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Investigation of the Effects of Eugenol on *Fusarium culmorum*[*]

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ABSTRACT: *Fusarium culmorum* is one of the causal agents lead to economic loses in small grain cereals. In this study, alterations in spore production, and linear growth rates in *F. culmorum* 15 isolate subjected to increased concentrations of eugenol (0, 200, 400, 800 µg mL-1) were examined. Additionally, the and expression of *FcMgv1* and *FcStuA* genes which are essential in asexual stage and cell wall structure formation were also examined tested in *F. culmorum* eugenol treated and non-treated fungal cultures. F15 isolate subjected to increased concentrations of eugenol (0, 200, 400, 800 µg mL-1). Minimum inhibitory concentration was determined as 400 µg mL-1 eugenol. In comparison of control and experiment sets, there were significantly different ($p<0.01$) the decrease in spore production and linear growth rate (LGR) were significantly different ($p<0.01$). In real time polymerase chain reaction (qPCR) analysis, β-tubulin was used as endogenous control and the expression of *FcMgv1* and *FcStuA* genes were determined by using cDNAs converted from total RNAs of control and experiment sets were used in Eva-Green fluorophore dyebased real time PCR to examine the *FcMgv1* and *FcStuA* expression. Significant differences ($p<0.05$) were also determined in fold changes in gene expression. Normalization results showed that fold changes in *FcMgv1* and *FcStuA* genes were as +4.35±0.25 and +2.04±0.13, respectively. qPCR results were also confirmed via reverse transcription PCR (RT-PCR) analysis. The late apoptosis was detected in the cells by using acridine orange/ethidium bromide staining. Findings obtained from this study showed that eugenol have the potential antifungal effects and it could be used in struggling with head blight disease caused by *F. culmorum* via its *in vitro* antifungal effects. This study presents the original data on about is first report to evaluatinge the phenotypic and transcriptomic effects of pure eugenol compound on *F. culmorum*.

Key words: Antifungal, *FcStua, FcMgv1*, Linear growth rate, qPCR

Eugenol'ün *Fusarium culmorum* Üzerindeki Etkilerinin İncelenmesi

ÖZET: *Fusarium culmorum* küçük daneli tahıllarda ekonomik kayıplara yol açan hastalık etmenlerinden birisidir. Bu çalışmada, arttırılmış eugenol konsantrasyonlarına (0, 200, 400, 800 µg mL^{-1}) maruz bırakılmış *F. culmorum* F15 izolatında spor üretimi ve doğrusal büyüme oranı belirlendi. İlaveten, eugenol uygulanmış ve uygulanmamış fungal kültürlerde aseksüel üreme ve hücre çeperi yapısının oluşumunda elzem olan *FcMgv1* ve *FcStuA* genlerinin anlatımı da test edildi. Üremeyi engelleyen minimum konsantrasyon 400 µg mL^{-1} eugenol olarak belirlendi. Kontrol ve deney setleri karşılaştırıldığında, spor üretimi ve doğrusal büyüme oranında bilimsel olarak anlamlı azalma belirlendi ($p<0.01$). Gerçek zamanlı polimeraz zincir reaksiyonu (qPCR) analizlerinde, β-tubulin endojen kontrol olarak kullanıldı ve *FcMgv1* ve *FcStuA* genlerinin anlatımı Eva-Green florofor boyası kullanılarak saptandı. Gen anlatımındaki oransal değişimlerde bilimsel olarak anlamlı farklılıklar ($p<0.01$) saptandı. Normalizasyon bulguları *FcMgv1* ve *FcStuA* genlerindeki oransal değişimlerin sırasıyla +4.35±0.25 and +2.04±0.13 olduğunu gösterdi. qPCR bulguları ayrıca ters transkripsiyon PCR (RT-PCR) analizleri ile doğrulandı. Akridin orange/Etidyum bromür dual boyamasında hücrelerde geç apoptoza rastlanmıştır. Çalışmadan elde edilen bulgular eugenolün potansiyel antifungal etkilere sahip olduğunu ve *F. culmorum*'un neden olduğu başak yanıklığı ile mücadelede kullanılabileceğini göstermiştir. Bu çalışma saf eugenol bileşiğinin *F. culmorum* üzerindeki fenotipik ve transkriptomik etkilerinin değerlendirilmesini açısından özgün bulgular sunmaktadır.

Anahtar Sözcükler: Antifungal, Doğrusal büyüme oranı, *FcStua, FcMgv1*, qPCR

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INTRODUCTION

Fusarium culmorum is major phytopathogenic species causing head blight and crown rot diseases worldwide. Epidemics result in severe yield loses and mycotoxin contamination of cereals and associated food products (Miedaner et al., 2008). The fungus was most frequently isolated from geographic regions with cooler climatic conditions (Pasquali and Migheli, 2014). This anomorphic species has ability to produce different hazardous myxotoxins including, nivalenol (NIV), deoxynivalenol (DON), 3-acetylated-deoxynilvalenol (3-AcDON) and zearalenon. The high level of phenotypic and genotypic variation among the *F. culmorum* populations of different geographic regions were present (Miedaner et al., 2008; Pasquali and Migheli, 2014). Moreover, co-presence of two mating type alleles and parasexual stage were also reported (Miedaner et al., 2008; Obanor et al., 2010). Thus, struggle with this quarantened species is obligatory for anywhere this phytopathogenic species is present in fields.

Management of *F. culmorum* diseases includes several critical stages including characterization the genetic structure of fungal pathogen populations, planting and development of cultivars with useful agronomic traits and approaches reducing fungal biomass in fields. Getting knowledge on genome is essential part of disease management. Preliminary output data associated with 3-AcDON producer *F. culmorum* FcUK99 strain is currently relased on databases (Scherm et al., 2011). Besides, hundreds of nucleotide sequences records for genes associated with mycotoxin production and pathogenicity have been formerly released NCBI database. In this manner, detailed analysis on genotype and chemotype diversity of *F. culmorum* isolates from different regions have been investigated (Pasquali and Migheli, 2014). However, the studies for getting knowledge on variation in genetic structures of *F. culmorum* populations is still limited. Additionally, the usage of genetically modified plants or naturally pathogen resistant cultivar and elite cereal lines (eg. *Triticum aestivum* L. cv. Sumai-3, *Hordeum vulgare* L. cv. Golden Promise) have importance in fight with *F. culmorum* diseases. However, these approaches have several disadvetages. These plants have restricted agronomic traits. The techniques and methods followed for *in vitro* and field treatments are time consuming (Anand et al., 2003; Bai and Shaner, 2004; Bernardo et al., 2007). The unexperienced and/or novel compounds which could have potential antifungal aganist *F. culmorum* usage could be another strategy. Thiophanate methyl, carbendazim and some other compound are known with antifungal effects with different levels against *Fusarium* isolates worldwide (Arslan ve Baykal, 2002; Bernardo et al., 2007; Chung et al., 2008). However, resistance to common antifungals, in particular demethylation inhibitors, have been reported worldwide (Arif et al., 2009). Thus, different antifungal compounds investigations would be useful in disease management.

Testing antifungal effects of novel compounds includes several different approaches such as macroconidia quantity, linear growth rate measurements, oxidative stress and apoptosis investigations, transcripts and metabolit alteration analysis. In this study, the effects of pure eugenol compound on *F. culmorum* 15 isolates obtained from diseased wheat kernels from Turkey was tested via phenotypic and gene expression analysis. Eugenol, with molecular formula of $C_{10}H_{12}O_2$, is chemical compound belonging to phenylpropanoid class. It has been extracted from several kind of plant species. It is known to be antifungal effects of several fungal species (Campaniello et al., 2010; Castillo et al., 2012). Even if the spore reproduction and growth rate effects of several plant essential oils extracts including eugenol are known for several fungi, effects of pure eugenol compound on *F. culmorum* is not investigated at viability associated gene expression level. Thus, in this study, it was aimed to investigate effects of pure eugenol compound on *F. culmorum* at phenotypic including spore production and linear growth rate analysis. Moreover, changes in expression of *FcMgv1* and *FcStuA* genes essential in several significant biological process such as sexual and/or asexual reproduction, cell wall stability and pathogenicity were also examined.

MATERIALS AND METHODS

Fungal Material and Phenotypic Tests

F. culmorum F15 isolate was provided from Prof. Dr. Berna Tunali, Samsun Ondokuz Mayıs University, Plant Protection Department, Agricultural Faculty. The fungal control sets were grown on potato dextrose agar (PDA: Hi-Media, India). *F. culmorum* F15 isolate was obtained from disesed wheat spike in the year

2006. The geographic origin and class B-trichothecene chemotype of the isolate were Boyabat-Sinop and 3-acetyldeoxynivalenol, respectively. *F. culmorum* F15 isolate was identified at species level by standard morphological and genetic tests previously (Yörük et al., 2016). Experimental groups were grown on media amended with increasing concentrations (0, 200, 400, 800 µg mL^{-1}) Eugenol (Sigma, U.S.A.) amended in PDA. *in vitro* growth conditions were maintained at 28°C for 7 days. The mycelial culture plugs of 0.25 cm^2 were used as a starting material. In this study, the agar dilution technique by using was used to determine the minimum inhibitory concentration (MIC) and inhibitory concentration 50% (IC_{50}). Linear growth rates (LGR) were calculated as mm day^{-1} at 4th and 7th days of incubation. In spore quantification tests, potato dextrose broth (PDB) and PDB amended with 200, and 400 µg mL^{-1} eugenol were used for control and experiment sets, respectively.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from seven days old culture using Tri-Reagent (Sigma, U.S.A.). 5-10 mg of mycelium was homogenized with 0.5 mL Tri-Reagent and then the manufacturer's protocol was followed. The obtained total RNA molecules were analyzed by visualization with 1% agarose gel electrophoresis under UV light. Quantitative analyzes of total RNA were measured using GelQuant Software (Biochemlabsolutions, A.B.D.) and spectrophotometer (Shimadzu, Japan).

Total RNA molecules were converterd to cDNA. cDNA molecules were obtained with two-step kit (Applied Biosystems, England) and then used in the qPCR (quantitative polymerase chain reaction; real time PCR) and RT-PCR (reverse transcription PCR) assays. cDNA synthesis was performed using 2µg RNA as the starting amount for all samples. Manufacturer's provided protocol was followed and cDNA molecules were diluted as ¼ for gene expression analysis.

Gene Expression Assays

qPCR and RT-PCR were used in expression analysis of the *FcMgv1* and *FcStuA* genes. β-tubulin gene was used as housekeeping gene. RT-PCR assay was carried out in a volume of 25µL including 1X PCR buffer, 0.25 mM dNTP mix, 10 pmol of each primer (Table 1), 2.5 mM $MgCl_2$, 5 µL of cDNA equivalent to 2 µg of RNA and 1 U *Taq* DNA polymerase enzyme (Thermo, U.S.A.). RT-PCR amplification was performed by pre-denautration at 94°C for 5 min, followed by 45 cycles of amplification at 94 °C for 20 s, 59 °C for 20 s, 72 °C for 40 s and final extension at 72 °C for 5 min. Amplification products were separeted with eletrophoresis on %1.7 agarose gel.

Table 1. Primers used in this study

Primer	Primer Sequence (5'-3')	Band Size (bp)
betaF/betaR	agggtcattacaccgagggt / gtaccaccaccaagagagtgg	121
MgvRTF/MgvRTR	aggttcaacgattccgacag / gaccattaccctgaggcaga	100
StuARTF/StuARTR	gcccctactggatacgatca / ttgccttctagggacattgg	100

In qPCR assays, target gene expressions were normalized according to β-tubulin expression. The Cp, ΔCT and ΔΔCT data were used for the analysis of the fold changes. LightCycler 480 II (Roche, Swiss) system was used in Eva Green I (Biorad, France) fluorescent dye accompanied assays. qPCR assay was carried out in a reaction volume of 20µL containing 1X Eva Green mix, 5 pmol each primer and amount of cDNA equivalent to 2 µg RNA. Cycling conditions were at 95ºC for 10 min, follwed as 45 cycles of 95ºC for 20 s, 59 ºC for 20 s, 72ºC for 40 s and cooling step at 40ºC for 30 s. Melting curve analysis and standard series consisting of 4 logarithmic phases, demonstrating qPCR activity and accuracy, were also maintained. Gene expression profiles were obtained according to $2^{-\Delta\Delta CT}$ normalization values (Livak and Schmittgen, 2001).

Experiments were replicated at least two times. Statistical analyzes were performed via One Way ANOVA Analysis with Tukey's post-hoc test (GraphPad Prism 5.0, USA).

Acridine orange/ethidium bromide (AO/EB) fluorescent staining

Effect of eugenol on *F. culmorum* F15 isolate was examined and displayed via fluorescent microscopy using AO/EB approval. *F. culmorum* F15 isolate cultivated on potato dextrose broth supplemented with Eugenol (IC_{50} concentration).

Cultures were developed using shaker at 28°C for 120 rpm. At the end of the 7 days the fungal culteres were centrifuged at 14.000 rpm for 3 minutes. The cells were washed 2 times with 1.5 ml 1X PBS. AO/EB (60µg mL^{-1} / 100µg mL^{-1}) mixture was used 5 µL for $2x10^5$ cells and incubated at 25°C for 5 minutes. After staining, the cells were washed and solved with 1X PBS.

Microscope imaging was managed with fluorescence microscope (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

F. culmorum F15 isolate showed mycelial growth (Figure 1.A) and spore reproduction in PDA/PDB media supplied with eugenol concentrations of 0, 200, 400 µg mL^{-1}. MIC concentration was determined to be 800 µg mL^{-1} eugenol. So, the IC_{50} concentration was calculated as 400 µg mL^{-1} and this set of experiments was used in gene expression studies.

There was a significant difference in LGR between experimental and control sets ($p<0.01$). LGR values were 9.64±0.39 in the control group whereas 4.73±1.21 and 4.29±0.58 for experiment sets (Figure 1.B). It was observed that the increased concentrations of eugenol resulted in decreased LGR values. Similarly, spore production was significantly decreased (up to 2.6 fold decrase, $p<0.05$) at increased concentrations of eugenol (Figure 1.C).

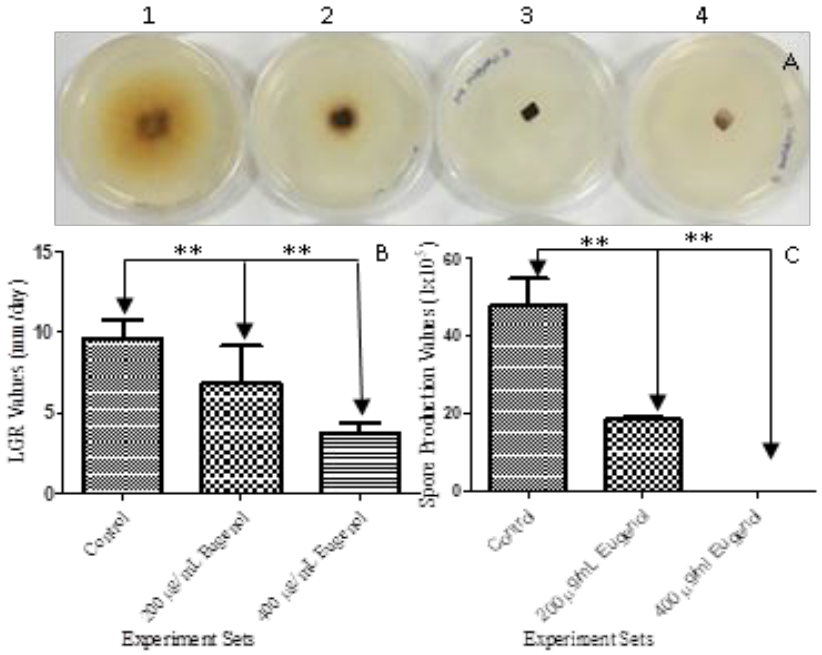

Figure 1. MIC test profile (A), LGR values (B) and spore reproduction values (C) of *F. culmorum* F15 isolate. 1, 2, 3 and 4 mean 0, 200, 400 and 800 µg mL^{-1} eugenol amended PDA media, respectively

Total RNA molecules of high quality ($\Delta 260\times 280^{-1}$ = 1.9-2.0) and amount (0.5-2 µg μL^{-1}) were obtained. cDNA molecules were converted from total RNAs and then used in RT-PCR and qPCR. In RT-PCR analysis, agarose gel electrophoresis yielded 121 bp fragment of the housekeeping gene and 100 bp of amplification product of the *FcMgv1* and *FcStuA* genes (data not shown). In qPCR analysis, mean E value was calculated as 2.2±0.06. The mean melting score for was caluculated as 0.92±0.009. Both E value and also melting scores showed that qPCR analysis were performed efficiently. ΔCp values for control and experiment sets of *FcMgv1/FcStuA* genes were calculated as 5.44±0.09/7.49±0.06 and 3.23±0.13/6.47±0.15, respectively. According to normalization results; expression level of *FcMgv1* and *FcStuA* genes were altered as +4.35±0.25 ($p<0.01$) and +2.04±0.13 ($p<0.01$) fold changes (Figure 2). These changes were found as significantly different in statistical analysis ($p<0.01$).

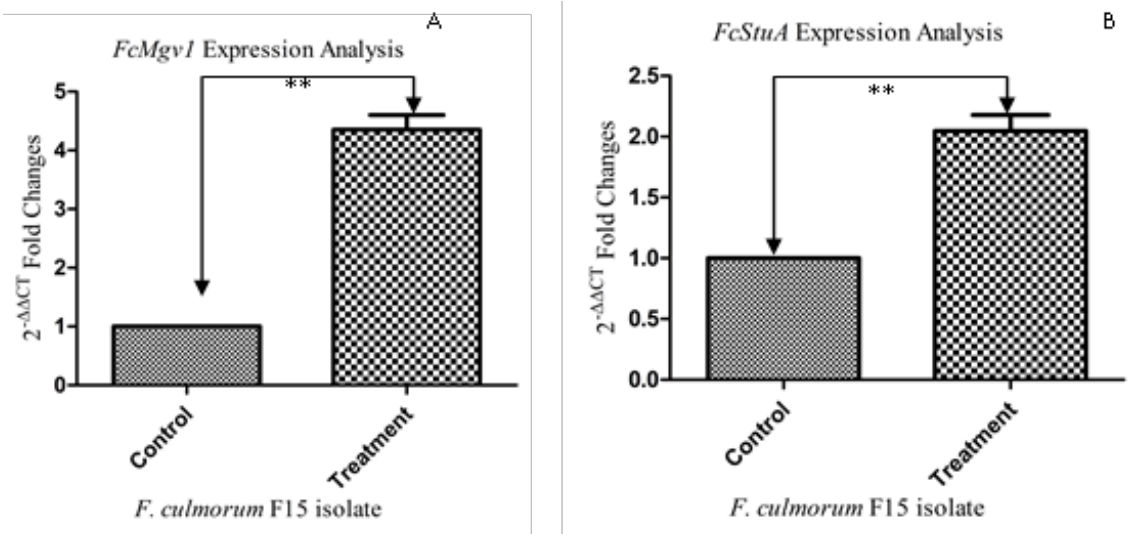

Figure 2. Fold changes in *FcMgv1* **(A)** and *FcStuA* **(B)** expressions

The effects of eugenol compound on phenotype and gene expression alterations have been determined in this study. The IC_{50} value 7-day-old cultures was determined as 400 µg mL^{-1} of eugenol and this results showed that Turkish *F. culmorum* F15 isolate could be accepted as relatively resistant strain when compared to common antifungal resistance levels (Chung et al., 2008; Arif et al., 2009). Nevertheless, incereased concentrations of eugenol led to scientifically significant decrease in the spore production and LGR values meaning that this compound could be evaluated as potential antifungal for *Fusaria* sp. Additionally, investigation the effects of this compound on plants could be useful for field treatments. Gene expression analysis supported the phenotypic investigations. Experiment set with IC_{50} value (400 µg mL^{-1} of eugenol) was investigated in qPCR and RT-PCR analysis revealing the expression of *FcMgv1* and *FcStuA* genes, responsible for cell wall integrity and sexual stage. In comparison to control groups, 3.23-7.49 fold increases in experiment sets were detected. These results showed that this compound could be evaluated as a potential abiotic stress factor for *F. culmorum*. In addition to LGR, macroconidia reproduction and qPCR assays, presence of potential apoptotic bodies was investigated via AO/EB staining. AO/EB stain nuclei green in healthy cells whereas EB penetrates cells which lost their integrity of cytoplasmic membrane and stains nuclei as orange-red in late apoptotic cells (McGahon et al., 1995). Figure 3 (A, B) shows that the both healthy and also red-orange stained nuclei and macroconidia were present in 7-day-old cultures treated with eugenol. No AOEB stained, green healthy cells were present in controls sets. Findins showed that eugenol could induce apoptosis in *F. culmorum*. In further studies, it is suggested that this agent with relatively high concentrations could be serve as a novel approach in disease control strategies in scientific works and field treatments.

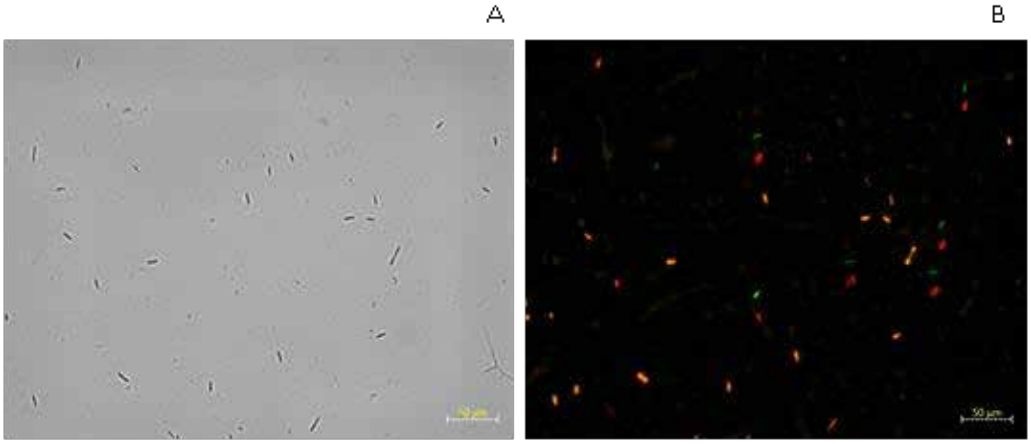

Figure 3. (**A**) Day-light and (**B**) GFP/AO/EB filter capturized profile of cells treated with eugenol

The potential antifungal effects of pure eugenol compound which was not formerly experienced on *F. culmorum* was investigated at phenotypic and transcriptomic levels in this study. Even if relatively high concentrations of eugenol is need for *F. culmorum* growth inhibition, the IC_{50} and MIC values for eugenol reported in this study has been involved in the IC_{50} and MIC value ranges for sensitive genotypes for common antigungals (Chung et al., 2008; Arif et al., 2009). The dermatophyte isolates of *Trichophyton rubrum* showed 256 µg mL^{-1} eugenol MIC value (Pereira et al., 2013). The MIC value for *Candida albicans* and *Aspergillus flavus* were recorded as 500 and 337.5 µg mL^{-1} eugenol (Braga et al., 2007; Trajano et al., 2012). It was shown that relatively high concentrations of eugenol inhibited the sexual and/or asexual reproduction via *in vitro* tests. Similarly, in this study, the increased concentration of eugenol significantly decreased the spore reproduction and linear growth rate gradually. It means that controlled usage of eugenol in fields could be useful in getting disease epidemics under control. At transcriptomic level, experiment sets of IC_{50} eugenol subjected showed significant alteration in *FcMgv1* and *FcStuA* genes whose expression are essential in fungal development and survival. Similarly, the upregulation of genes associated with cell viability and cell wall integrity due to several antimicrobial chemicals have been also reported for other fungal species and all these findings are important for plant pathology research area (Sanglard et al., 1995; Karababa et al., 2004; Sefer-Yörük et al., 2017). However, further studies on antifungal effects of eugenol would include higher number of fungal samples, belonging to teleomorphic and/or anomorphic species, from different regions worldwide and investigation the mycotoxin gene expression alterations. Findings obtained from current study are important in terms of presenting preliminary output for investigation antifungal effects of eugenol on head blight and root rot disease pathogens.

CONCLUSION

The struggle with *F. culmorum* includes several kinds of approaches. The usage of naturally disease resistant cultivars or genetically modified plants, antogonistic interactions and fungucide application are the most popular disease management strategies. Restricted agronomic traits, relatively low success, time-intensivity need and resistance development in fungi are some important disadventages of these approaches. Novel combinations and solutions are needed to be developed and used. In particular, usage of different and specific chemical compounds on phytopathogens provides efficient strategy in comparison to remaining approaches. In this study, effects of eugenol is investigated on *F. culmorum*. Findings showed that usage of this compound could decrease *in vitro* growth, mycotoxin biosynthesis and lead to apoptosis. Further studies could involve *in vitro* comibined usage of eugenol with some other major plant essential oils compounds, usage against some other necrotrophic and biotrophic pathogens and *in planta* usage.

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