25 (OPM1). Figure 2.A shows CRED-RA band profiling of OPG13 primer.

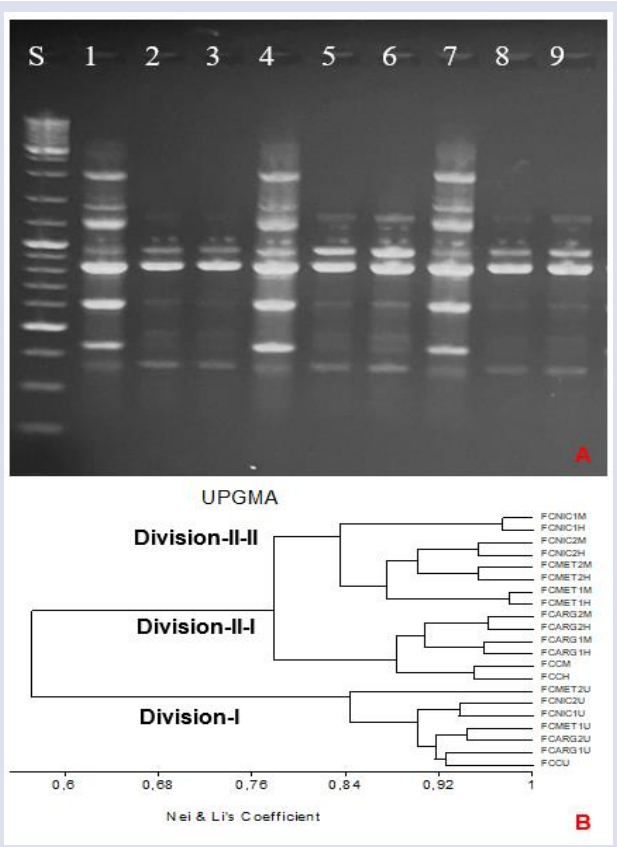

Figure 2. Agarose gel profiling of OPG13 primer (A) and UPGMA dendrogram of CRED-RA assay. U, M, and H means undigested, *Msp*I-digested, and *Hap*II-digested samples, respectively. S: 1kb plus DNA ladder (Thermo, U.S.A.). Samples from "1" to "9" mean FCCU, FCCH, FCCM, FCNIC1U, FCNIC1H, FCNIC1M, FCNIC2U, FCNIC2H, FCNIC2M, respectively. U, H, and M also mean "undigested", "*Hap*II-digested", and "*Msp*I-digested" samples.

The GTS values were recorded between 75.5% (FCMET2) and 100% (FCARG1). These findings were very consistent with the LGR analysis. L-arginine and L-methionine yielded contrasting GTS values. In comparison to previous studies, a relatively moderate level of genomic template stability level was present in this study. Prednisone and Nair reported that prednisone treatment yielded up to a 66.6% decrease in GTS for *F. oxysporum* [35]. Similar findings with more than 50% GTS decrease were reported from studies including biotic and abiotic stress factors against *Fusarium* spp. [36,37].

Genetic diversity analysis combined with similarity matrix and UPGMA dendrogram showed that digestion type is decisive for the distribution and clustering of experimental sets into sub-divisions. According to the similarity matrix, the minimum genetic similarity value was 46.5% for FCARG2U, and FCNIC2H. The maximum genetic similarity value was recorded as 98% for FCMET1H and FCMET1M (data not shown). The almost homogenous distribution of enzyme-digestion profiling for experimental sets was detected via the UPGMA dendrogram (Figure 2.B). All the undigested samples were co-clustered in division I, while a nearly homogenous distribution of *Hpa*II and *Msp*I digested samples were clustered in sub-divisions II-I and II-II. In comparison to previous studies including *Fusarium* spp., nearly perfect, consistent, and very clear clustering of *F. culmorum* experimental sets present in this study [32-35]. However, in comparison to those investigations, methylation changes were analyzed via MSAP in this study.

MSAP Analysis

FcStuA and *tri6* genes were amplified using the MSAP technique in undigested, *Hap*II-digested, and *Msp*I-digested *F. culmorum* FcUK99 strains. The *FcStuA* gene was amplified from each experimental set at approximately 2 kb (Figure 3).

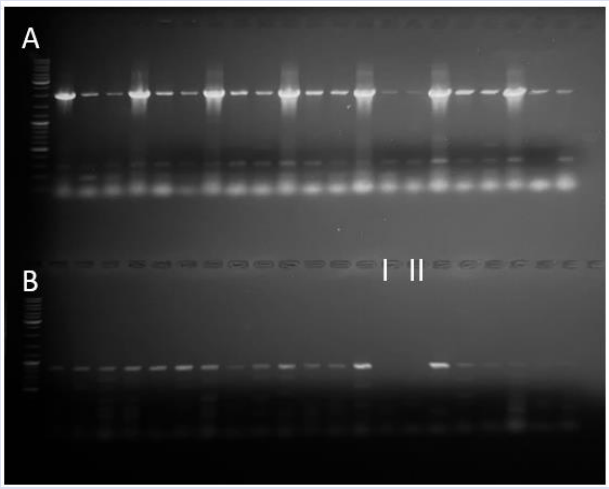

Figure 3. Agarose gel profiling of *FcStuA* (A) and *tri6* (B) MSAP assays. I: FCMET2H and II: FCMET2M. Samples were run on agarose gels with the same loading order as illustrated on Figure 2.

However, each set excluding FCMET2H and FCMET2M yielded amplicon for the *tri6* gene. This data is very important in terms of supporting our findings obtained

from LGR analysis. Increased concentrations of L-methionine resulted in an alteration in Type-II and Type-III methylation in the *F. culmorum* FcUK99 strain. However, no methylation alteration was detected for L-arginine-treated fungal samples. Type-II and Type-III methylation changes were detected in fungal genomes subjected to biotic or abiotic stress factors in previous studies[35–37]. Similar to the findings from a previous report of Albayrak et al. (2023), mycotoxin-related gene *tri5* showed Type-III methylation alteration in response to myrcene treatment [38]. It could be concluded that Type-II or Type-III methylation differentiation studies on fungal organism which are subjected to biotic and/or abiotic stress factors would be evaluated in terms of mycotoxin biosynthesis related genes.

Gene Expression Analysis

After quality and quantity control of total RNAs extracted from experimental sets, qRT-PCR analysis was performed. *tri6* and *FcStuA* expression were normalized to the expression of *β-tubulin* (Table 3). *FcStuA* expression resulted in significantly altered expression between control and experimental sets (Figure 4).

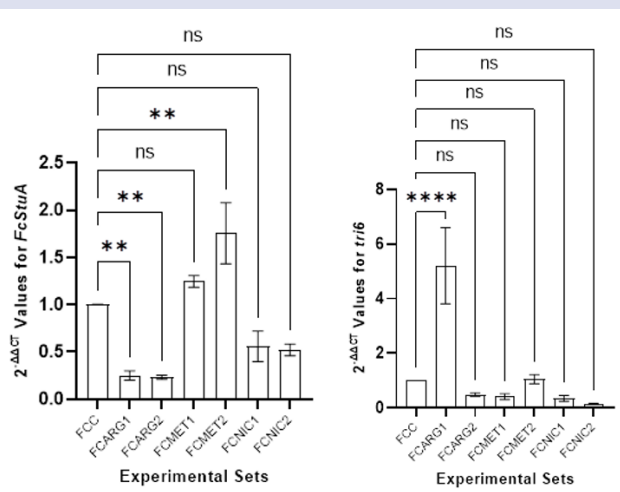

Figure 4. Fold changes in *FcStuA* (left-sided) and *tri6* (right-sided) gene expression levels in 7 experimental sets of *F. culmorum* treated with L-arginine, L-methionine, and nicotinamide. "ns" means "no significant changes-p>0.05", "**" means "significant changes with p<0.01", and "****" means "significant changes with p>0.0001".

The upregulation in *FcStuA* expression could be evaluated as a positive regulator for stress factor presence in *Fusarium* spp [39,40]. Our findings revealed that L-methionine treatment led to increased expression of *FcStuA* gene in *F. culmorum* FcUK99 strain. In concordance with LGR data, it can be concluded that FcStuA, as a transcription factor, plays a crucial role in regulating responses to environmental changes and stress factors. Additionally, tri6, a zinc finger transcription factor involved in regulating DON biosynthesis, exhibited contrasting expression profiles between the FCARG and FCMET groups.. As a result of gene expression analysis for *tri6* toxin gene, it was determined that *tri6* expression level decreased with increasing concentrations of L-arginine, while the *tri6* expression level increased with increasing concentrations of L-methionine. It seems that 1 mg mL^{-1} of L-methionine treatment *in vitro* and/or *in vivo* would be enough to decrease the DON biosynthesis. This concentration could be accepted as a relatively low or moderate level of potential antifungal treatment in comparison to previous investigations [38,41–43].

Conclusion

F. culmorum reduces the quality and yield of the product in the cereal economy. In an effort to prevent this situation, the number of studies carried out to increase the quality and yield of the product is increasing. In this study, it was observed that L-arginine amino acid had a growth promoting effect on *F. culmorum*, while L-methionine had a growth suppressing effect. The potential antifungal effect of L-methionine treatment has been confirmed at morphological, epigenetic, and transcriptional levels with scientifically significant data. However, *in planta* studies are needed to confirm the usefulness of L-methionine supplements or combinations in the control of phytopathogenic organisms. Studies conducted on wheat and barley plants have demonstrated that the amino acids L-arginine and L-methionine can enhance plant growth and physiological responses under abiotic stress conditions such as drought. These amino acids appear to support the stress response mechanisms of plants, thereby exhibiting potential to improve agricultural productivity.

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Conflict of Interest

The authors declare that they have no conflicts of interest for this work.

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