ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CHARACTERIZATION OF PATHOGENICITY TRAITS AND TRICHOTHECENE PRODUCTION IN THE WHEAT PATHOGEN *Fusarium culmorum*

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BUĞDAY PATOJENİ *Fusarium culmorum*'UN PATOJENİK ÖZELLİKLERİ VE TRİKOTESEN ÜRETİMİNİN KARAKTERİZASYONU

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MAYIS 2014

Naz Kanıt, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID 521121146, successfully defended the **thesis** entitled "**Characterization of Pathogenicity Traits and Trichothecene Production in the Wheat Pathogen *Fusarium culmorum*** ", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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May 2014

Naz KANIT
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ABBREVIATIONS

β-CD	**:** Beta cyclodextrin
cDNA	**:** Complementary deoxyribo nucleic acid
CFR	**:** Crown and Foot Rot
cm	**:** centimetre
CMC	**:** Carboxy Methyl Cellulose
C_T	**:** Threshold Cycle
DON	**:** Deoxynivalenol
et al	**:** And others
FHB	**:** *Fusarium* Head Blight
FRR	**:** Foot and Root Rot
FUS	**:** Fusarenon X
g	**:** gram
h	**:**hour
L	**:** liter
M	**:** molar
mg	**:** milligram
mL	**:** milliliter
mm	**:** millimetre
mM	**:** millimolar
µg	**:** microgram
µL	**:** microliter
µM	**:** micromolar
ng	**:** nanogram
nm	**:** nanometre
NIV	**:** Nivalenol
OD	**:** Optical Density
PCR	**:** Polymerase Chain Reaction
PDA	**:** Potato Dextrose Agar
ppm	**:** Part per million
Q-RT PCR	**:** Quantitative Real Time Polymerase Chain Reaction
RNA	**:** Ribo nucleic acid
SDS	**:** Sodium dodecyl sulfate
3-ADON	**:** 3-acetylated deoxynivalenol
15-ADON	**:** 15-acetylated deoxynivalenol

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CHARACTERIZATION OF PATHOGENICITY TRAITS AND TRICHOTHECENE PRODUCTION IN THE WHEAT PATHOGEN *Fusarium culmorum*

SUMMARY

Fusarium culmorum is a soil-borne plant pathogen and it is a very important factor in the development of diseases *Fusarium* Head Blight (FHB), Foot and Root Rot (FRR) and Crown and Foot Rot (CFR) in small grain cereals such as wheat and maize, leading to important yield losses. During infection, *F. culmorum* produces a wide range of mycotoxins, such as trichothecenes, which accumulate on the plant. These mycotoxins can also cause serious diseases in humans and animals when consumed alongside the cereals. Even though *F. culmorum* is an important concern worldwide, the whole genome of this fungus has not been sequenced yet. There are several possibilities to prevent fungal growth and mycotoxin production on plant. It was previously shown that application of phenolic compounds on the plants leads to less mycotoxin production, even though the mechanisms are unknown.

The aim of this thesis is to investigate the mechanisms of pathogenicity of *F. culmorum* and investigate the effects of several phenolic compounds on mycotoxin production of the fungus.

Firstly, three genes with unknown function; *FGSG_08817*, *FGSG_16572* and *FGSG_16588* were investigated through previously obtained knock-out mutants. These mutants were studied under cell wall, osmotic, oxidative and thermal stress conditions and it was shown that tested genes were not related to mechanisms which the cells require to cope with these stresses. *In vitro* pathogenicity tests were also carried out for these strains and it was established that these genes do not affect the pathogenicity of the fungus.

Additional experiments were carried out where the fungus was grown in Liquid Vogel's medium in the presence of phenolic compounds ferulic acid (0.25mM) and ferulic acid dimer (0.25mM), as well as four other phenolic compounds referred to as compound 1 (0.25mM), compound 2 (0.25mM), compound 3 (0.25mM) and compound 4 (0.25mM, 0.10mM, 0.05mM). All media were prepared with beta-cyclodextrin (3mM) to enhance solubility. Gene expression levels of five day old mycelia were tested by Quantitative Real Time PCR. Genes regarding trichothecene production pathway; *TRI5, TRI4, TRI11, TRI3, TRI13, TRI6, TRI10* and *FPP* were tested with *18S* gene as the reference. Fourteen day old mycelia were separated from the liquid media, dried and weighed; whereas the liquid portions were tested by liquid chromatography-mass spectrometry (LC-MS) method to establish the amount of toxins produced by the fungus. It was observed that the presence of ferulic acid and compound 2 leads to downregulation of trichothecene pathway genes and toxin content. Ferulic acid dimer led to downregulation of tested genes, but the toxin production increased. Compound 1 led to increase in both gene expression and toxin

levels. Compounds 3 and 4 both led to downregulation of the genes and inhibition of toxin production. For three concentrations tested in compound 4, it was found that 0.25mM and 10mM concentrations were sufficient to downregulate gene expression levels, whereas 0.05mM of the compound was not sufficient to decrease them. However, all three concentrations of compound 4 led to inhibition of toxin production, where higher concentrations of the compound decreased toxin production more effectively.

BUĞDAY PATOJENİ *Fusarium culmorum*'UN PATOJENİK ÖZELLİKLERİ VE TRİKOTESEN ÜRETİMİNİN KARAKTERİZASYONU

ÖZET

Fusarium culmorum tek hücreli bir ipliksi mantardır ve en çok bitki patojeni olmasıyla tanınır. Genomu henüz sekanslanmamıştır ve bu nedenle bir çok genin işlevi ve patojenik özellikleri detaylı olarak bilinmemektedir. Mısır ve buğday gibi küçük tohumlu tahılları enfekte edebilen *F. culmorum,* özellikle buğdayda kök ve kökboğazı çürüklüğü, buğday başak yanıklığı gibi bir çok hastalığa neden olabilir. Bu hastalıklar sonucu buğday tohumlarında beyaz renkli fenotip, buruşuk taneler, kısırlık gibi problemler görülür. Bu durum ise üretimde verimin ve kazancın ciddi miktarda düşmesine neden olmaktadır. Bunun haricinde, *F. culmorum* tarafından enfekte edilmiş bitkilerde, mantarlar tarafından sentezlenen ve salgılanan mikotoksinlerin birikimi gözlenmektedir. Mikotoksinler, *Fusarium* türleri tarafından üretilen ikincil metabolitlerdir ve binlerce farklı çeşidi bulunmaktadır. Temel olarak *Fusarium* türleri tarafından üretiken mikotoksinler trikotesen ve fumonisin olarak iki gruba ayrılırlar. Mikotoksinler bitkiler üzerinde üretilir, birikir ve bitkiler yoluyla insanlar ve diğer hayvanlara geçerler. Trikotesenler ökaryotlarda protein sentezini inhibe edebilirken, fumonisinler sfingolipid üretimine engel olarak insan ve hayvanların sağlığını tehdit ederler.*F. culmorum* B tipi trikotesenler olan deoksinivalenol (DON), 15-asetildeoksinivalenol (15-ADON), 3-asetildeoksinivalenol (3-ADON) gibi mikotoksinleri üretebilmektedir. Bu mikotoksinlerin üretimi trikotesen yol izi ile gerçekleşir. Bu yolizinde 14 gen tarafından üretilen farklı proteinler yer almaktadır. Tek bir molekül farnesil pirofosfatın (FPP) girdiği tepkimeler serisi sonucu toksinler üretilir. Toksinlerin üretimini engellemek ya da azaltmak amacıyla fenolik bileşikler kullanmak çözüm yollarından biridir.

Bu tezin amacı, *F. culmorum*'un patojenik özelliklerinin genetik karakterizasyonunun yapılmasıdır. Bu amaçla, tezin ilk kısmında, fonksiyonu bilinmeyen üç genin işlevi, önceden elde edilmiş delesyon mutantları ve ektopik türler kullanılarak araştırılmıştır. Tüm türler öncelikle 2M Sorbitol, 1M NaCl, 0.02% SDS, 0.5 ppm Tebuconazol, 30mM $K_2S_2O_8$, sıcak (37°C) ve soğuk (8°C) stresleri varlığında fenotipik olarak test edilmiş ve büyüme miktarları kaydedilmiştir. Ardından patojenitelerindeki değişikliğin tespiti için, miseller üzerine yerleştirilen buğday tohumlarının filizlenmeleri gözlemlenmiştir.

FGSG_08817 geninin araştırılmasında 2 ektopik, 4 delesyon türü kullanılarak fenotip ve patojenite testleri yapılmıştır. Büyüme miktarları yaban tip ile kıyaslandığında, delesyon mutantlarının oluşan streslere karşı daha dayanıklı ya da zayıf olduğu görülmemiş, patojenite ise değişmemiştir.

FGSG_16588 geni araştırılırken, önceden elde edilen bir delesyon mutantı ve bir ektopik tür incelenmiştir. Bu türlere yapılan testler sonucu, delesyon mutantının stres koşulları altında yaban tip ile aynı şekilde ürediği, buğday patojenitesinin de benzer şekilde kaldığı görülmüştür. Ancak bu genin, 8-E olarak adlandırılan ektopik türünde NaCl ve sorbitol varlığında daha fazla üreme görülmüştür. NaCl ve sorbitol test edilen konsantrasyonlarda ortamda bulunduklarında hücrelerde ozmotik strese neden olurlar. Sonuç olarak 8-E ektopik türün ozmotik strese direnç kazandığı görülmüştür. Patojenite testlerinde ise 8-E'nin patojenik özelliklerinin arttığı görülmüştür. Ektopik türde istenilen gen silinmediğinden bulgular genin fonksiyonları ile ilişkilendirilmemiştir.

Üçüncü incelenen gen *FGSG_16572* beş farklı delesyon mutantı kullanılarak fenotipik ve patojenik testler yardımıyla araştırılmıştır, ancak bu genin de patojeniteyle ilintili olmadığı ve test edilen stres koşullarına direnç sağlamadığı görülmüştür.

Tezin ikinci kısmında ise, fenolik bileşiklerin *F. culmorum*'un mikotoksin üretimine etkisi araştırılmıştır. Bu amaçla ferülik asit ve dimerik ferülik asit bileşiklerinin, bunların yanı sıra, isimleri patent başvurusu sebebiyle gizli tutulan ve bileşik 1, 2, 3 ve 4 olarak adlandırılan dört farklı fenolik bileşiğin *F. culmorum* üzerindeki etkileri gen anlatım, hücresel üreme ve toksin üretim düzeylerinde test edilmiştir. Kimyasallar, toksin üretimini tetikleyen Vogel besiyerine, çözünmeyi arttırması için beta siklodekstrin ilavesiyle eklenmiştir. Tüm bileşikler 0.25mM konsantrasyon ile test edilmiştir. Bileşik 4 için 0.10mM ve 0.05mM konsantrasyonları da araştırmaya katılmıştır. Belirtilen besiyerlerinde 5 gün boyunca üreyen hücrelerden 3 biyolojik tekrar ile örnekler toplanıp, sıvı azot ile hücreler parçalanmış, RNA izole edilip, tüm RNAlar cDNA'ya çevrilmiştir. cDNA'lar kullanılarak trikotesen yolizinde yer alan *FPP, TRI5, TRI4, TRI11, TRI3, TRI13, TRI6* ve *TRI10* genlerinin anlatım düzeyleri Gerçek Zamanlı Polimeraz Zincir Reaksiyonu ile belirlenmiştir. ΔΔCT yöntemi ile yorumlanan sonuçlar, kontrol grubunda araştırılan genin anlatımı 1 olarak kabul edilerek düzenlenmiştir.

Ferülik asit, literatürdeki sonuçlarla uyumlu olarak, test edilen genlerin anlatımını 0.6-0.8 düzeyinde azaltan bir etki göstermiştir. Dimerik ferülik asidin 0.25mM konsantrasyon ile varolduğu ortamda üreyen hücrelerin genanlatımlarının 0.5-0.8 arasında olduğu görülmüştür. Bileşik 1'in varlığı kimi genlerin anlatımlarında artışa neden olurken, bazılarında değişiklik gözlemlenmemiştir. Bileşik 2, test edilen genlerin büyük bir çoğunluğunun anlatımının yaklaşık iki kat daha fazla artmasına neden olmuştur. Bileşik 3'ün ise 0.25mM konsantrasyonda 0.6-1.0 arasında değişen, çoğunlukla azalmış gen anlatımına sebep olduğu görülmüştür.Bileşik 4'ün test edilen üç farklı konsantrasyonu karşılaştırıldığında, 0.25mM ve 0.10mM konsantrasyonlarda, *FPP* dışındaki trikotesen yolizi genlerinin tümünün anlatımlarının azaldığı gözlemlenmiştir. 0.05mM konsantrasyonun gen anlatımlarını, diğer yüksek konsantrasyonlara kıyasla daha az etkilediği görülmüştür. Yalnızca gen anlatım değerleri üzerine yapılan birincil yorumlarda bileşik 4'ün 0.10mM'ının gen anlatımını azaltmaya yeterli olduğu belirtilmiştir.

Gen anlatımlarındaki etkileri test edilen tüm bileşiklerin toksin üretim ve hücre üremesiüzerindeki etkileri araştırılmıştır. Bu amaçla, Vogel besiyerinde gen anlatımı için bahsedildiği şekilde, 5 biyolojik tekrar ile hazırlanan hücreler 14. gün sonunda toplanmıştır. Sıvı kısımdan toksinler ayrılarak sıvı kromatografi-kütle spektrometri

(LC-MS)yöntemi ile analiz edilmiştir. Hücrelerden oluşan katı kısım ise kurutularak tartılmıştır.

0.25 mM ferülik asidin hücre üremesini fazla engellemediği, ancak toplam toksin üretimini %30'a kadar düşürebildiği görülmüştür. Gen anlatım sonuçları ile tutarlı olan toksin analizi, ferülik asidin başarılı bir inhibitör olduğunu göstermiştir.

Aynı konsantrasyondaki dimerik ferülik asidin de hücre üremesine etkisinin az olduğu görülmüştür. Her ne kadar test edilen genlerin anlatımları bu kimyasalın varlığında azalmış olarak bulunmuş olsa da, dimerik ferülik asidin toksin üretimini %380 oranında arttırdığı görülmüştür.

Bileşik 1'in test edilen konsantrasyonda hücre üremesine etkisi olmadığı görülmüştür. Gen anlatım düzeylerinin azalmış olmasına rağmen, toksin üretiminin %130 civarında olduğu tespit edilmiştir.

Bileşik 2 hücre üremesini %20 oranında azaltmış ve test edilen kimyasallar arasında en etkili mantar ilacı olarak belirlenmiştir. Toksinler bu kimyasalın varlığında % 40 oranında üretilebilmiştir.

Bileşik 3 varlığında hücrelerin üremesi yaklaşık %80 olarak görülmüştür. Bu bileşik, test edilen konsantrasyonda, toplam toksin üretimini %35 azaltmıştır ve bu sonuçlar, gen anlatım sonuçları ile tutarlı bulunmuştur.

Bileşik 4'ün üç farklı konsantrasyonu olan 0.25mM, 0.10mM ve 0.05mM varlığında toksin üretimlerinin, bileşiğin konsantrasyonuyla ters orantılı olarak azaldığı görülmüştür. Test edilen en yüksek konsantrasyonda toplam toksin üretimi %33 olarak bulunmuştur. 0.10mM konsantrasyonda toksinlerin %43 miktarında üretildiği, 0.05mM konsantrasyonda ise üretimin %47 oranında gerçekleştiği gösterilmiştir. Bulunan bu sonuçlar, gen anlatım sonuçları ile uyumlu görünmektedir.

Test edilen fenolik bileşiklerden istenilen sonuca benzer sonuçlar veren ferülik asit, bileşik 2 ve bileşik 4 için *in planta* deneyler önerilmiştir.

1. INTRODUCTION

1.1 General Information on *Fusarium* Species

Genus *Fusarium* is a branch of Kingdom Fungi, Phylum Ascomycota, Subphylum Pezizomycotina, Class Sordariomycetes, Subclass Hypocremycetidae, Order Hypocreales, and Family Nectriaceae (Scherm *et al.*, 2013).

Fusarium genus was first introduced in 1809. It consists of fungi that are mostly plant-pathogenic, but can also cause diseases in humans and domesticated animals. They produce secondary products (toxins) that are related to plant diseases, cancer and growth defects in humans. Mostly, they lead to various diseases in different parts of the plants such as root, stems, fruits and leaves (Leslie and Summerell, 2006).

Members of this genus are chemoorganotrophic aerobes that form structures called hyphae which are described as a network of filaments formed by tubular cell walls surrounding the cytoplasm. They grow across a surface with other hyphae. These structures can be visualized macroscopically and are called as mycelia. On aerial branches of hyphae, asexual spores called conidia are formed. This structure is an essential part of dispersion of the fungi to new habitats since conidia germinate into new hyphae (Clark *et al.*, 2009).

Sexual reproduction is observed in some species of *Fusarium*. There are two styles of sexual reproduction; homothallic or heterothallic. Homothallic mating system is the most common system in this genus where sexual spores, called ascospores, can be produced in a culture that was originating from a single spore. Hence, homothallic strains of *Fusarium* are defined as self-fertile. Heterothallic species, on the other hand, require a sexual interaction with another, physiologically distinct strain in order to carry on their life cycles. The physiological difference between two heterothallic strains arises from mating types. All *Fusarium* heterothallic species show dimictic mating system which includes a single mating-type locus called *MAT*. This locus may contain one of the two functional alleles, termed *MAT-1* and *MAT-2*, which are at the same position on the chromosome but their sequences are not similar

to each other. Even though this dimictic mating system is seen in most ascomycetes, these alleles can be very different, for example no similarity in mating type alleles of *Saccharomyces cerevisiae* is ever encountered in any of the *Fusarium* species. However, some species such as *F. oxysporum* and *F. culmorum* do not have a known sexual stage (Leslie and Summerell, 2006).

Some *Fusarium* species are named differently depending on different life cycles they go through. *Fusarium* has two states; the asexual state called anamorph and a sexual state called teleomorph (Guadet *et al.*, 1989). Mostly, teleomorphs are only generated under laboratory conditions; however, there are exceptions. Because of this difference, some species are known with two different names for different states. Anamorphic *Fusarium* can have teleomorphs in genera *Gibberella, Haematonectria* or *Albonectria.*When referring to species in *Fusarium* with teleomorphs in different genera, both names are mentioned to prevent confusion (Leslie and Summerell, 2006).

Mycotoxins are secondary products produced by several *Fusarium* species. They consist of two families; trichothecenes and fumonisins. Mycotoxins are produced and accumulated in crop plants which are infected by the fungi (Alexander *et al.*, 2009). When accumulated, mycotoxins can induce apoptosis in eukaryotic cells which leads to concerns about food and feed safety of animals and humans (Bhat *et al.*, 2010; Peraica *et al.*, 1999).

Trichothecenes are tetracyclic sesquiterpenoid mycotoxins produced during isoprenoid metabolism of several species of *Fusarium* including *F. graminearum* and *F. culmorum.*These mycotoxins consist of a tricyclic nucleus with an epoxide function (Figure 1.1). There are over 150 different toxins, classified in four groups (type A, B, C and D). T-2 and HT-2 toxins are two of many Type A trichothecenes; whereas Type B trichothecenes consist of nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and fusarenon-X (FUS). Trichothecenes induce several health problems, which can be the result of toxins' ability to inhibit protein synthesis in humans and animals (Alexander *et al.*, 2009; Langseth and Rundberget, 1998).

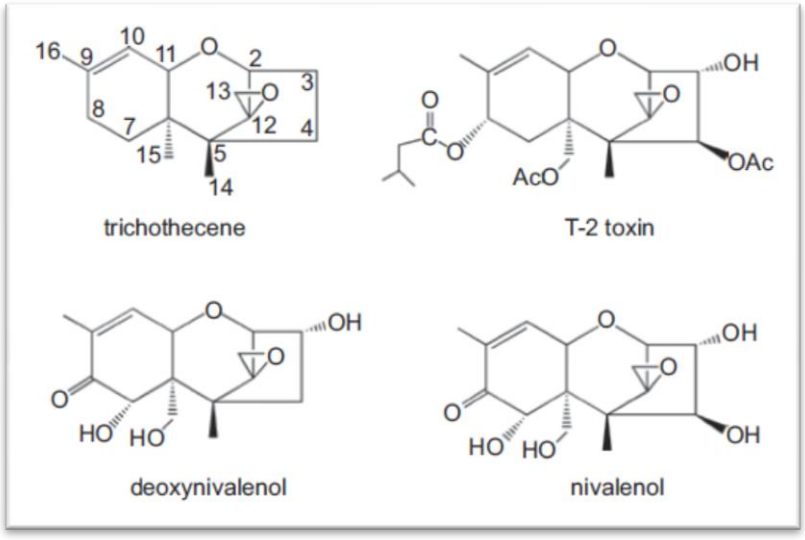

Figure 1.1: Trichothecenes of *Fusarium* (Alexander *et al.*, 2009).

Fumonisins are produced during polyketide and amino acid metabolisms of certain *Fusarium* species such as *F. proliferatum* and *F. verticillioides*. These molecules have a linear structure with amine and tricarballylic ester functions. There are more than 30 kinds of known fumonisin analogs, but the best known and most common fumonisins are the B-series fumonisins; B_1, B_2, B_3 and B_4(Figure 1.2). The most abundant type is the B_1 series that take up 70-80% of the total fumonisin content. Fumonisins have the ability to disrupt sphingolipid biosynthesis, leading to multiple health problems (Alexander *et al.*, 2009).

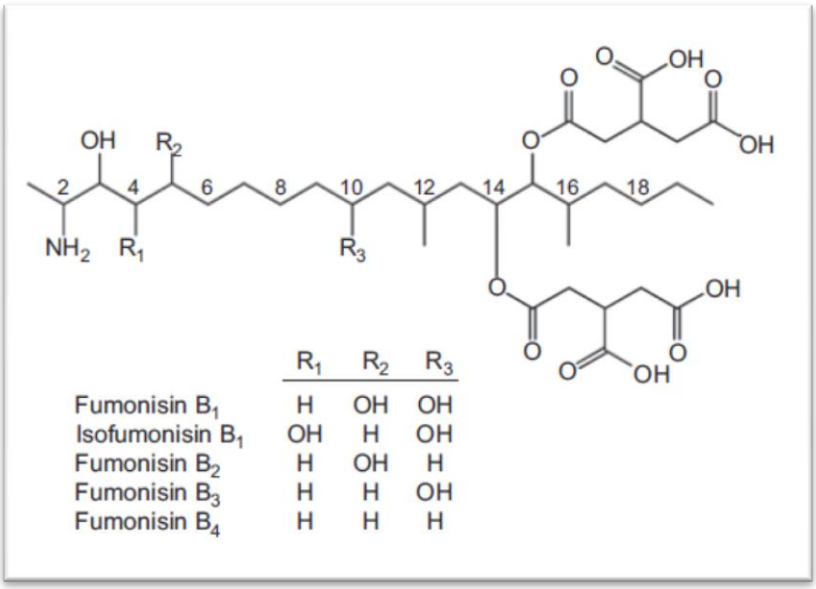

Figure 1.2: Fumonisins of *Fusarium* (Alexander *et al.*, 2009).

1.2 *Fusarium graminearum*

Fusarium graminearum Schwabe, also known as *Gibberella zeae* Schwein (Petch) in teleomorph stage, is a haploid pathogenic fungus (Miedaner *et al.*, 2008). *F. graminearum* can reproduce sexually and asexually. When reproduced asexually, it forms macroconidia that contain 5-6 septa. Optimum growth temperature of *F. graminearum* is 25°C and on Potato Dextrose Agar (PDA) it grows rapidly, 4.7-6.1 cm in three days. *F. graminearum* is the most dominant plant pathogen in Europe causing Fusarium Head Blight (FHB) primarily on maize, barley and wheat (Leslie and Summerell, 2006). The genome of *F. graminearum* was sequenced and announced in 2003. Genome size was stated to be 36.1 megabase long, consisting of four chromosomes with genes predicted to be coding for 13937 proteins (Trail, 2009).

1.3 *Fusarium culmorum*

Fusarium culmorum is another member of the genus *Fusarium.* Despite the fact that *F. culmorum* contains transcribing mating genes, teleomorph state is still unknown. Itdoes not form any microconidia and macroconidia are formed by 3-5 septates (Figure 1.3). Macroconidia are blunt, thick-walled and generally 30-50 x 5.0-7.5 µm in size (Scherm *et al.*, 2013).

Figure 1.3: Macroconidia of *F. culmorum* (Scherm *et al.*, 2013).

F. culmorum has an optimum temperature of 25°C and shows rapid growth (1.6-2.2 cm/day) when grown on PDA, with formation of light yellow to red mycelium

(Scherm *et al.*, 2013). Genome of *F. culmorum* has not been sequenced yet, but it is known that it is a haploid microorganism and has five chromosomes with over 10000 estimated genes (Mishra *et al.*, 2003; Spanu *et al.*, 2012). Since *F. culmorum* is closely related to *F. graminearum* in phylogenetic terms, genetic analysis of this microorganism is mostly carried out in regard to the genomic sequence of *F. graminearum* (Mulé *et al.*, 2004).

1.3.1. Diseases caused by *F. culmorum*

F. culmorum is best known as a soil-borne pathogen of small-grain cereals such as wheat and barley. It is one of the most important factors in development of several diseases such as Fusarium Head Blight (FHB), Foot and Root Rot (FRR) and Crown and Foot Rot (CFR) (Scherm *et al.*, 2013; Balmas *et al.*, 2006).

Infection of the seed initiates with the germination of seed and primary root emerge and the fungus penetrates through the lesions. It can also penetrate through the stomata, from the insertion point of the basal leaf sheath. After penetration, fungus starts to colonize; first at the intercellular apoplastic pathway which is between cells of epidermis and cortex, and next it moves on to intracellular area where it colonizes the tissues. If the fungus grows further along the stem, basal browning occurs, which is a result of plant's response to the infection (Scherm *et al.*, 2013).

1.3.1.1 *Fusarium* Head Blight (FHB)

FHB, also known as scab, is an economically important, worldwide disease of wheat caused by *Fusarium* species (Windels, 2000; McMullen *et al.*, 1997). Symptoms of FHB, shown in Figure 1.4, include highly reduced yields with formation of discolored and shriveled kernels referred to as tombstones, depressed stems and a loss in seed quality and viability (Windels, 2000). In addition, spore accumulation on infected spikelets can be seen after longer wet periods, leading to a pink to salmon-orange color on infected head (Goswami and Kistler, 2004). FHB is spread by air-borne chlamydospores of *F. graminearum* or soil-borne sporodochia of *F. culmorum* by splash-dispersal to the heads of cereals and it is favored by high humidity or continuous rain (Leslie and Summerell, 2006; Parry *et al.*, 1995). Upon infection by *F. culmorum* at anthesis, the flowering period of the plant, FHB starts developing by germination of macroconidia and penetration into host tissue and it spreads until harvesting of the grain. During spreading, host is contaminated by mycotoxins such

as type B trichothecenes, fusarins and zearalenone, which may inhibit the defense response of the host (Scherm *et al.*, 2013).

Figure 1.4: Symptoms of Fusarium Head Blight (FHB) A: discolored kernels, B: accumulation of orange sporodochia (Scherm *et al.*, 2013).

1.3.1.2 Foot and Root Rot (FRR)

FRR, also known as Fusarium Crown Rot, shows different symptoms according to the time of infection (Figure 1.5). In early stages, attack of the fungus can lead to death, with roots, coleoptiles or pseudostem discolored to brown. In later stages it leads to shriveled grains on the white head and tiller abortion, with brown lesions on the second or third internode of the main stem. High humidity leads to accumulation of sporulating mycelium on the nodes, showing a reddish-pink coloration on this area. (Scherm *et al.*, 2013).

Figure 1.5: Symptoms of Foot and Root Rot (FRR). A: pink discoloration on basal nodes, B: wheat with white heads (Scherm *et al.*, 2013).

1.3.1.3 Crown and Foot Rot (CFR)

CFR is another disease caused by *F. culmorum.* It is seen mostly in dry soils where temperature is warmer, such as central and southern Italy (Cariddi and Catalano, 1990; Scherm *et al.*, 2011). In early stages, infection leads to seedling death before or just after emerge. In later stages, brown lesions on the stem are seen, alongside tiller abortion, whiteheads, shriveled grains or absence of grain. As a consequence, CFR leads to significant yield loss in wheat production (Balmas *et al.*, 2006; Scherm *et al.*, 2011).

1.3.2 Mycotoxin production by *F. culmorum*

F. culmorum can produce different mycotoxins of the type B trichothecenes and is divided into two chemotypes, depending on the toxin they produce. Members of chemotype I can produce DON and its acetylated forms; 3-ADON and 15-ADON. On the other hand, members of chemotype II produce NIV and/or FUS (Scherm *et al.*, 2013).

Genetic basis of toxin production was established in *F. graminearum* and it was shown that in the genome, there is one core gene cluster, and two other loci that are functioning in production of different toxins. It was also seen that in the genome of DON producing *F. graminearum* strains, several genes were nonfunctional, when these genes were found to be functional in NIV-producing *F. graminearum* strains. There are 12 genes on the core gene cluster, referred to as *TRI3, TRI4, TRI5, TRI6, TRI7, TRI8, TRI9, TRI10, TRI11, TRI 12, TRI13* and *TRI14*. The other two loci consist of *TRI16* and *TRI1* genes. For T-2 toxin production, products of all mentioned genes should be active, whereas in NIV-producing strains, *TRI16* is blocked and in DON-producing strains *TRI7, TRI13* and *TRI16* are blocked; either by multiple insertions or deletions on these regions (Brown *et al.*, 2002; Lee *et al.*, 2002; Alexander *et al.*, 2009).

For production of mentioned toxins, a pathway was proposed by combination of previous data and it can be seen in Figure 1.6 (Alexander *et al.*, 2009).

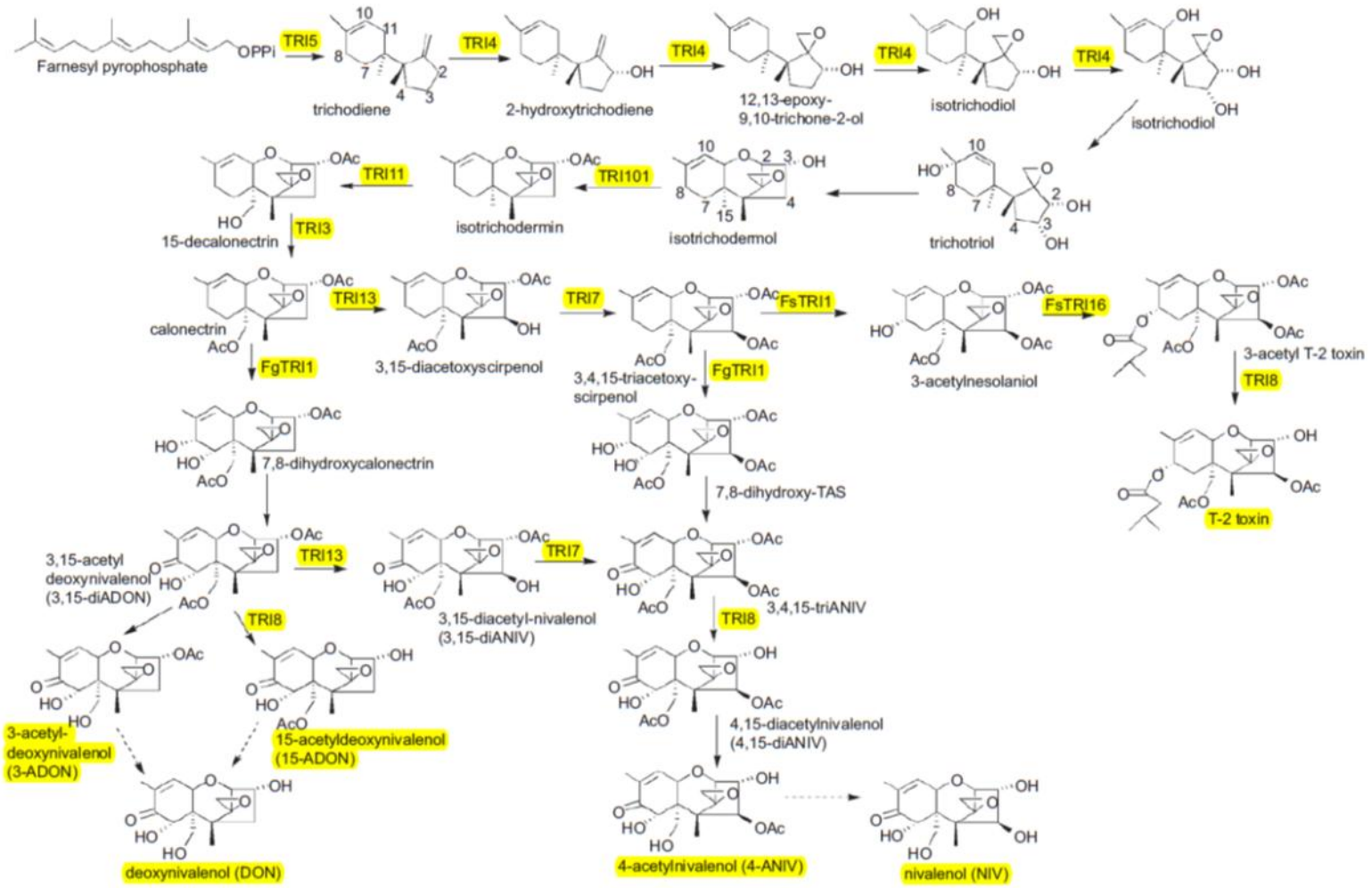

Figure 1.6: Trichothecene pathway proposed by Alexander *et al.* (2009). Genes and final products are highlighted.

The pathway displayed in Figure 1.6 starts with farnesyl diphosphate, which is an isoprenoid intermediate in primary metabolism. Farnesyl diphosphate is converted to trichodiene by trichodiene synthase enzyme coded by *TRI5* gene (Hohn and Vanmiddlesworth, 1986). Next, trichodiene undergoes four consecutive oxygenation reactions by cytochrome P450 monooxygenase which is encoded by *TRI4* gene (McCormick *et al.*, 2006). The next two steps occur nonenzymatically, leading to formation of isotrichodermol (Kimura *et al.*, 2007; McCormick *et al.*, 2006). The next reaction is isotrichodermol esterification of acetyl to the C-3 hydroxyl, catalyzed by the enzyme C-3 acetyltransferase, encoded by *TRI101* (Kimura *et al.*, 1998; McCormick *et al.*, 1999). The carbon at 15^{th} position is then hydroxylated by cytochrome P450 monooxygenase, encoded by *TRI11* (Alexander *et al.*, 1998). Next, the *TRI3*-encoded acetyltransferase transfers an acetyl moiety to the C-15 oxygen and the pathway reaches to a branching point where either T-2 or NIV or DON can be the end product (McCormick *et al.*, 1996).

In T-2 and NIV producing *Fusarium* species, reaction continues with C-4 hydroxylation and this reaction is catalyzed by a cytochrome P450 monooxygenase encoded by gene *TRI13* (Brown *et al.*, 2002; Lee *et al.*, 2002). This reaction is followed by esterification of C-4 oxygen by an acetyltransferase, encoded by *TRI7* (Lee *et al.*, 2002). Then, depending on the *Fusarium* species, product of gene *TRI1* can hydroxylate C-8 or both C-8 and C-7. In T-2 toxin producing strains, C-8 is hydroxylated and 3-acetylnesolaniol is formed and the next reaction is esterification of C-8 by C-8 acetyltransferase encoded by *TRI16* (Brown *et al.*, 2003). In NIV producing strains, both C-8 and C-7 are hydroxylated by C-7,8 oxygenase encoded by *TRI1* (McCormick *et al.*, 2006). For DON producing strains, after *TRI3*-mediated reaction, since *TRI13* does not produce a functioning enzyme, *TRI1* encoded C-7,8 oxygenase hydroxylates carbons at 7^{th} and 8^{th} position (Brown *et al.*, 2003; McCormick *et al.*, 2006). The final step for all mentioned toxins is the same where deacetylation by trichothecene-3-O-esterase encoded by *TRI8* is observed, leading to final formation of the toxins (McCormick and Alexander, 2002).

Other genes of the core cluster that are not included in the trichothecene pathway have various properties. The function of *TRI9* is not yet known. *TRI12* encodes a transporter protein that acts like a trichothecene efflux-pump. *TRI14* gene product is a putative protein, whose function is still unknown. *TRI6* and *TRI10* genes are the

regulatory genes of the pathway, coordinating the expression of other trichothecene genes. It was shown that silencing of *TRI6* leads to lower *TRI5* expression and lower toxin production (Alexander *et al.*, 2009; Scherm *et al.*, 2011).

1.3.3 Control Strategies

In order to prevent yield losses in wheat and health problems caused by *Fusarium* species, several control strategies are suggested. Ideally, the best choice is to choose wheat cultivars that are already resistant to the fungus. For example, wheat cultivar Sumai3 has the ability to detoxify DON, resulting in a natural FHB resistance. However, there are not a lot of naturally resistant wheat cultivars. Therefore other approaches are suggested (Scherm *et al.*, 2013; Lemmens *et al.*, 2005).

Usage of fungicides such as azoles (e.g. bromuconazole and tebuconazole) and strobin (e.g. azoxystrobin) showed a 70% success rate in controlling the disease in wheat cultivars with a moderate resistance. However, the concentration of the fungicide applied to the field is crucial, since lower doses of fungicide that is not enough to prevent mycelial growth may lead to increased toxin production. Additionally, emerge of some resistant*Fusarium* species was observed due to usage of molecules with similar mode of action (Scherm *et al.*, 2013).

In order to prevent development of resistance, the new focus is to find and develop new molecules that are similar to natural or natural-like inhibitors of the fungus and which can avert the pathogenicity and mycotoxins production of *Fusarium* or stimulate response by the host plant (Scherm *et al.*, 2013). For this reason, phenolic and polyphenolic compounds are under investigation, since these compounds have shown inhibitory activity against the fungus' pathogenicity (Boutigny *et al.*, 2010; Bakan *et al.*, 2003). Ferulic acid and its dehydrodimers are the most common phenols in wheat bran and hydrocinnamic acids are known to be the most abundant components of the primary cell wall of cereals (Bily *et al.*, 2003; Bakan *et al.*, 2003). Ferulic acid is also known to reinforce the primary cell wall of the plant and it was shown to decrease DON and ADON biosynthesis by *F. culmorum* in *in vitro* studies, although the mechanism of action is still unknown (Boutigny *et al.*, 2010).

1.4 Chemicals Utilized in This Thesis Study

Effects of chemicals on toxin production and trichothecene pathway of *F. culmorum* were investigated with six different chemicals throughout the thesis study. These chemicals are ferulic acid, ferulic acid dimer and four other phenolic compounds whose names are not relieved and referred to as Compound 1, 2, 3 and 4. Each compound was dissolved in the toxin inducing Vogel's Medium (Vogel, 1956), with addition of beta-cyclodextrin (β-CD) to increase solubility of the chemicals tested. β -cyclodextrin has a ring structure which shows hydrophobic behavior inside and hydrophilic behavior outside(Figure 1.7), and it forms complexes with hydrophobic compounds, leading to a higher solubility in water-based solutions for such compounds (Del Valle, 2004).

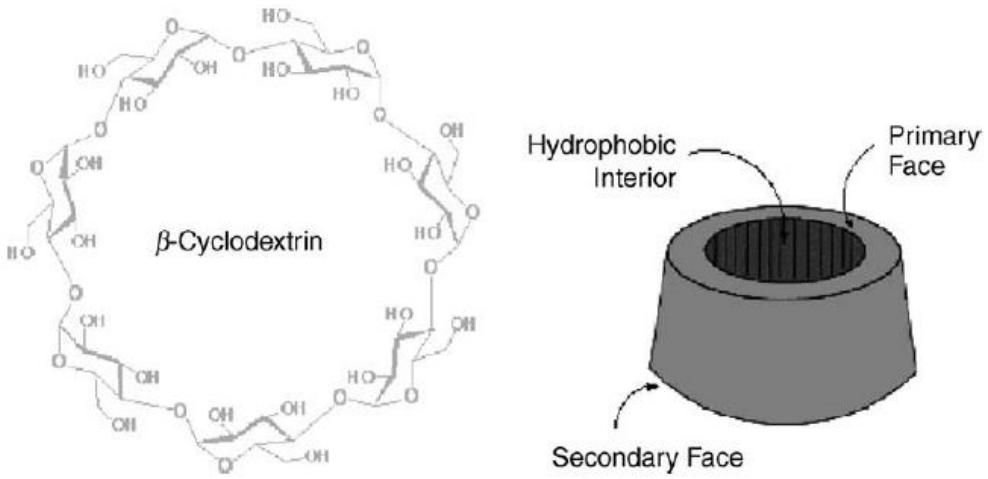

Figure 1.7: Structure of β-cyclodextrin (Del Valle, 2004).

1.5 Genes Studied in This Thesis Study

Throughout this thesis study, gene expression profiles of several genes were investigated via Quantitative Real Time PCR method. For this purpose, gene *FGSG_01102* (referred to as *18S*) was chosen as the reference gene. *18S* gene encodes 18S rRNA biogenesis protein RCL1 (Broad Institute *Fusarium*Comparative Database, 2010, a). Genes investigated were *FPP*, *TRI5*, *TRI4*, *TRI11*, *TRI3*, *TRI13*, *TRI6* and *TRI10*. Gene *FPP* encodes protein farnesyl pyrophosphate (FPP) synthetase, which uses geranyl pyrophosphate to produce FPP, the precursor of trichothecene production pathway (Boutigny *et al.*, 2009). Functions of the remaining genes were described in Section 1.3.2.

Additionally, previously obtained knock-out strains of the three genes *FGSG_08817*, *FGSG_16572*, and *FGSG_16588* were studied to characterize how these genes function in stress conditions phenotypically and how they affect the pathogenicity in more detail. *FGSG_08817* of *F. graminearum* strainPH-1 (FG3) is located on chromosome 2, supercontig 5, and consists of 1196 nucleotides. *FGSG_08817* encodes a hypothetical protein of 216 amino acids. This putative protein contains 5 domains: a binding domain, a domain showing similarity to ADP-ribosylation factor family, a miro-like protein domain, a domain with Ras-family protein properties and a small GTP-binding protein domain. This hypothetical protein is classified as a Ras-family protein, described as a probable novel protein of Ras superfamily KREV-1 (Broad Institute *Fusarium*Comparative Database, 2010, b; Munich Information Center for Protein Sequences, Fusarium Graminearum Database, n.d., a). Proteins in Ras family are in charge of translating extracellular signals to the nucleus for proper regulation of the cells. They are activated when coupled with a GTP, and they use the phosphate energy to carry out reactions (Herrmann *et al.*, 1996). In order to investigate gene *FGSG_08817*,four knock-out strains (referred to as 7B, 9B, 16B and 17B) and two ectopic strains (referred to as 3B-E and 12B-E) originating from *Fusarium culmorum UK 99* (NRRL54111) (Rothamsted Research, UK) (referred to as FcUK99) have been used throughout the studies.

FGSG_16572, also referred to as *FGSG_05904*, is defined as a DNA repair protein in *F. graminearum* strainPH-1 (FG3) and is located on chromosome 3, supercontig 3. The gene is 4422 nucleotides long and encodes a protein of 896 amino acids that contain three domains: an SNF2 family N-terminal domain, a zinc finger domain and a helicase conserved C-terminal domain. This gene is classified in the adenosine triphosphate gene family and its product shows similarity to the RAD16 protein of *Saccharomyces cerevisiae* that functions mostly in DNA repair mechanisms (Broad Institute *Fusarium*Comparative Database, 2010, c; Munich Information Center for Protein Sequences, Fusarium Graminearum Database, n.d., b). ATP-binding cassette (ABC) transporters are integral membrane proteins that hydrolyze ATP to transport molecules across cellular membranes, and are known to confer resistance to a wide range or organisms such as bacteria, fungi and mammalian cells (Borges-Walmsley *et al.*, 2003). For investigation of gene *FGSG_16572*, five knock-out strains (referred to as 2N, 3N, 5N, 6N and 7N) that are originating from *Fusarium culmorum* (W. G.

Smith) Sacc. (Strain ISPaVe MCf21; syn. Strain INRA 117), referred to as Fc21WT, have been used.

F. graminearum gene *FGSG_16588* or alternatively *FGSG_06013* encodes a hypothetical protein. This gene is 1377 nucleotides long and is located on chromosome 3, supercontig 3 (FG3). The hypothetical protein consists of 407 amino acids and it is described to be related to RING Finger protein Dorfin (Broad Institute *Fusarium*Comparative Database, 2010, d; Munich Information Center for Protein Sequences, Fusarium Graminearum Database, n.d., c). Dorfin (Double ring finger protein) was shown to be localized in the centrosomal region of human cells where it leads to formation of aggregates composed of misfolded ubiquitylated proteins that cannot be further processed by the cell (Niwa *et al.*, 2002). In order to analyze the role of gene *FGSG_16588,*one knock-out strain (referred to as 9) and one ectopic strain (referred to as 8-E), bothoriginating from Fc21WT were used in experiments.

1.6 Aim of this Thesis Study

In general, the aim of this study was to better understand of the pathogenic behavior of *F. culmorum.* For this purpose, three genes with unknown function were investigated to see their effects on the pathogenicity of the fungus by using previously obtained knock-out mutants. Additionally, *F. culmorum* was treated with six phenolic compounds in order to observe how the gene expression and toxin production were altered when incubated in the presence of these compounds. Ferulic acid, ferulic acid dimer and compounds 1, 2 and 3 were tested at 0.25mM concentration whereas compound 4 was tested in three different concentrations (0.25mM, 0.10mM and 0.05mM) to observe the effect of the concentration of this compound.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Microorganisms and seeds

In this thesis work, *Fusarium culmorum UK 99* (NRRL54111) (Rothamsted Research, UK) referred to as FcUK99 and *Fusarium culmorum* (W. G. Smith) Sacc. (Strain ISPaVe MCf21; syn. Strain INRA 117), referred to as Fc21WTwere used as the wild type fungal strains.

Previously obtained *FGSG_08817*knock-out strains (referred to as 7B, 9B, 16B and 17B) and ectopic strains (referred to as 3B-E and 12B-E) originating from FcUK99; *FGSG_16572*knock-out strains (referred to as 2N, 3N, 5N, 6N and 7N) originating from Fc21WT; *FGSG_16588*knock-out strain (referred to as 9) and ectopic strain (referred to as 8-E) originating from Fc21WT were used for further characterization of these genes by *in vitro* phenotypic bioassays and *in vitro* pathogenicity assays.

For the *in vitro* pathogenicity assays, durum wheat seeds (*Hathor cultivar*) were used.

2.1.2. Fungal media compositions

Potato Dextrose Agar (PDA) was prepared as indicated by the manufacturer (Sigma-Aldrich, St. Louis, MO, USA). For preparation of Carboxymethylcellulose (CMC) liquid media, 15 gL^{-1} of carboxymethylcellulose sodium salt, 1 gL^{-1} $(NH_4)NO_3$, 1 gL^{-1} KH_2PO_4, 0.5 $gL^{-1}MgSO_4.7H_2O$ and 1 gL^{-1} yeast extract were added to distilled water.

For *in vitro* phenotypic bioassays, different culture media were prepared to create different stress environments. Substrate basis of all media was PDA and five different media were prepared by amending PDA with either 2M Sorbitol, or 1M NaCl or 30mM $K_2S_2O_8$ or 0.02% [w/v] Sodium dodecylsulfate (SDS) or 0.5 ppm tebuconazole, respectively. All media were amended with 50 µg/mL tetracycline, 50 µg/mL streptomycin and 50 µg/mL chloramphenicol in order to prevent bacterial

contaminations. For *in vitro* pathogenicity assays, as control 2% [w/v] water agar (WA) plates were prepared as control. All mentioned media were prepared by weighing the components, dissolving in distilled water and mixing by a magnetic stirrer, unless stated otherwise. All media were autoclaved at 121°C for 20 minutes after preparation.

Toxin-inducing liquid Vogel's Medium was prepared as follows.Firstly, 1 L of 2% [w/v] glucose solution was prepared and autoclaved. A Trace Element Solution with 100mL final volume was then prepared by dissolving 5.3 g citric acid x H_2O, 5.0 g $ZnSO_4$ x 7 H_2O, 0.05 g $MnSO_4$ x H_2O, 0.25 g $CuSO_4$ x H_2O, 1.0g Fe $(NH_4)_2(SO_4)_2$ x 6 H_2O, 0.05 g boric acid and 0.05 g Na_2MoO_4 x 2 H_2O in distilled water. Then, a 50x Salts Solution with a 1L final volume was prepared by adding 25g KH_2PO_4, 30 g K_2HPO_4, 0.85 g $MgSO_4$ x $7H_2O$, 50 g $(NH_4)_2SO_4$, 5mg Biotin and 5mL of the previously prepared trace element solution, filled up with distilled water and filter sterilized. For each liter of 2% [w/v] glucose solution, 20mL sterile Salts Solution was added.

Vogel's Media were added with several phenolic compounds at different concentrations. Ferulic acid, ferulic acid dimer and compounds 1, 2 and 3 were tested separately with a final concentration of 0.25mM in Vogel's Medium. Compound 4 was studied at three different concentrations; 0.25mM, 0.10mM and 0.05mM. Each medium was also added with the dissolving agent beta-cyclodextrin to a final concentration of 3mM. For control groups, Vogel's media without and with beta-cyclodextrin (final concentration 3mM) were prepared. Chemicals were completely solubilized and mixed by sonication at 60 Hz at room temperature for 90 minutes.

2.1.3. Kits, chemicals and laboratory equipment

Several kits were used in this thesis study. Nucleospin RNA Plant Kit (MACHEREY-NAGEL) was used for RNA isolation, iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories) for cDNA synthesis, and iQ SYBR Green Supermix kit (Biorad) was used for Quantitative Real Time PCR experiments.

Chemicals used in this thesis study are listed in Table 2.1 along with their suppliers.

Table 2.1: Chemicals utilized in the thesis work.

Chemical	Supplier
$(NH_4)_2SO_4$	Sigma-Aldrich (St. Louis, MO, USA)
$(NH_4)NO_3$	Sigma-Aldrich (St. Louis, MO, USA)
15-acetyl-deoxynivalenol	Sigma Chemicals (St. Louis, MO, USA)
3-acetyl-deoxynivalenol	Sigma Chemicals (St. Louis, MO, USA)
Agar	OXOID Ltd. (Basingstoke, Hampshire,England)
beta-cyclodextrin	(CAVAMAX®7 PHARMA)
Biotin	Sigma-Aldrich (St. Louis, MO, USA)
Boric acid	Sigma-Aldrich (St. Louis, MO, USA)
carboxylmethylcellulose sodium salt	Fluka Analytical Sigma-Aldrich (St. Louis, MO, USA)
Citric acid x H_2O	Sigma-Aldrich (St. Louis, MO, USA)
$CuSO_4$ x H_2O	Sigma-Aldrich (St. Louis, MO, USA)
D-(+)-glucose	Sigma-Aldrich (St. Louis, MO, USA)
Deoxy Nivalenol	Sigma Chemicals (St. Louis, MO, USA)
Fe $(NH_4)_2(SO_4)_2$ x 6 H_2O	Sigma-Aldrich (St. Louis, MO, USA)
Ferulic acid	Sigma-Aldrich (St. Louis, MO, USA)
K_2HPO_4	Sigma-Aldrich (St. Louis, MO, USA)
$K_2S_2O_8$	Sigma-Aldrich (St. Louis, MO, USA)
KH_2PO_4	Sigma-Aldrich (St. Louis, MO, USA)
Liquid Nitrogen (LN_2)	Sapio (Caponago, Italy)
$MgSO_4$ x $7H_2O$	Sigma-Aldrich (St. Louis, MO, USA)
$MgSO_4.7H_2O$	Sigma-Aldrich (St. Louis, MO, USA)
$MnSO_4$ x H_2O	Sigma-Aldrich (St. Louis, MO, USA)
Na_2MoO_4 x 2 H_2O	Sigma-Aldrich (St. Louis, MO, USA)
NaCl	Sigma-Aldrich (St. Louis, MO, USA)
Nivalenol	Sigma Chemicals (St. Louis, MO, USA)
Sodium dodecylsulfate	Sigma-Aldrich (St. Louis, MO, USA)
Yeast extract	Sigma-Aldrich (St. Louis, MO, USA)
$ZnSO_4$ x 7 H_2O	Sigma-Aldrich (St. Louis, MO, USA)

Tested compounds 1, 3 and 4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compound 2 was previously synthesized in the laboratory.

A list of laboratory equipment used in the experiments is given in Table 2.2.

Table 2.2: Laboratory equipment used in the thesis work.

Equipment	Supplier
Micropipettes	Gilson 1000 µl, 200 µl, 20 µl, 10 µl, 2 µl (France)
Pipette tips	Biohit (Finland)
Pipette tips with filter	Alpha Laboratories (USA)
Petri Dishes	Aptaca (Italy)
Disposable Tubes	15mL, 50mL (Eppendorf)
Laminar Flow	ASALAIR 1200
Autoclave	ASAL S.R.L (Italy)
Deep Freezers and Refrigerators	+4°C Iberna (Italy) -20°C Indesit (Italy) -80°C Sanyo (Italy)
Vortex Mixer	Techno Kartell
Magnetic Stirrer	VELP Scientifica (Europe)
Real Time PCR System	CFX-Connect RealTime System (Bio-Rad, USA) MyIQ Single Color Real Time PCR Detection System (BioRad, USA)
96-well Plates	Bio-Rad (USA)
PCR Sealers	Bio-Rad (GB)
Microcentrifuge Tubes (1.5 ml)	Eppendorf (Germany)
Balance	KERN EW
Sonicator	Branson Model 3510 OPTO-LAB
Incubators	Intercontinental (Italy)
LC-MSD 1100	Agilent Technologies (Palo Alto, CA, USA)
Luna C18 Column	Phenomenex (Castelmaggiore, BO, Italy)

In addition to the equipment listed in Table 2.2, several other tools such as pestle and mortars, cork borers (5 mm and 11 mm), and wire needles were used throughout the experiments.

2.1.4. Primers used in Quantitative Real Time PCR experiments

A list of primers and their sequences, along with their efficiencies as forward-reverse couples, are shown in Table 2.3.

Table 2.3: Primers, sequences (5'=>3') and efficiencies.

Primer name	Sequence (5'=>3')	Efficiency (%)
***18S*-For**	TTGACCCGTTCGGCACCTTAC	90.6
***18S*-Rev**	AAGTTTCAGCCTTGCGACCATAC	
***FPP*-For**	CATGATCGCCATTAACGAC	97.2
***FPP*-Rev**	CAATGTGTTCGGGCTT	
***TRI3*-For**	GTTCGAGCTGGCTTTGGTAG	101.2
***TRI3*-Rev**	ATTCGTCGCTCTCTGGTGAT	
***TRI4*-For**	CCGACTTCCACTTTGTCGTT	99.5
***TRI4*-Rev**	TCAGAATGGTGACCAGACCA	
***TRI5*-For**	ACCCTCAATTCCTTCGTCGTAGTG	95.7
***TRI5*-Rev**	CCCAAACCATCCAGTTCTCCATC	
***TRI6*-For**	TTATCGCCCTTCCCACCTTCAC	96.0
***TRI6*-Rev**	TAAAGTCCCGTCCGCTCTCAAAG	
***TRI10*-For**	TCTGAACAGGCGATGGTATGGA	103.9
***TRI10*-Rev**	CTGCGGCGAGTGAGTTTGACA	
***TRI11*-For**	GGACCACGGAATTGTATTGG	94.9
***TRI11*-Rev**	TGGTACCAATTGCGGCTACT	
***TRI13*-For**	GAGATGATTTCCTGCGTGGT	98.8
***TRI13*-Rev**	AGCACCATCTGTTCCAAAGC	

2.2 Methods

2.2.1. Characterization of genes by testing knock-outstrains

2.2.1.1 *In vitro* phenotypic bioassays

Strains to be analyzed, along with the control strains were cultured on PDA at 25°C in the dark. After 7 days, 5 mm plugs covered with mycelia were cut out by a cork borer (5mm) and placed on new PDA plates containing Sorbitol, NaCl, $K_2S_2O_8$, SDS and tebuconazole, as described previously in Section 2.1.2. Inoculated plates were incubated at 25°C for 3 days. For additional thermal stress tests, plugs were placed on fresh PDA plates and incubated for 14 days either at 37°C or at 8°C. All experiments were done in triplicates. After incubation, diametric growths of mycelia in all conditions were measured with a ruler and recorded.

2.2.1.2 *In vitro* pathogenicity assays

Plugs of 11 mm diameter were cut from seven days old *Fusarium* strain cultures previously grown on PDA. Ten plugs covered with mycelia of each strain were placed in empty Petri dishes. Additionally, plugs were cut from non-inoculated 2% [w/v] WA and PDA and ten plugs were placed in empty Petri dishes as control. One

durum wheat seed was added on the top of each plug. Plates were incubated at 25°C in the dark for 5 days. After incubation, growth of seeds was observed for each strain and control group and pathogenicity levels were determined depending on the number of seeds that managed to grow.

2.2.2 Genetic and molecular aspects of toxin production in the presence of phenolic compounds

2.2.2.1 Culture conditions

Liquid Vogel's Media containing the substrates were prepared as described in Section 2.1.2. Eight ml of each type of substrate was added to 60 mm Petri dishes. 10^4 conidia/mL of Fc21WT were inoculated into each Petri dish, all plates were closed carefully by using parafilm and were incubated at 25°C without shaking in the dark. For each chemical and control group, ten biological repeats were carried out to be used later on for RNA extraction, dry weight and toxin analysis.

For practical necessities, the experiment was carried out in two groups. In the first group phenolic compounds ferulic acid, ferulic acid dimer, compounds 1 and 2 were tested. In the second group, other phenolic compounds 3 and 4 (three different final concentrations) were tested. Each group was added with two control conditions; Vogel's media without and with β-cyclodextrin (3mM).

2.2.2.2 Isolation of RNA and reverse transcription

After 5 days, 3 culture plates from each chemical group alongside three plates from control group containing 3mM β-cyclodextrin were chosen. Fungi (Fc21WT) were collected from the liquid cultures by using sterile RNase free pipette tips and dried with sterile filter paper. Dry mycelia were carved from the paper and placed in RNase free tubes. Tubes were immediately flash-frozen in liquid nitrogen and then stored at -80°C.

For the following cell disruption, samples were grinded to powder by using a mortar and a pestle, previously cooled with liquid nitrogen. Powder-like samples were collected in fresh RNase-free tubes, flash-frozen in liquid nitrogen and stored at -80°C until further use.

RNA extraction was carried out by using the Nucleospin RNA Plant Kit (MACHEREY-NAGEL) according to the manufacturer's instructions, including an on-column DNAse treatment. Total RNA concentrations were determined by spectrophotometry (GeneQuant, Amersham Biosciences) and then adjusted to 100 ng/µL. All RNA samples were reverse transcribed to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) as described by the manufacturer. For each reaction, 1 µg of the RNA template (= 10 µL) was added to 4 µL of 5x iScript Reaction mix, 1 µL of iScript reverse transcriptase and 5 µL of nuclease free water, enabling 20 µL final volumes. Samples were incubated in Thermal Cycler (MyIQ Single Color Real Time PCR Detection System, BioRad, USA) with the suggested reaction protocol of the kit (5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, final hold at 4°C). Resulting cDNA concentrations were estimated to be 50 ng/µL. In quantitative Real-Time experiments 5 ng/µL final cDNA concentration were applied. For RNA samples with an initial concentration lower than 100 ng /µL, 50 ng/µL stocks were prepared, cDNA synthesis was carried out with 0.5 µg of RNA template and the resulting cDNA was diluted 5 times to resulting in the same 5ng/µL final cDNA concentration.

2.2.2.3 Quantitative Real Time PCR analysis

Quantitative Real Time PCR experiments were carried out using the iQ SYBR Green Supermix (Biorad) on a 96-well plate. The sample mix consisted of 1 µL of cDNA (5 ng/ µL), 10 µL 2x iQ SYBR Green Supermix, 0.4 µL of each primer (10 µM stock) and 8.2 µL sterile water with a 20 µL final volume. For gene expression analysis, following PCR protocol was applied: one cycle at 95°C for 4 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Melting curve analysis covered a temperature range of 50°C to 85°C with gradual heating steps increasing 0.5°C every 30 seconds. Results were analyzed by the operating Bio-Rad CFX Manager Program to obtain the expression data calculated with primer efficiencies. Efficiencies were obtained previously by applying the same primer concentration and five serial 1:5 dilutions of Fc21WT cDNA where the slopes of the forming standard curves were analyzed and the efficiency of each primer combination was calculated by the Bio-Rad CFX Manager Program.

2.2.2.4 Dry weight analysis and toxin extraction

After 14 days of incubation at 25°C in the dark, 5 biological repeats from each tested compound were subjected to toxin analysis. Liquid media and mycelia were separated by using a vacuum pump and a Hirsch filter system where mycelia were washed with 1mL distilled water three times. Sampled mycelia were dried at 80°C oven for 48 hours and mycelial dry weights were determined. Culture filtrates, containing 3-ADON, DON and 15-ADON toxins, were stored at -20°C for further analysis.

In order to extract trichothecenes from the culture filtrates, 3 mL of ethyl acetate were added to circa 9 mL of culture filtrates, vortexed for 1 minute at maximum speed and then centrifuged at 2700 x g for 10 minutes. The organic phase containing the toxins was transferred into a new sterile 15 mL-tube and the extraction step was repeated two more times. 6-7 mL of ethyl acetate and extracted toxins mixture were stored at -20°C for further processing.

2.2.2.5 Toxin analysis of Type B trichothecenes with LC-MS

Quantification of trichothecenes was established in the presence of NIV, DON, Fumonisin, 15-ADON and 3-ADON standards. Using particles with 3μm diameter on the LC column, samples were analyzed at 40°C with a flow rate of 0.4 mL/min. Mobile phase consisted of Eluent A (0.01% acetic acid [v/v] with water) and Eluent B (acetonitrile). Twenty μL of each sample were injected and analyzed for 20 minutes, with the detector DAD reading at wavelengths 214nm and 270nm. The spectrometer was programmed for reading, in Positive mode [M + H +], the molecular ion of DON (297 m/z) and molecular ions of 15-ADON and 3-ADON (339 m/z). The energy of fragmentation was 50 eV and the Ion Spray was set to 3200 Vcap in Pos. Nitrogen temperature was 350° C under a pressure of 42 psig with a flow rate of 9.5 L/min.

3. RESULTS AND DISCUSSION

3.1 Characterization of *FGSG_08817*

3.1.1 *In vitro* phenotypical bioassays of *FGSG_08817*knock-outmutants

After incubation at 25°C for 3 days, plates containing stress elements were collected and mycelium growth values of wild type FcUK99, two ectopic strains (3B-E & 12B-E) and four deletion mutants of *FGSG_08817* (7B, 9B, 16B & 17B) were measured and listed in Table 3.1.

Table 3.1: Diameters (cm) of mycelia growth for *FGSG_08817* strains (± stands for standard deviation of three biological replicates) under stress caused by 2M Sorbitol, 1M NaCl, 0.02% SDS, 0.5 ppm Tebuconazole, 30mM $K_2S_2O_8$, heat (37°C) and cold (8°C).

	Sorbitol	**NaCl**	**SDS**	**Tebuc.**	**PersK**	**37°C**	**8°C**
FcUK 99	1.85 ± 0.07	3.82 ± 0.35	3.03 ± 0.51	1.30 ± 0.00	1.46 ± 0.45	0.00	1.93 ± 0.06
3B-E	1.47 ± 0.60	4.44 ± 0.47	3.74 ± 0.31	1.63 ± 0.47	2.77 ± 0.25	0.00	2.06 ± 0.17
12B-E	1.66 ± 0.67	3.64 ± 0.30	2.73 ± 0.29	1.97 ± 0.31	1.84 ± 0.39	0.00	1.64 ± 0.02
7B	1.93 ± 0.46	4.09 ± 0.37	3.21 ± 0.37	1.70 ± 0.26	1.91 ± 0.24	0.00	2.02 ± 0.05
9B	2.10 ± 0.20	4.31 ± 0.38	3.37 ± 0.32	1.54 ± 0.05	2.20 ± 0.56	0.00	1.87 ± 0.06
16B	2.25 ± 0.07	3.87 ± 0.09	2.67 ± 0.31	1.43 ± 0.15	1.73 ± 0.19	0.00	1.96 ± 0.08
17B	1.73 ± 0.64	3.97 ± 0.24	2.62 ± 0.32	1.40 ± 0.20	1.78 ± 0.25	0.00	1.89 ± 0.05

Using the values of mycelial growth listed in Table 3.1, Figure 3.1 was plotted to visualize the difference in growth in the presence of each stress element.

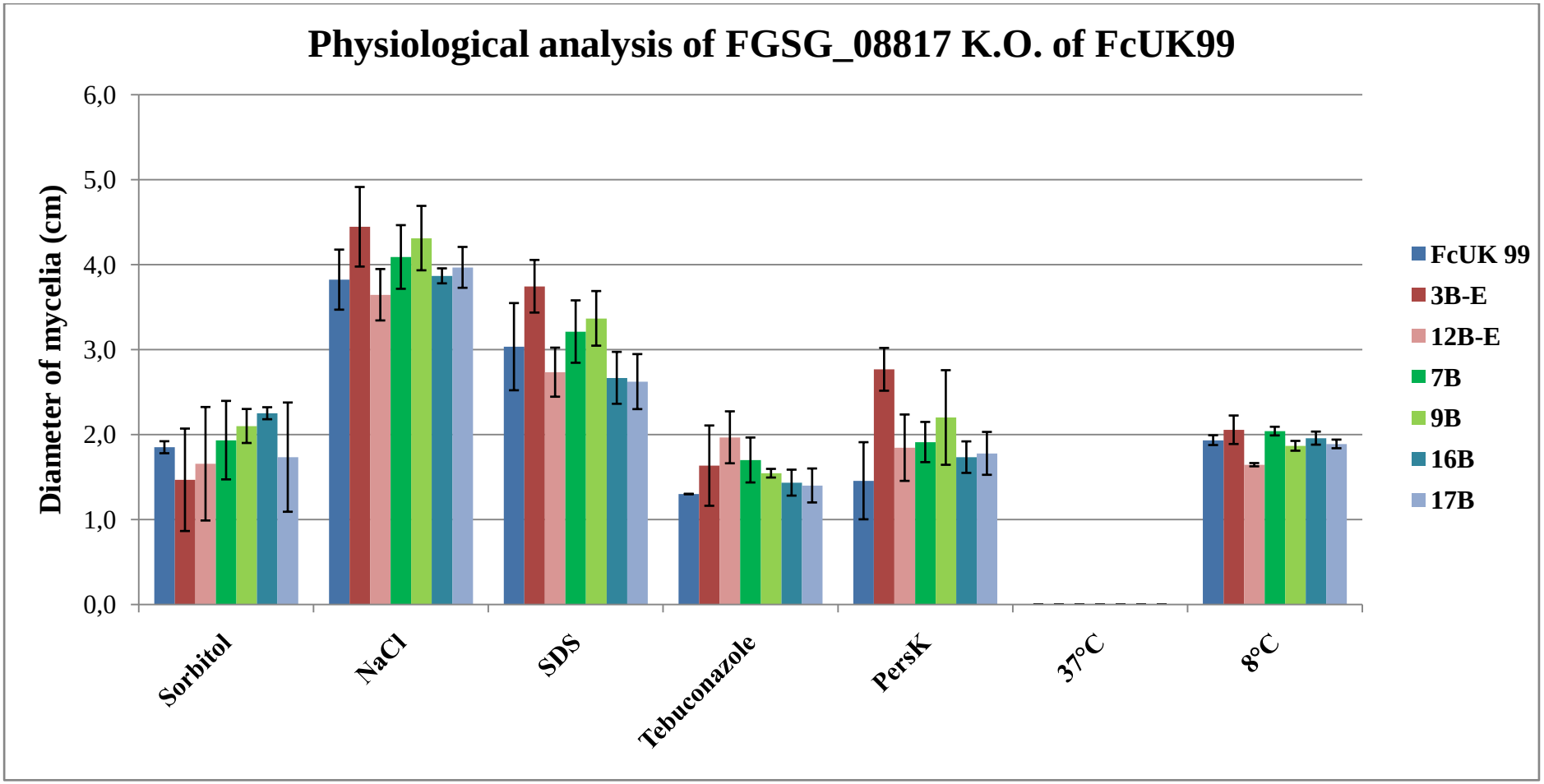

Figure 3.1: Growth of mycelium (*FGSG_08817*) in the presence of stresses caused by 0.02% SDS, 1M NaCl, 2M Sorbitol, 30 mM Potassium Persulfate, 0.5 ppm Tebuconazole, 37°C heat and 8°C cold.

It was observed that in the presence of 2M sorbitol, overall growth of all strains were very similar and around 2 cm for each strain (Figure 3.1). In the presence of 1M NaCl, the best growing strain was 3B-E, however, growth of all strains still remained similar. The presence of sorbitol and sodium chloride at these concentrations lead to osmotic stress in fungi (Hirasawa, *et al.*, 2006). The results suggested that there was no difference between the growth of the wild type and the ectopic and mutant strains.Therefore,the deletion of gene *FGSG_08817* does not seem to contribute to resistance or sensitivity against osmotic stress.

In the presence of 0.02 % SDS, the wild type strain FcUK99 showed an average of 3.03 cm growth, where the ectopic strain 3B-E shows a slightly higher growth. The other ectopic strain and the four knock-out mutants also showed similar growth behaviour. In addition, it was shown that in the presence of 0.5 ppm tebuconazole, the least growing strain was FcUK99; however, growth did not change dramatically between the six strains and the control. SDS and tebucanozole both lead to cell wall damage. SDS is an ionic detergent that solubilizes membrane proteins and was shown to be harmful for the cell wall composition (Filip, *et al.*, 1973). Tebuconazole is a well-known fungicide and it inhibits ergosterol biosynthesis of fungal phytopathogens (Hewitt, 1998). In previous studies, it was shown that cell wall stress on filamentous fungi leads to activation of chitin biosynthesis genes and an increased level of chitin in the cell wall (Ram, *et al.*, 2004). In Figure 3.1, it is shown that cell wall stress in the environment did not change the growth of mutant and ectopic strains. Deletion of gene *FGSG_08817*did neither confer resistance, nor sensitivity against cell wall damaging compounds, and hence, this gene is not related to any pathway concerning cell wall stability in *F. culmorum*.

In the presence of 30mM potassium persulfate in the media, it was observed that 3B-E ectopic strain shows the highest growth. Remaining ectopic strain and the four knock-outs showed a similar growth value, which was slightly higher than that of FcUK99. Potassium persulfate is a very strong oxidative agent, and it chemically degrades chitosan which is a deacetylated form of chitin and is located on cell wall and septa of yeast and filamentous fungi (Vlasov, *et al.*, 2009; Muzzarelli, *et al.*, 2012). The presence of potassium persulfate leads to oxidative stress on fungi. Additionally, upon infection of plants by phytopathogenic fungi, it was shown that plants generate hydrogen peroxide and superoxide as a defense mechanism against

pathogens (Kachroo, *et al.*, 2003). Therefore, if deletion of gene *FGSG_08817* leads to a higher growth rate under oxidative stress, it will provide a better chance at infecting the plant, hence increasing the pathogenicity; or vice versa. Since all ectopic and mutant strains display growth somewhat similar to that of the wild type; it can be stated that gene *FGSG_08817* is not involved in any pathways of *F. culmorum* regarding resistance to oxidative stress.

Under thermal stress at 37°C, no mycelial growth was observed for any of the wild type, mutant or ectopic strains. Alternatively, at 8°C thermal conditions, all strains grew around 2cm in diameter, approximately in the same manner. Therefore, it was shown that gene *FGSG_08817* does not contribute to any sensitivities or resistances against any of the tested thermal stresses.

3.1.2 *In vitro* pathogenicity assays of *FGSG_08817*knock-out mutants

In order to test the effects of gene knock-out on pathogenicity of *F. culmorum*, *in vitro* pathogenicity tests were carried out. Growths of seeds placed on plugs covered with mycelia were observed after 5 days of incubation in the dark. It was observed that 10 out of 10 seeds that were planted on control plugs (2%WA and PDA without mycelia) managed to grow successfully. Eight out of ten seeds placed on plugs containing FcUK99 were found to be infected, whereas the ectopic strains 3B-E and 12B-E showed a similar effect in pathogenicity, by infecting nine and ten out of ten seeds, respectively. Knock-out mutants also showed similar pathogenicity, where mutants 7B and 17B prevented the growth of all ten seeds, 16B prevented growth of nine out of ten seeds and 9B prevented eight out of ten seeds. These results suggest that deletion of this gene did not lead to a less pathogenic strain and hence, gene *FGSG_08817* does not play a very important role in pathogenicity of *F. culmorum.*

3.2 Characterization of *FGSG_16588*

3.2.1 *In vitro* phenotypical bioassays of *FGSG_16588*knock-out mutants

Deletion of gene *FGSG_16588* was investigated through analysis of one ectopic (8-E) and one mutant knock-out (9) strain, along with Fc21WT, the original strain. Results of growth under different stress conditions are shown in Table 3.2 and Figure 3.2.

Table 3.2: Diameters (cm) of mycelia growth for *FGSG_16588* strains (± stands for standard deviation of three biological replicates) under stress caused by 2M Sorbitol, 1M NaCl, 0.02% SDS, 0.5 ppm Tebuconazole, 30mM $K_2S_2O_8$, heat (37°C) and cold (8°C).

	Sorbitol	NaCl	SDS	Tebuc.	PersK	37°C	8°C
Fc21wt	1.74 ± 0.25	3.52 ± 0.21	3.7 ± 0.26	3.42 ± 0.59	1.37 ± 0.15	0.00	1.37 ±0.32
8-E	4.30 ± 0.20	4.99 ± 0.34	3.99 ± 0.14	2.63 ± 0.23	1.83 ± 0.2	0.00	1.27 ±0.06
9	1.40 ± 0.20	3.03 ± 0.06	3.43 ± 0.06	3.13 ± 0.06	0.87 ± 0.21	0.00	1.10 ±0.10

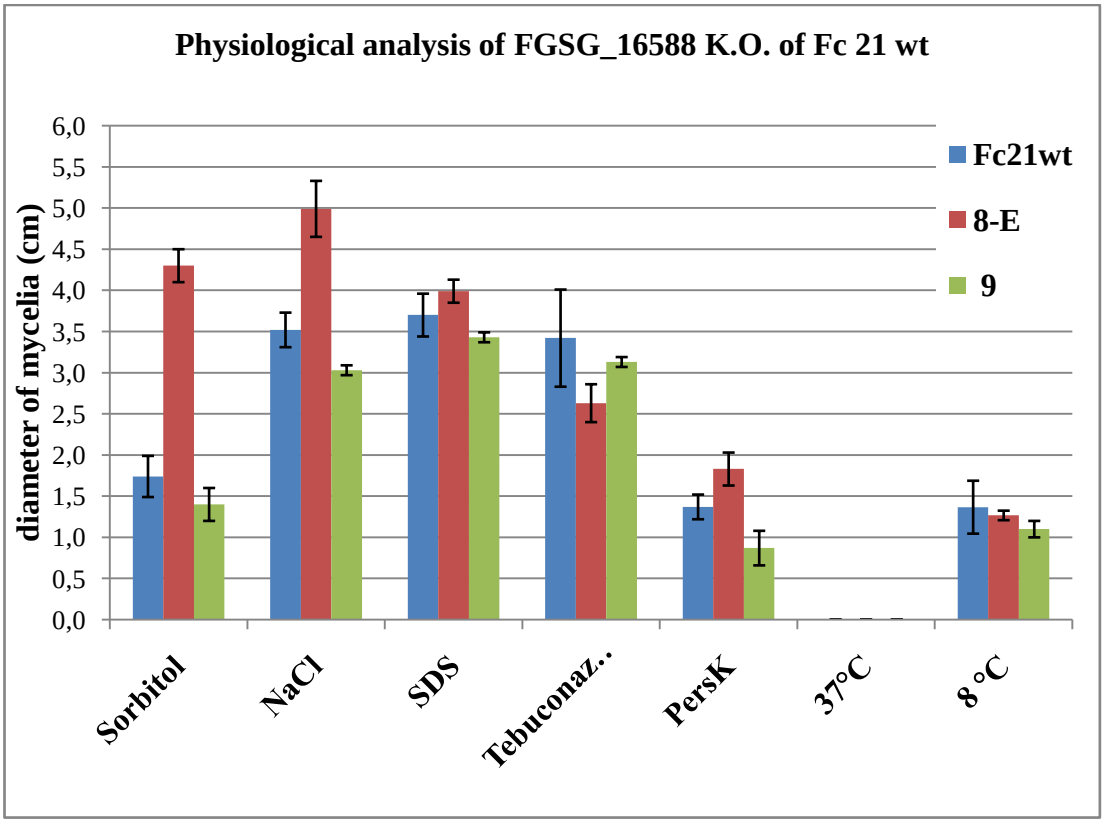

Figure 3.2: Growth of mycelium (*FGSG_16588*) in the presence of stresses caused by 0.02% SDS, 1M NaCl, 2M Sorbitol, 30 mM Potassium Persulfate, 0.5 ppm Tebuconazole, 37°C heat and 8°C cold.

When results in Figure 3.2 were observed, it was found that the ectopic strain 8-E showed significantly increased growth in the presence of oxidative stress elements; sorbitol and sodium chloride. In the presence of sorbitol, Fc21WT and 9 grew around 1.5 cm whereas 8-E grew approximately three times more compared to these two strains with 4.3 cm growth in diameter. Furthermore, the presence of NaCl showed a similar effect with around 1.5 cm increased growth of 8-E. Since 9 and Fc21WT showed similar growth, it can be said that deletion of this gene does not lead to any change in osmotic stress resistance. On the other hand, 8-E strain contains a DNA fragment (selective marker) which is randomly inserted in the genome, as a result of

previously carried out knock-out experiments. The selective marker may have been integrated in another gene, leading to a slight resistance to osmotic stress by disturbing this random gene which may be involved in pathways related to this stress. Addition of SDS or tebuconazole apparently did not affect the growth of these three strains, with each strain approximately having the same mycelial diameter. Hence, it can be stated that this gene does not contribute to resistance pathways against cell wall stress factors. The presence of the oxidative stress factor potassium persulfate led to different results, all of which were clustered around 1.4 cm mycelial growth. It was observed that the best growth rate was that of the 8-E strain, whereas strain 9 showed the lowest growth rate. However, differences in growth between these three strains were considered minor and it was concluded that this gene is not involved in mechanisms of environmental oxidative stress. Under thermal stress at 37°C, no mycelial growth was observed for any strain and at 8°C, all strains showed a similar growth, suggesting that gene *FGSG_16588* does not contribute to any sensitivities or resistances against any of the tested heat stresses.

3.2.2 *In vitro* pathogenicity assays of *FGSG_16588*knock-out mutants

Experiments were carried out in the same manner as described previously, for all three strains, along with the two control plates. An image of the results is shown in Figure 3.3.

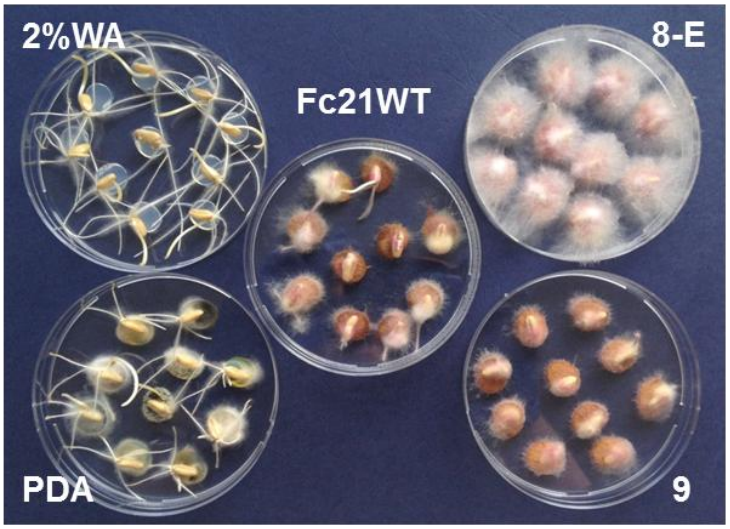

Figure 3.3: *In vitro* pathogenicity results for 2%WA and PDA control plates (on the left), Fc21WT (in the center), 8-E (upper right) and 9 (lower right).

The results in Figure 3.3 showed that all ten seeds planted on control plugs were grown successfully. Wild type Fc21WT was shown to fully infect 6 out of 10 seeds,

and the knock-out mutant 9 allowed growth of 7 out of 10 seeds. On the other hand, it was observed that 8-E strain successfully infected all ten seeds. Furthermore, this strain showed a higher growth compared to other strains, even growing strongly on seeds.

Considering the results of *in vitro* phenotypical bioassays, it can be stated that deletion of *FGSG_16588* does not lead to any changes in pathogenic behavior of *F. culmorum.* However, it is clear that pathogenicity of 8-E strain was enhanced in comparison to other strains, which may be related to its increased resistance to osmotic stress. Since insertion of selective marker is random, location of the DNA fragment is unknown. Further experiments can be suggested to determine the exact location of the insert to have a better understanding of this behavior.

3.3 Characterization of *FGSG_16572*

3.3.1 *In vitro* phenotypical bioassays of *FGSG_16572*knock-out mutants

After incubation at 25°C for 3 days, diametric mycelial growth of wild type strain Fc21WT and five knock-out mutants (2N, 3N, 5N, 6N & 7N) of *FGSG_16572* were measured.The results are shown in Table 3.3 and Figure 3.4.

Table 3.3: Diameters (cm) of mycelial growth for *FGSG_16572* strains (± stands for standard deviation of three biological replicates) under stress caused by 2M Sorbitol, 1M NaCl, 0.02% SDS, 0.5 ppm Tebuconazole, 30mM $K_2S_2O_8$, heat (37°C) and cold (8°C).

	Sorbitol	**NaCl**	**SDS**	**Tebuc.**	**PersK**	**37°C**	**8°C**
Fc21wt	2.13 ± 0.84	3.53 ± 0.12	3.09 ± 0.14	2.93 ± 0.17	0.50 ± 0.00	0.00	0.87 ± 0.06
2N	2.80 ± 0.70	4.57 ± 0.06	4.00 ± 0.35	3.37 ± 0.17	1.40 ± 0.17	0.00	1.78 ± 0.04
3N	2.23 ± 0.68	3.93 ± 0.12	3.01 ± 0.15	3.00 ± 0.26	0.80 ± 0.10	0.00	1.00 ± 0.17
5N	2.93 ± 0.21	3.53 ± 0.12	2.78 ± 0.52	3.23 ± 0.12	0.67 ± 0.15	0.00	1.17 ± 0.06
6N	2.70 ± 0.52	3.06 ± 0.19	2.87 ± 0.49	3.00 ± 0.00	0.63 ± 0.12	0.00	1.13 ± 0.12
7N	2.90 ± 0.17	3.57 ± 0.21	3.16 ± 0.42	3.38 ± 0.04	0.60 ± 0.10	0.00	1.01 ± 0.10

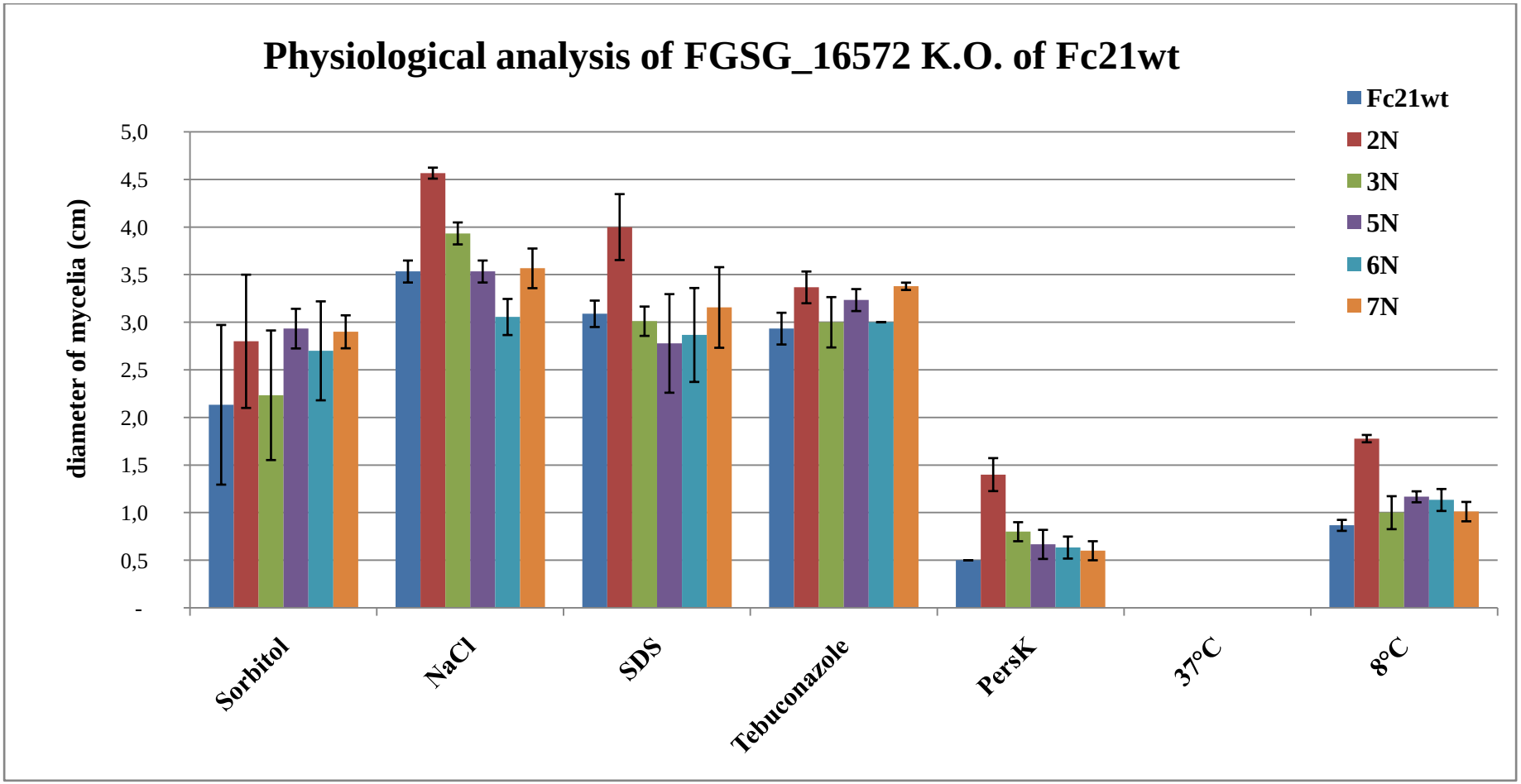

Figure 3.4: Growth of mycelium (*FGSG_16572*) in the presence of stresses caused by 0.02% SDS, 1M NaCl, 2M Sorbitol, 30 mM Potassium Persulfate, 0.5 ppm Tebuconazole, 37°C heat and 8°C cold.

Deletion of gene *FGSG_16572* was shown not to affect mycelial growth in the presence of sorbitol in culture media. When average values are considered, least growing strain at this condition was Fc21WT, whereas 5N and 7N knock-out mutants showed a greater diametric growth, where the difference is approximately 80mm. This difference can be considered not very important according to the standard deviation values. Additionally, the presence of NaCl led to varying growth levels clustered around 3.5cm. Strain 2N showed the highest growth in this case, followed by 3N. However, not all knock-out strains showed a higher growth compared to Fc21WT; 6N showed less and 5N and 7N showed almost the same growth. There is not an obvious pattern of difference between all strains when grown in the presence of osmotic stress factors sorbitol and sodium chloride. Therefore, it is suggested that gene *FGSG_16572* does not affect growth of *F. culmorum* when osmotic stress is present. Existence of SDS in media leads to the highest growth in strain 2N followed by all remaining strains with around a 1cm difference in diametric growth. On the other hand, presence of tebuconazole does not cause a difference in growth of any of the strains, where all strains show a similar growth, around 3cm. Tebuconazole and SDS are the cell wall stressing conditions that are applied to all strains. It was observed that when in the presence of SDS, 2N strain grew much more than all the remaining strains, but when in the presence of tebuconazole, it grew in the same manner with other strains. Therefore, it can be stated that deletion of gene *FGSG_16572* does not lead to any resistances or sensitivities on cell wall. In the presence of oxidative stress factor potassium persulfate it was seen that, once again, strain 2N shows an increased growth compared to other strains which have almost the same growth values, around 0.5cm in diameter. Therefore, it can be stated that only strain 2N gained a somewhat considerable resistance against oxidative stress, however, since remaining knock-out mutants don't show a meaningful difference in growth in comparison to Fc21WT, it was concluded that *FGSG_16572* is not related to any pathways considering oxidative stress response. In thermal stress conditions, at 37°C, no growth was observed for any strain, as well as at 8°C, all strains showed a similar growth overall, with again strain 2N leading in growth. Therefore, gene *FGSG_16572* is not an essential gene when coping with thermal stress at these tested temperatures. But strain 2N showed an increased, over-average growth in almost all tested conditions.

3.3.2 *In vitro* pathogenicity assays of *FGSG_16572*knock-out mutants

In vitro pathogenicity assays were done as described previously, testing strains Fc21WT, 2N, 3N, 5N, 6N and 7N. All ten seeds at each control (PDA and WA 2%) group were seen to be growing, whereas Fc21WT prevented growth of 7 out of 10 seeds. 5N and 6N strains allowed growth of 2 out of 10 seeds and remaining strains 2N, 3N and 7N strains prevented the growth of all ten seeds. These results suggest that deletion of gene *FGSG_16572* does not decrease the level of pathogenicity of *F. culmorum*; therefore it can be stated that this gene is not related to pathogenic behavior of *F. culmorum.*

3.4 Genetic and Molecular Aspects of Toxin Production in the Presence of Chemicals

3.4.1 RNA isolation and Quantitative Real Time PCR experiments

In order to carry out Q-RT PCR experiments, RNA was isolated after 5 days of fungal incubation.RNA concentrations for all three biological repeats of each compound group were measured with 1:16 diluted RNA samples and concentration of stock was calculated. RNA isolation of first group was carried out where ferulic acid, ferulic acid dimer, Compound 1 and 2 were tested in final concentration 0.25mM, alongside control plates containing 3mM β-cyclodextrin (referred to as β-CD). Resulting RNA concentrations along with OD 260/280 ratio were listed in Table 3.4. The second group of this experiment consisted of compound 3 at a final concentration 0.25mM and Compound 4 at three different concentrations; 0.25mM, 0.10mM and 0.05mM, along with a control group containing 3mM β-cyclodextrin (β-CD). RNA concentrations and OD 260/280 ratio are shown in Table 3.5.

Table 3.4: RNA concentrations, 260 nm values and OD 260/280 ratio in the presence of ferulic acid, ferulic acid dimer, compound 1, compound 2 and β-CD (control).

Compound		Concentration (ng/µL)	260 nm	OD 260/280
β-CD	Set 1	471.7	0.737	1.8
	Set 2	517.1	0.808	1.81
	Set 3	621.4	0.971	1.86
ferulic acid	Set 1	526.1	0.822	1.92
	Set 2	682.2	1.066	1.85
	Set 3	572.8	0.895	1.81
ferulic acid dimer	Set 1	617	0.964	1.69
	Set 2	599	0.936	1.84
	Set 3	345.6	0.54	1.77
Compound 1	Set 1	510.1	0.797	1.79
	Set 2	591.4	0.924	1.8
	Set 3	394.9	0.617	1.69
Compound 2	Set 1	348.8	0.545	1.69
	Set 2	518.4	0.81	1.75
	Set 3	496.6	0.776	1.72

Table 3.5: RNA concentrations, 260 nm values and OD 260/280 ratio in the presence of compounds 3 and 4 at given final concentrations and β-CD (control).

Compound			Concentration (ng/µL)	260 nm	OD 260/280
β-CD		Set 1	273.9	0.428	1.74
		Set 2	60.2	0.094	2.18
		Set 3	206.7	0.323	2.02
Compound 3	0.25 mM	Set 1	166.4	0.26	2.03
		Set 2	154.2	0.241	2.01
		Set 3	239.4	0.374	1.82
Compound 4	0.25 mM	Set 1	204.2	0.319	1.85
		Set 2	188.2	0.294	1.81
		Set 3	221.4	0.346	1.9
	0.10 mM	Set 1	186.2	0.291	1.8
		Set 2	296.3	0.463	1.98
		Set 3	345.6	0.54	1.76
	0.05 mM	Set 1	222.1	0.347	1.77
		Set 2	201	0.314	1.86
		Set 3	137.6	0.215	1.79

Tables 3.4 and 3.5 show that highly concentrated RNA were isolated for most samples. Quality of each RNA was verified by including OD 260/280 absorbance ratio where most samples showed ideal values (between 1.8 and 2.0) or values close to this interval.

Using these RNA as stocks, real time PCR experiments were carried out, again in two groups. Genes *FPP*, *TRI5*, *TRI4*, *TRI11*, *TRI3*, *TRI13*, *TRI6* and *TRI10* were analyzed for all samples and *18S* gene was contributed as the reference gene. In each consecutive real time PCR experiment, a different gene was analyzed; hence, melting curve and amplification curves were plotted with all tested samples for each gene, individually. Amplification and melting curves for the first group where compounds ferulic acid, ferulic acid dimer, compound 1 and 2 were tested can be seen in Figure 3.5 and Figure 3.6, respectively.

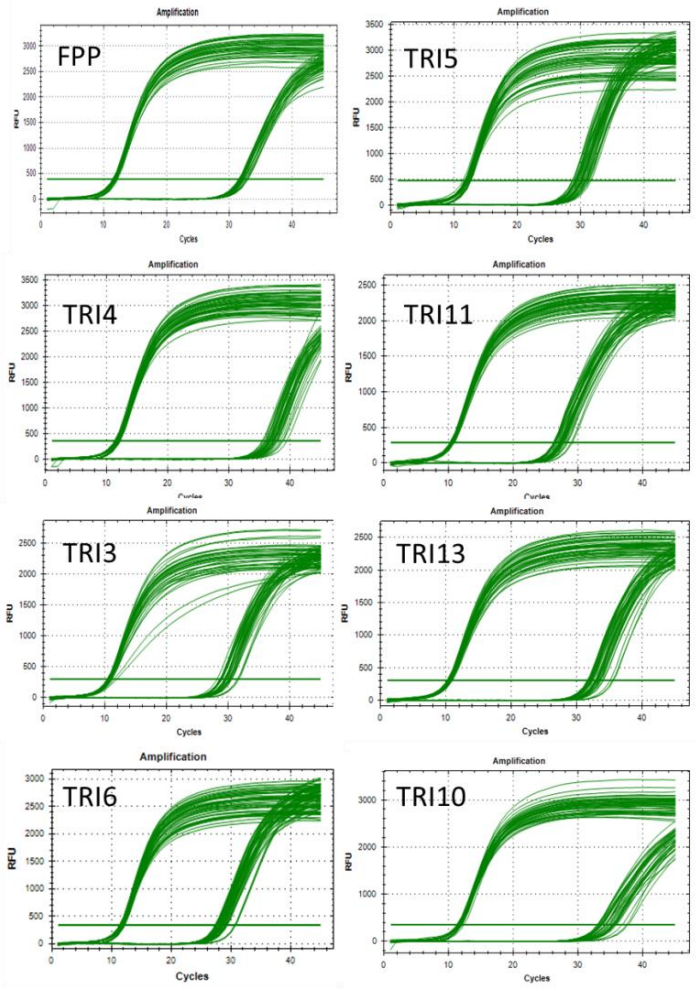

Figure 3.5: Amplification curves for indicated genes in the presence of ferulic acid, ferulic acid dimer, compounds 1 and 2, and β-CD (control).

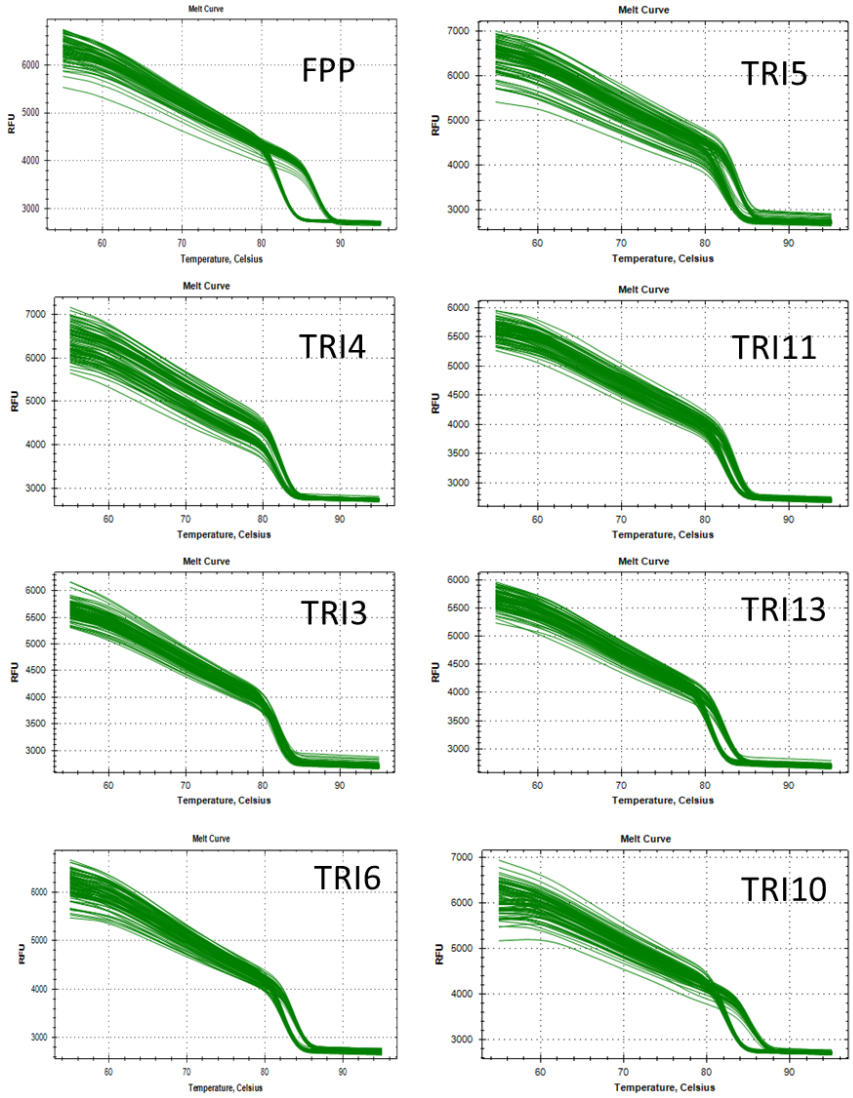

Figure 3.6: Melting Curve Analysis for indicated genes in the presence of ferulic acid, ferulic acid dimer, compounds 1 and 2, and β-CD (control).

Figure 3.5 shows that in all repeats carried out for each gene to be analyzed, reference gene amplification was shown to get to the exponential phase around 11th cycle, which shows that the presence of chemicals does not affect the expression of the reference gene *18S* and that it is a reliable reference gene. Melting curve analysis(Figure 3.6) showed that melting temperatures of genes were similar to that of *18S*, and denaturation of double stranded nucleic acids formed during amplifications were in unity.

Amplification curves and melting curve analysis of second group (compounds 3 and 4 (three different concentrations) and β-CD) can be seen in Figure 3.7 and Figure 3.8, respectively.

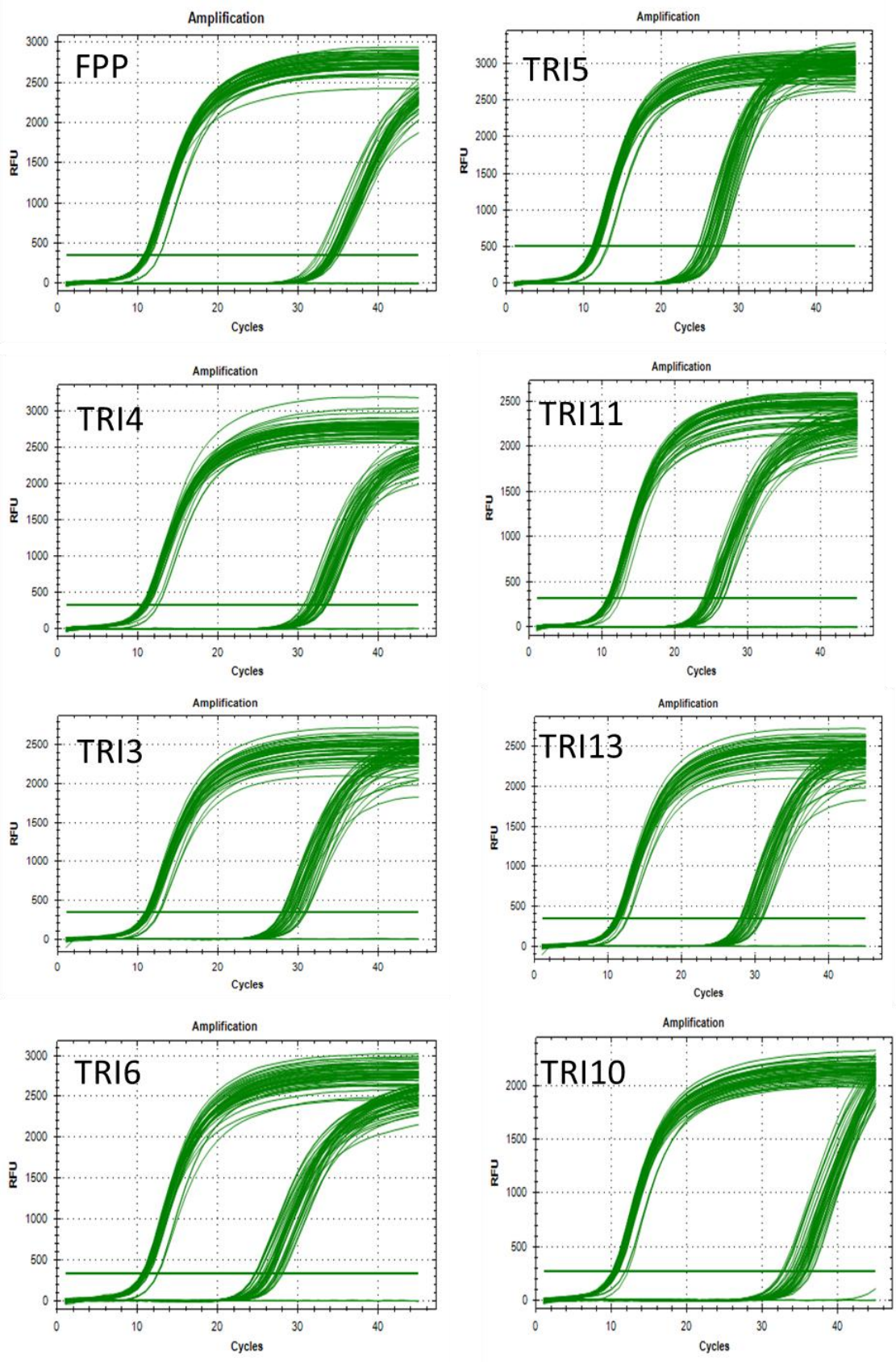

Figure 3.7: Amplification curves for indicated genes in the presence of compounds 3 and 4, and β-CD (control).

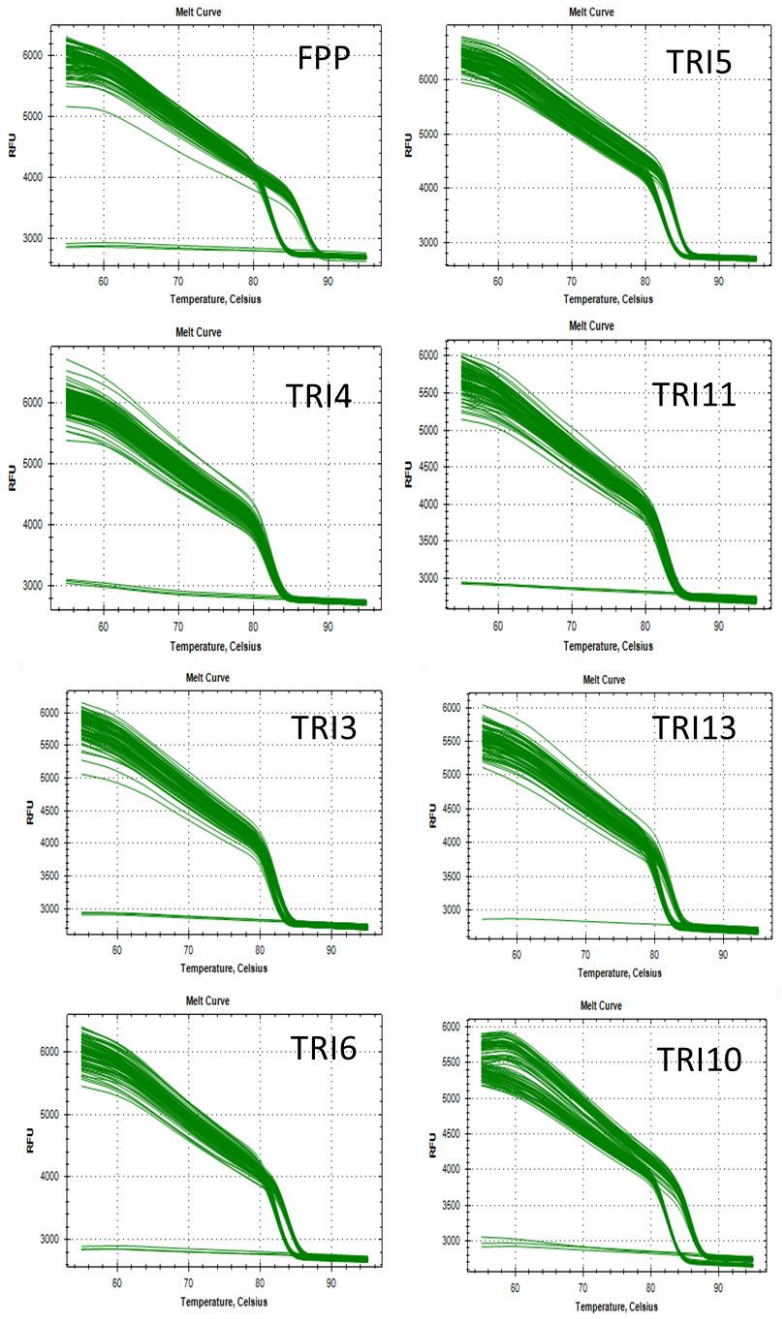

Figure 3.8: Melting Curve Analysis for indicated genes in the presence of compounds 3 and 4, and β-CD (control).

Results shown in Figure 3.7 and Figure 3.8 were seen to be in unity with the previously displayed results of the first group of chemicals. Amplifications and melting curves showed similar changes, leading to confirmation of reliability of *18S* as the reference gene and of the real time polymerization reaction.

Threshold cycle (C_T) values were determined after Real Time Experiments. By using C_T values, normalized expressions of each gene were calculated by using CFX Manager Software (Bio-Rad, USA). This software uses a derivative of $\Delta\Delta C_T$ method

where primer efficiencies are taken into consideration. Obtained C_T values and relative expression data for the early-trichothecene pathway genes *FPP*, *TRI5* and *TRI4* for the first group of chemicals are shown in Table 3.6.

Table 3.6: C_T, $\Delta\Delta C_T$ and standard error values for genes *FPP*, *TRI5* and *TRI4*in the presence of ferulic acid, ferulic acid dimer, compounds 1 and 2, and β-CD (control) (± : Standard deviation).

		FPP		***TRI5***		***TRI4***	
		C_T^{18S}	**C_T^{Fpp}**	**C_T^{18S}**	**C_T^{Tri5}**	**C_T^{18S}**	**C_T^{Tri4}**
B-CD	**Set 1**	11.60±0.11	31.70±0.25	11.97±0.20	29.34±0.07	11.40±0.05	36.41±0.72
	Set 2	11.96±0.24	31.60±0.11	11.99±0.15	29.98±0.23	12.00±0.06	37.06±0.36
	Set 3	11.88±0.14	32.47±0.19	11.75±0.38	30.31±0.14	11.85±0.25	37.32±0.41
	ΔΔCT	**1**		**1**		**1**	
	Std.Err.	**0.11**		**0.11**		**0.15**	
		C_T^{18S}	**C_T^{Fpp}**	**C_T^{18S}**	**C_T^{Tri5}**	**C_T^{18S}**	**C_T^{Tri4}**
ferulic acid	**Set 1**	11.65±0.07	32.31±0.18	11.89±0.15	29.40±0.15	11.63±0.25	36.69±0.64
	Set 2	11.48±0.05	31.88±0.34	11.72±0.03	31.10±0.09	11.51±0.21	38.33±0.63
	Set 3	11.44±0.05	32.66±0.27	11.85±0.23	30.78±0.10	11.66±0.24	37.01±0.41
	ΔΔCT	**0.65**		**0.64**		**0.68**	
	Std.Err.	**0.06**		**0.11**		**0.14**	
		C_T^{18S}	**C_T^{Fpp}**	**C_T^{18S}**	**C_T^{Tri5}**	**C_T^{18S}**	**C_T^{Tri4}**
ferulic acid dimer	**Set 1**	11.60±0.23	32.92±0.15	11.67±0.05	31.35±0.20	11.40±0.03	37.81±0.47
	Set 2	11.95±0.07	32.49±0.26	12.16±0.08	30.18±0.08	11.90±0.31	37.50±0.35
	Set 3	11.48±0.05	31.81±0.03	11.68±0.08	28.68±0.07	11.26±0.07	35.74±0.36
	ΔΔCT	**0.66**		**0.83**		**0.81**	
	Std.Err.	**0.08**		**0.22**		**0.2**	
		C_T^{18S}	**C_T^{Fpp}**	**C_T^{18S}**	**C_T^{Tri5}**	**C_T^{18S}**	**C_T^{Tri4}**
Compound 1	**Set 1**	11.68±0.10	32.04±0.05	12.04±0.21	30.70±0.17	11.89±0.12	37.99±1.42
	Set 2	11.82±0.21	31.87±0.26	11.92±0.04	30.10±0.04	11.60±0.03	36.65±0.43
	Set 3	12.09±0.37	32.08±0.19	12.08±0.05	30.07±0.09	11.79±0.28	36.51±0.02
	ΔΔCT	**0.93**		**0.8**		**0.92**	
	Std.Err.	**0.06**		**0.06**		**0.22**	
		C_T^{18S}	**C_T^{Fpp}**	**C_T^{18S}**	**C_T^{Tri5}**	**C_T^{18S}**	**C_T^{Tri4}**
Compound 2	**Set 1**	11.98±0.154	31.99±0.20	12.46±0.01	29.06±0.08	11.83±0.20	36.02±0.44
	Set 2	11.96±0.24	31.97±0.22	12.43±0.06	29.99±0.14	12.02±0.01	37.12±0.02
	Set 3	12.10±0.24	31.50±0.05	12.59±0.04	29.24±0.10	11.87±0.26	35.99±0.15
	ΔΔCT	**1.18**		**1.94**		**1.61**	
	Std.Err.	**0.09**		**0.19**		**0.23**	

C_T values and relative expression ($\Delta\Delta C_T$) data for the middle-trichothecene pathway genes *TRI11*, *TRI3* and *TRI13* for the first group of chemicals are shown in Table 3.7.

Table 3.7: C_T, $\Delta\Delta C_T$ and standard error values for genes *TRI11*, *TRI3* and *TRI13*in the presence of ferulic acid, ferulic acid dimer, compounds 1 and 2, and β-CD (control) (± : Standard deviation).

		TRI11		***TRI3***		***TRI13***	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
B-CD	**Set 1**	10.32±0.13	26.33±0.14	10.63±0.32	28.83±0.14	10.26±0.16	31.67±0.15
	Set 2	10.41±0.05	27.27±0.01	10.47±0.18	29.75±0.03	10.31±0.14	33.05±0.12
	Set 3	10.27±0.15	27.55±0.12	10.4±0.08	29.84±0.21	10.31±0.06	33.37±0.15
	ΔΔCT	**1**		**1**		**1**	
	Std.Err.	**0.13**		**0.12**		**0.18**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
ferulic acid	**Set 1**	10.43±0.10	26.73±0.10	10.60±0.06	29.15±0.19	10.52±0.12	32.13±0.07
	Set 2	10.51±0.05	28.25±0.10	10.66±0.10	30.52±0.06	10.59±0.10	33.83±0.32
	Set 3	10.24±0.04	27.55±0.15	10.43±0.07	30.26±0.11	10.29±0.09	33.48±0.18
	ΔΔCT	**0.76**		**0.73**		**0.82**	
	Std.Err.	**0.11**		**0.11**		**0.15**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
ferulic acid dimer	**Set 1**	10.48±0.07	28.99±0.13	10.57±0.14	31.58±0.13	10.43±0.04	35.35±0.25
	Set 2	10.44±0.04	27.53±0.09	11.37±0.64	30.12±0.07	10.58±0.10	33.32±0.31
	Set 3	10.44±0.13	26.34±0.14	10.51±0.05	28.81±0.15	10.43±0.15	32.06±0.15
	ΔΔCT	**0.83**		**0.75**		**0.62**	
	Std.Err.	**0.21**		**0.23**		**0.21**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
Compound 1	**Set 1**	10.55±0.02	27.82±0.16	10.79±0.01	30.13±0.05	10.56±0.08	33.01±0.14
	Set 2	10.46±0.04	27.06±0.02	10.81±0.16	29.39±0.03	10.51±0.17	32.18±0.09
	Set 3	10.61±0.03	27.29±0.16	10.97±0.15	29.43±0.09	10.80±0.03	32.34±0.16
	ΔΔCT	**0.91**		**1.11**		**1.41**	
	Std.Err.	**0.07**		**0.1**		**0.14**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
Compound 2	**Set 1**	10.73±0.17	26.22±0.05	10.97±0.20	28.41±0.31	10.79±0.22	31.78±0.10
	Set 2	10.53±0.10	27.19±0.09	10.75±0.10	29.62±0.19	10.67±0.12	32.86±0.19
	Set 3	10.58±0.11	26.30±0.09	11.08±0.11	28.82±0.08	10.72±0.12	31.68±0.09
	ΔΔCT	**1.65**		**1.79**		**1.99**	
	Std.Err.	**0.18**		**0.25**		**0.27**	

Finally, C_T values and relative expression ($\Delta\Delta C_T$) data for the regulatory genes of trichothecene pathway; *TRI6* and *TRI10*, of the first group of chemicals are shown in Table 3.8.

Table 3.8: C_T, $\Delta\Delta C_T$ and standard error values for genes *TRI6* and *TRI10*in the presence of ferulic acid, ferulic acid dimer, compounds 1 and 2, and β-CD (control) (± : Standard deviation).

		TRI6		***TRI10***	
		C_T^{18S}	C_T^{Tri6}	C_T^{18S}	C_T^{Tri10}
control	**Set 1**	11.50 ± 0.02	27.66 ± 0.06	11.41 ± 0.03	33.62 ± 0.11
	Set 2	11.75 ± 0.04	28.50 ± 0.08	11.78 ± 0.13	34.70 ± 0.57
	Set 3	11.71 ± 0.16	28.59 ± 0.02	11.82 ± 0.20	35.52 ± 0.18
	ΔΔCT	**1**		**1**	
	Std.Err.	**0.1**		**0.21**	
		C_T^{18S}	C_T^{Tri6}	C_T^{18S}	C_T^{Tri10}
ferulic acid	**Set 1**	11.55 ± 0.07	27.68 ± 0.16	11.45 ± 0.04	33.53 ± 0.14
	Set 2	11.34 ± 0.02	29.31 ± 0.06	11.33 ± 0.03	35.35 ± 0.23
	Set 3	11.51 ± 0.26	28.96 ± 0.15	11.67 ± 0.57	35.65 ± 0.27
	ΔΔCT	**0.68**		**0.75**	
	Std.Err.	**0.12**		**0.19**	
		C_T^{18S}	C_T^{Tri6}	C_T^{18S}	C_T^{Tri10}
ferulic acid dimer	**Set 1**	11.41 ± 0.05	30.69 ± 0.08	11.36 ± 0.05	37.32 ± 0.47
	Set 2	11.96 ± 0.20	29.10 ± 0.13	11.94 ± 0.18	35.60 ± 0.17
	Set 3	11.52 ± 0.22	27.74 ± 0.20	11.30 ± 0.10	33.30 ± 0.45
	ΔΔCT	**0.53**		**0.52**	
	Std.Err.	**0.16**		**0.22**	
		C_T^{18S}	C_T^{Tri6}	C_T^{18S}	C_T^{Tri10}
Compound 1	**Set 1**	11.53 ± 0.08	28.53 ± 0.15	11.53 ± 0.09	34.51 ± 1.08
	Set 2	11.70 ± 0.16	28.14 ± 0.10	11.57 ± 0.28	34.32 ± 0.23
	Set 3	11.73 ± 0.08	28.19 ± 0.22	11.78 ± 0.19	34.47 ± 0.39
	ΔΔCT	**0.98**		**1.11**	
	Std.Err.	**0.06**		**0.16**	
		C_T^{18S}	C_T^{Tri6}	C_T^{18S}	C_T^{Tri10}
Compound 2	**Set 1**	11.95 ± 0.21	27.39 ± 0.06	11.85 ± 0.20	33.18 ± 0.11
	Set 2	12.10 ± 0.02	28.25 ± 0.01	11.72 ± 0.21	34.27 ± 0.03
	Set 3	12.14 ± 0.05	27.47 ± 0.07	11.71 ± 0.31	33.36 ± 0.35
	ΔΔCT	**1.86**		**2.18**	
	Std.Err.	**0.2**		**0.3**	

For the second group of chemicals, C_T values and relative expression ($\Delta\Delta C_T$) data of early-trichothecene production pathway genes *FPP*, *TRI5* and *TRI4*are shown in Table 3.9.

Table 3.9: C_T, $\Delta\Delta C_T$ and standard error values for genes *FPP*, *TRI5* and *TRI4*in the presence of compounds 3 and 4 (all tested concentrations) and β-CD (control) (± : Standard deviation).

		FPP		***TRI5***		***TRI4***	
		C_T^{18S}	C_T^{Fpp}	C_T^{18S}	C_T^{Tri5}	C_T^{18S}	C_T^{Tri4}
control	**Set 1**	10.84±0.01	34.21±0.25	11.26±0.03	25.31±0.01	10.76±0.17	31.78±0.23
	Set 2	11.47±0.05	34.00±0.24	11.99±0.07	25.34±0.07	11.34±0.04	32.55±0.18
	Set 3	10.71±0.01	34.28±0.14	11.23±0.09	24.61±0.03	10.66±0.11	31.23±0.08
	ΔΔCT	**1**		**1**		**1**	
	Std.Err.	**0.09**		**0.11**		**0.15**	
		C_T^{18S}	C_T^{Fpp}	C_T^{18S}	C_T^{Tri5}	C_T^{18S}	C_T^{Tri4}
Compound 3	**Set 1**	10.79±0.07	33.87±0.26	11.25±0.05	25.66±0.23	10.62±0.06	32.63±0.13
	Set 2	10.80±0.07	34.09±0.71	11.19±0.04	25.49±0.03	10.63±0.10	32.15±0.12
	Set 3	10.67±0.07	33.74±0.12	11.08±0.04	25.51±0.01	10.46±0.10	31.51±0.19
	ΔΔCT	**0.82**		**0.59**		**0.67**	
	Std.Err.	**0.11**		**0.02**		**0.08**	
		C_T^{18S}	C_T^{Fpp}	C_T^{18S}	C_T^{Tri5}	C_T^{18S}	C_T^{Tri4}
Compound 4 0.25mM	**Set 1**	10.96±0.05	33.74±0.12	11.36±0.07	25.85±0.07	10.76±0.05	31.50±0.13
	Set 2	11.22±0.07	33.98±0.62	11.63±0.07	26.32±0.06	11.11±0.02	32.61±0.16
	Set 3	11.06±0.04	34.41±0.12	11.53±0.05	27.56±0.03	10.96±0.07	33.51±0.06
	ΔΔCT	**1.14**		**0.37**		**0.63**	
	Std.Err.	**0.12**		**0.06**		**0.13**	
		C_T^{18S}	C_T^{Fpp}	C_T^{18S}	C_T^{Tri5}	C_T^{18S}	C_T^{Tri4}
Compound 4 0.10mM	**Set 1**	10.73±0.03	34.28±0.095	11.07±0.05	26.57±0.08	10.57±0.06	32.38±0.26
	Set 2	10.83±0.06	34.18±0.26	11.24±0.06	27.02±0.05	10.65±0.06	33.35±0.17
	Set 3	10.74±0.07	34.01±0.17	11.17±0.01	27.28±0.09	10.52±0.04	32.37±0.09
	ΔΔCT	**0.86**		**0.23**		**0.45**	
	Std.Err.	**0.04**		**0.02**		**0.05**	
		C_T^{18S}	C_T^{Fpp}	C_T^{18S}	C_T^{Tri5}	C_T^{18S}	C_T^{Tri4}
Compound 4 0.05mM	**Set 1**	11.05±0.01	32.52±0.30	11.50±0.06	25.05±0.04	10.83±0.13	30.71±0.18
	Set 2	11.26±0.02	33.84±0.10	11.76±0.04	25.50±0.08	11.16±0.03	31.65±0.25
	Set 3	12.62±0.01	34.82±0.20	13.03±0.09	26.18±0.03	12.38±0.22	33.22±0.18
	ΔΔCT	**2.05**		**1.06**		**1.4**	
	Std.Err.	**0.64**		**0.2**		**0.42**	

C_T values and relative expression ($\Delta\Delta C_T$) data for the middle-trichothecene pathway genes *TRI11*, *TRI3* and *TRI13* obtained with the second group of chemicals are listed in Table 3.10.

Table 3.10: C_T, $\Delta\Delta C_T$ and standard error values for genes *TRI11*, *TRI3* and *TRI13*in the presence of compounds 3 and 4 (all tested concentrations) and β-CD (control) (± : Standard deviation).

		TRI11		***TRI3***		***TRI13***	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
control	**Set 1**	10.85±0.17	24.34±0.08	11.04±0.03	28.53±0.13	10.73±0.15	32.04±0.29
	Set 2	11.57±0.20	24.58±0.07	11.77±0.17	27.96±0.14	11.29±0.13	30.35±0.02
	Set 3	10.73±0.09	23.94±0.14	10.82±0.05	28.29±0.04	10.45±0.15	32.30±0.10
	ΔΔCT	**1**		**1**		**1**	
	Std.Err.	**0.11**		**0.12**		**0.24**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
Compound 3	**Set 1**	10.74±0.60	24.74±0.15	28.25±0.33	11.03±0.05	10.66±0.10	30.76±0.33
	Set 2	10.94±0.08	24.58±0.06	27.85±0.02	11.06±0.11	10.70±0.02	30.29±0.08
	Set 3	10.67±0.10	24.64±0.18	29.15±0.04	10.92±0.07	10.49±0.11	33.33±0.16
	ΔΔCT	**0.66**		**0.8**		**0.94**	
	Std.Err.	**0.03**		**0.11**		**0.31**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
Compound 4 0.25mM	**Set 1**	11.06±0.07	25.07±0.07	11.18±0.04	29.42±0.11	10.91±0.10	34.09±0.26
	Set 2	11.13±0.11	25.20±0.01	11.34±0.03	29.13±0.02	10.94±0.10	31.81±0.30
	Set 3	11.18±0.02	26.6±0.05	11.47±0.04	30.83±0.16	11.06±0.07	34.04±0.27
	ΔΔCT	**0.43**		**0.38**		**0.35**	
	Std.Err.	**0.07**		**0.07**		**0.09**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
Compound 4 0.10mM	**Set 1**	10.66±0.04	25.60±0.02	11.05±0.05	30.20±0.13	10.54±0.25	35.31±0.07
	Set 2	10.83±0.07	26.04±0.12	11.04±0.03	29.90±0.12	10.71±0.05	32.54±0.46
	Set 3	10.78±0.11	26.12±0.06	11.01±0.07	30.68±0.29	10.76±0.05	35.29±0.32
	ΔΔCT	**0.28**		**0.22**		**0.14**	
	Std.Err.	**0.02**		**0.02**		**0.04**	
		C_T^{18S}	C_T^{Tri11}	C_T^{18S}	C_T^{Tri3}	C_T^{18S}	C_T^{Tri13}
Compound 4 0.05mM	**Set 1**	11.10±0.09	24.17±0.13	11.21±0.11	28.61±0.25	11.06±0.08	34.07±0.24
	Set 2	11.35±0.12	24.62±0.14	11.50±0.12	28.98±0.11	11.29±0.15	32.36±0.33
	Set 3	12.25±0.32	25.17±0.02	12.58±0.10	29.47±0.06	12.24±0.74	32.26±0.16
	ΔΔCT	**1.03**		**0.86**		**0.67**	
	Std.Err.	**0.15**		**0.14**		**0.17**	

Finally, C_T values and relative expression ($\Delta\Delta C_T$) data for the regulatory genes of trichothecene pathway; *TRI6* and *TRI10*, of the second group of tested chemicals are shown in Table 3.11.

Table 3.11: C_T, $\Delta\Delta C_T$ and standard error values for genes *TRI6* and *TRI10*in the presence of compounds 3 and 4 (all tested concentrations) and β-CD (control) (± : Standard deviation).

		TRI6		***TRI10***	
		C_T^{18S}	**C_T^{Tri6}**	**C_T^{18S}**	**C_T^{Tri10}**
control	**Set 1**	10.72 ± 0.03	25.62 ± 0.11	10.27 ± 0.07	34.38 ± 0.38
	Set 2	11.42 ± 0.02	24.64 ± 0.07	10.92 ± 0.03	32.63 ± 0.18
	Set 3	10.63 ± 0.03	25.82 ± 0.01	10.20 ± 0.03	34.53 ± 0.28
	ΔΔCT	**1**		**1**	
	Std.Err.	**0.15**		**0.24**	
		C_T^{18S}	**C_T^{Tri6}**	**C_T^{18S}**	**C_T^{Tri10}**
Compound 3	**Set 1**	10.66 ± 0.14	25.13 ± 0.04	10.17 ± 0.03	33.72 ± 0.29
	Set 2	10.63 ± 0.02	24.73 ± 0.02	10.17 ± 0.08	33.13 ± 0.66
	Set 3	10.54 ± 0.04	26.44 ± 0.08	10.01 ± 0.00	34.45 ± 1.01
	ΔΔCT	**0.78**		**0.87**	
	Std.Err.	**0.14**		**0.17**	
		C_T^{18S}	**C_T^{Tri6}**	**C_T^{18S}**	**C_T^{Tri10}**
Compound 4 0.25mM	**Set 1**	10.90 ± 0.02	27.21 ± 0.05	10.30 ± 0.08	35.42 ± 0.19
	Set 2	11.21 ± 0.05	26.38 ± 0.14	10.66 ± 0.05	34.41 ± 0.11
	Set 3	11.06 ± 0.06	27.98 ± 0.17	10.56 ± 0.06	35.93 ± 0.24
	ΔΔCT	**0.32**		**0.38**	
	Std.Err.	**0.05**		**0.06**	
		C_T^{18S}	**C_T^{Tri6}**	**C_T^{18S}**	**C_T^{Tri10}**
Compound 4 0.10mM	**Set 1**	10.67 ± 0.06	27.47 ± 0.06	10.23 ± 0.07	35.74 ± 0.10
	Set 2	10.65 ± 0.03	26.72 ± 0.04	10.30 ± 0.08	34.71 ± 0.29
	Set 3	10.56 ± 0.07	27.50 ± 0.06	10.12 ± 0.07	36.24 ± 0.42
	ΔΔCT	**0.23**		**0.25**	
	Std.Err.	**0.02**		**0.04**	
		C_T^{18S}	**C_T^{Tri6}**	**C_T^{18S}**	**C_T^{Tri10}**
Compound 4 0.05mM	**Set 1**	10.97 ± 0.06	26.09 ± 0.04	10.42 ± 0.06	34.75 ± 0.08
	Set 2	11.17 ± 0.06	26.17 ± 0.02	10.76 ± 0.011	34.89 ± 0.12
	Set 3	12.52 ± 0.06	26.30 ± 0.01	11.91 ± 0.22	35.48 ± 0.28
	ΔΔCT	**0.86**		**0.62**	
	Std.Err.	**0.14**		**0.24**	

To observe the effects of each chemical separately, using data from Table 3.6, Table 3.7 and Table 3.8, graphs were plotted for each chemical tested in the first group. Expression of all genes investigated in this study in the presence of 0.25mM ferulic acid are shown in Figure 3.9, as fold expression of control group where each individual gene expression was estimated as 1.

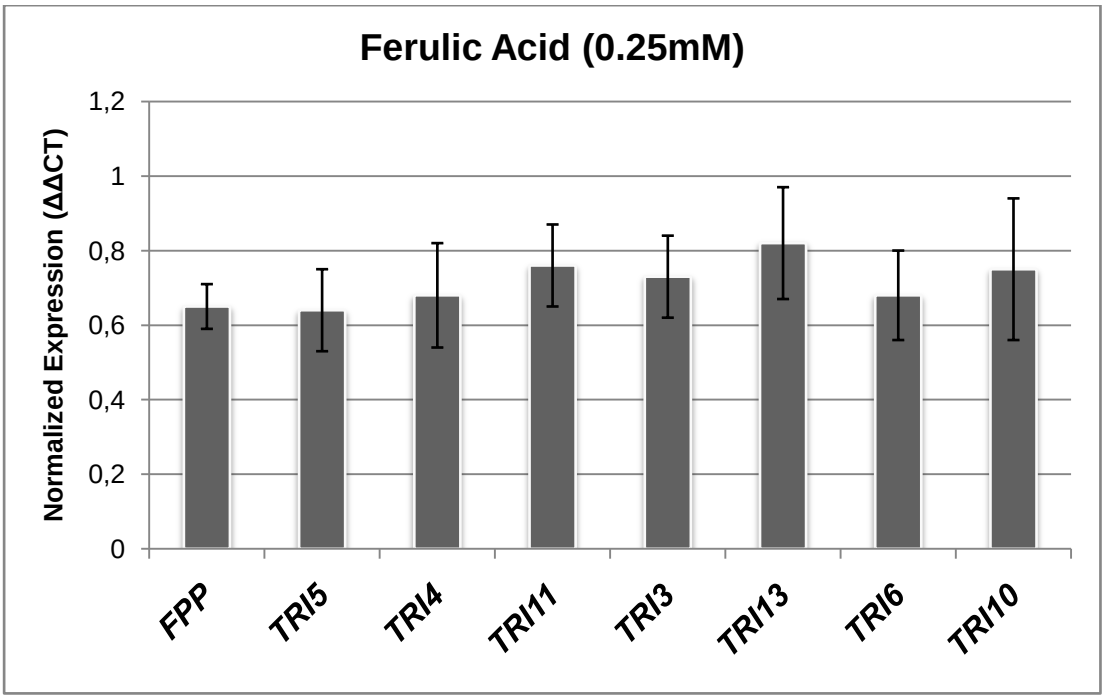

Figure 3.9: Expression levels of genes *FPP*, *TRI5*, *TRI4*, *TRI11*, *TRI3*, *TRI13*, *TRI6* and *TRI10*in the presence of 0.25mM ferulic acid, as fold of control.

Ferulic acid is the most abundant phenolic compound in wheat (Tian, *et al.*, 2004). In Figure 3.9, it can be seen that in the presence of 0.25mM ferulic acid, expression of all genes were lower than 1, meaning that they were all downregulated. Ferulic acid is a very important monomer in plant cell wall composition and its presence is often related to plant's defense mechanism against pathogens. It is known that ferulic acid has fungistatic effects on *F. graminearum* and can also be in relation to severity of plant diseases; however, in previous studies it was shown that these effects can vary between different strains of *Fusarium* (Assabgui, *et al.*, 1993; Bily, *et al.*, 2003). It was also shown that at this concentration, toxin production levels decreased to 57% in *F. graminearum* (Bily, *et al*., 2003). In another study, trichothecene pathway genes were investigated in the presence of 0.5mM ferulic acid where genes *FPP*, *TRI5*, *TRI4*, *TRI11*, *TRI6* and *TRI10* were shown to be expressed 1.5, 4.1, 4.8, 5.4, 2 and 2.3 times less, respectively (Boutigny, *et al.*, 2009). When compared to previous findings, it was found that the presence of 0.25mM ferulic acid showed a similar result, where gene expression was lowered in tested trichothecene pathway genes. However, lower concentrations of ferulic acid led to a less decreased gene expression level, compared to previous data.

Results of gene expression in the presence of 0.25mM ferulic acid dimer are shown in Figure 3.10 as fold expression of control.

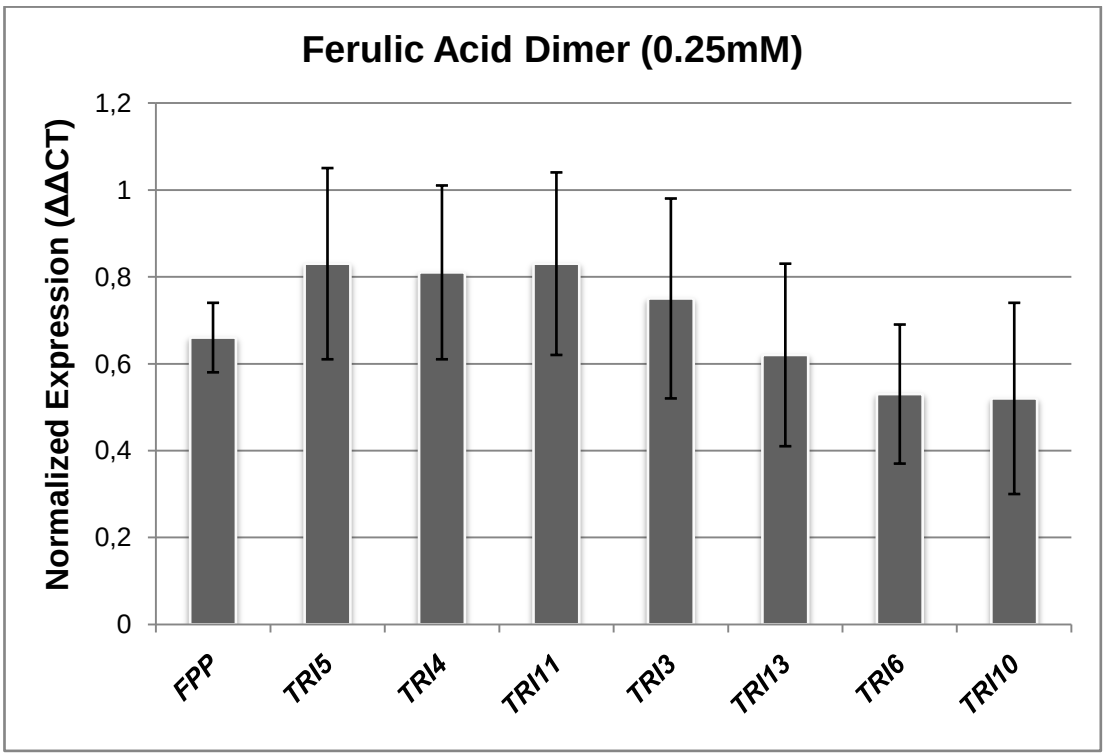

Figure 3.10: Expression levels of genes *FPP, TRI5, TRI4, TRI11, TRI3, TRI13, TRI6* and *TRI10* in the presence of 0.25mM ferulic acid dimer, as fold of control.

The presence of 0.25mM ferulic acid dimer was tested in this study. It was observed that this compound led to an overall downregulation in tested trichothecene pathways genes (Figure 3.10). In comparison to results of ferulic acid shown in Figure 3.9, it was observed that regulatory genes of this pathway were more downregulated, whereas the early and middle trichothecene pathway genes tested showed less downregulationin the presence of ferulic acid dimer (Figure 3.10). It was previously shown that naturally occurring wheat bran extract containing ferulic acid and its dimeric forms inhibit toxin production in *F. culmorum*. However, the content of this mixture has not been fully identified yet (Boutigny, *et al.*, 2010). Here, it was shown that diferulic acid also contributed to downregulation of toxin production and it is another important component of plant defense mechanism against pathogens.

Changes in gene expression in the presence of compound 1 (0.25mM) are shown in Figure 3.11 as fold of control gene expression.

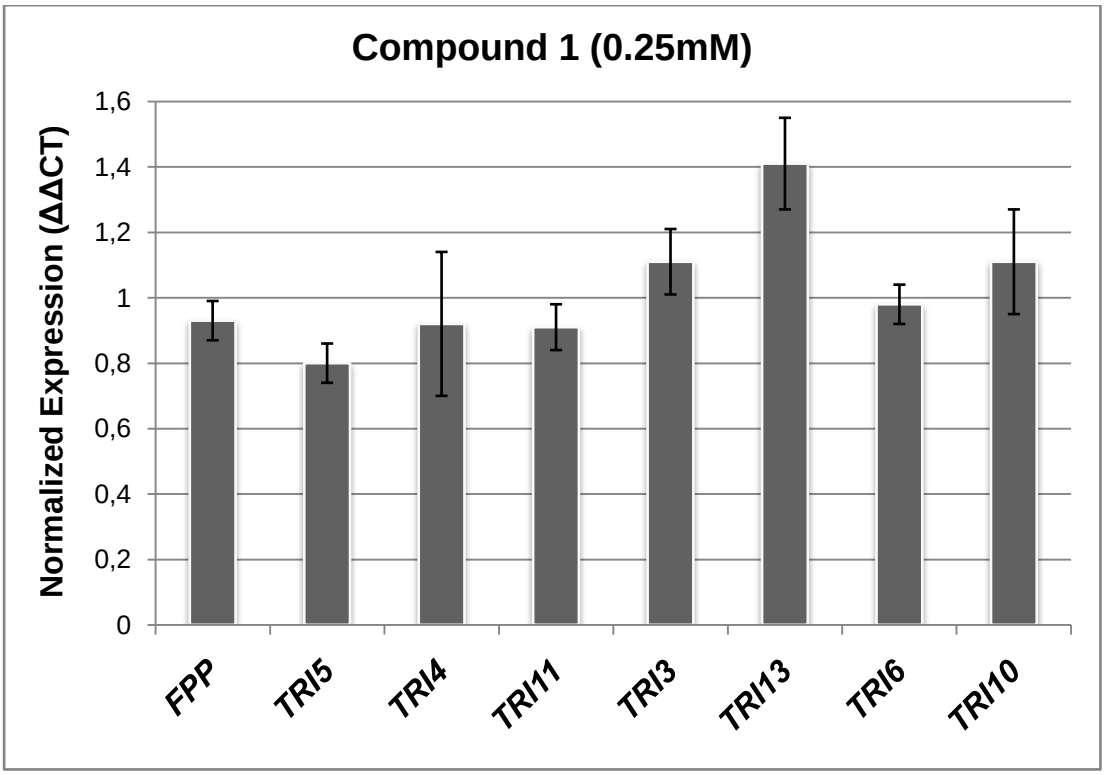

Figure 3.11: Expression levels of genes *FPP, TRI5, TRI4, TRI11, TRI3, TRI13, TRI6* and *TRI10*in the presence of compound 1 (0.25mM), as fold of control.

In Figure 3.11, it was observed that the expression levels ofall genes studied did not change dramatically when compound 1 was present in the culture media. Both regulatory genes *TRI6* and *TRI10* remained around 1, as well as *FPP*, *TRI4*, *TRI11* and *TRI3*. There was a slight downregulation in *TRI5* with an average fold-expression of 0.8. Additionally, *TRI13* showed a slightly increased expression level with 1.4-fold expression when compared to that of control. However, when the pathway was considered, these changes were not very significant, since the gene expression levels in the studied part of the pathway did not show an increase or decrease, which could have shown a significant result in gene expression level. On the other hand, this compound was tested at a very low concentration in this study, and the results could be different at higher concentrations.

Figure 3.12 shows the gene expression levels in comparison to control in the presence of compound 2 (0.25mM) in culture media.

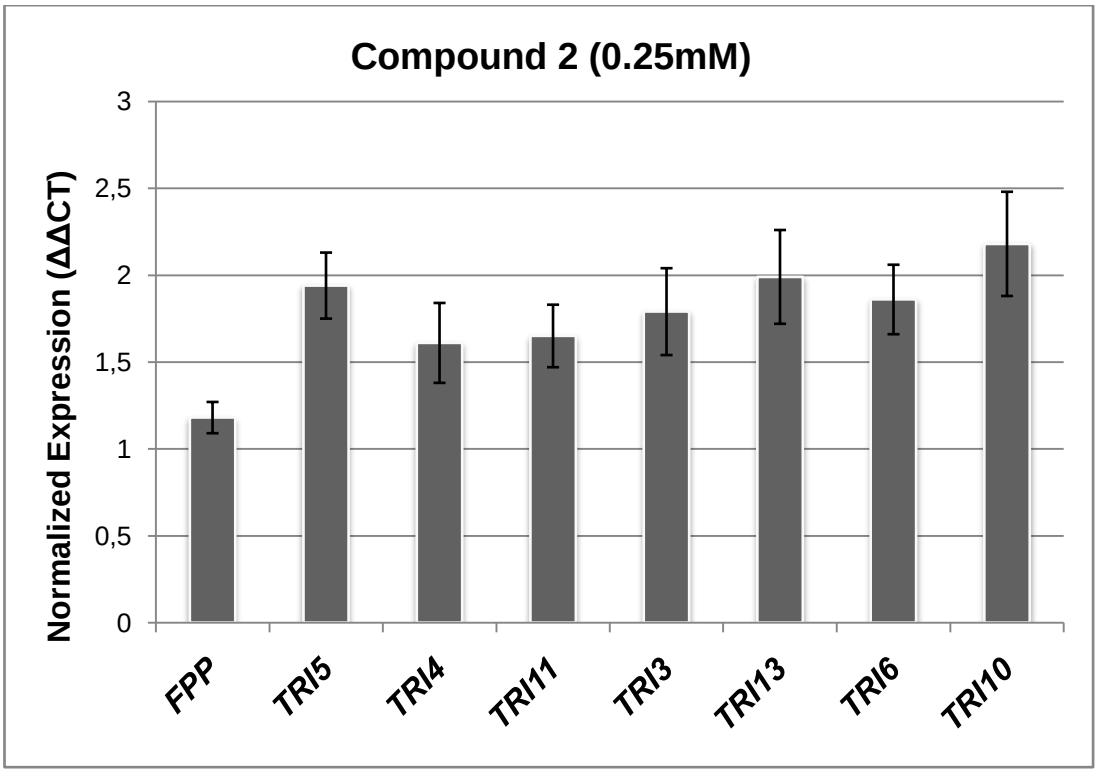

Figure 3.12: Expression levels of genes *FPP, TRI5, TRI4, TRI11, TRI3, TRI13, TRI6* and *TRI10*in the presence of compound 2 (0.25mM), as fold of control.

Figure 3.12 shows that all genes tested, other than *FPP*, showed that the presence of compound 2 led to upregulation of trichothecene pathway genes. Only the expression of *FPP* did not change, which can be explained by upregulation of regulatory genes *TRI6* and *TRI10*. These two genes are positively regulating *TRI* genes (Boutigny, *et al.*, 2009), but they are not associated with *FPP* expression.

For the second group of chemicals, two graphs were plotted by using data in Table 3.9, Table 3.10 and Table 3.11, which can be seen in Figure 3.13 and Figure 3.14. All values in these graphs were determined as fold of control, where gene expression levels in control groups were shown as 1. Figure 3.13 shows the gene expression levels in the presence of compound 3 (0.25mM).

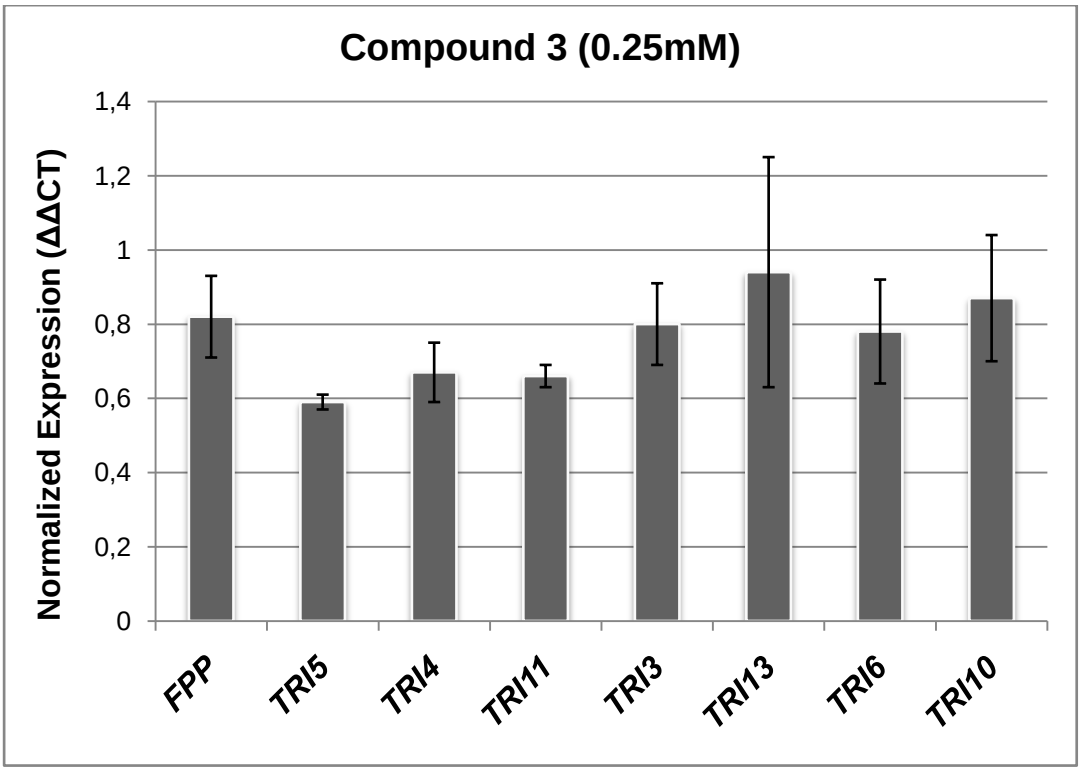

Figure 3.13: Expression levels of genes *FPP*, *TRI5*, *TRI4*, *TRI11*, *TRI3*, *TRI13*, *TRI6* and *TRI10*in the presence of compound 3 (0.25mM), as fold of control.

In Figure 3.13, it is shownthat all tested genes were downregulated, similar to the results shown in Figure 3.9. The presence of compound 3 led to a slight downregulationof all genes tested, varying from 0.6 to 0.9 in average.

Compound 4 was tested at three different concentrations; 0.25mM, 0.10mM, 0.05mM. In order to provide a better understanding in how the concentration changes the gene expression levels, Figure 3.14 was plotted where all three concentrations were included and compared.

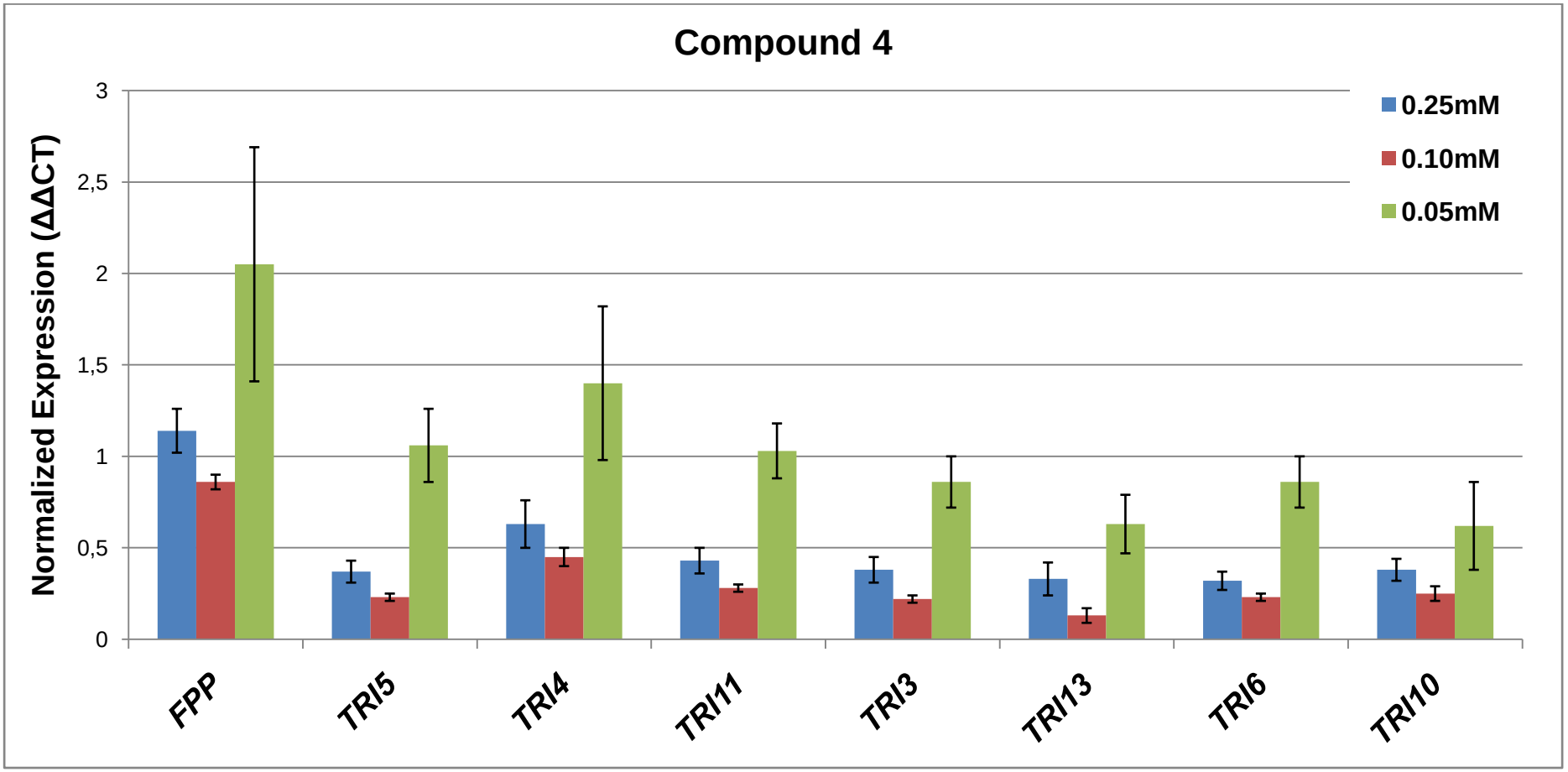

Figure 3.14: Expression levels of genes *FPP*, *TRI5*, *TRI4*, *TRI11*, *TRI3*, *TRI13*, *TRI6* and *TRI10*in the presence of 0.25mM, 0.10mM and 0.05mM of compound 4, as fold of control.

In Figure 3.14, it can be seen that in the presence of 0.25mM and 0.10mM compound 4, there was a similar pattern in gene expression. When gene expression levels were observed individually, it was observed that *FPP* expression remained around 1, not changing, in the presence of 0.25mM or 0.10mM of the compound, but *FPP* expression increased by two-fold in the presence of 0.05mM of the compound. *TRI5* expression dramatically decreased when mycelia were grown with 0.25mM or 0.10mM of the compound. However, this change was not observed in the presence of only 0.05mM of the compound, gene expression remained unchanged, which could be a result of the very low concentration. *TRI4* expression was again shown to be decreasing in the presence of higher concentrations, and was slightly upregulated in the presence of 0.05mM of the compound. The remaining five genes; *TRI11, TRI3, TRI13, TRI6* and *TRI10* showed a similar pattern, where they were highly downregulated at 0.25mM and 0.10mM concentrations of the compound. Interestingly, it was observed that even though the compound concentration was less (0.10mM), gene expression levels were lower compared to a higher concentration (0.25mM). It may be that at 0.10mM concentration, this compound is at its most effective concentration, even though the mechanism for this result still remains unknown. At the lowest concentration tested (0.05mM), it was found that for these five genes, expression levels remained around 1, where this compound showed only an insignificant effect on the tested genes. At this concentration, *TRI13* and *TRI10*were slightly downregulated, but expressions were still higher, compared to other tested concentrations. Overall, in Figure 3.14 it can be obviously seen that compound 4 downregulated the tested genes of trichothecene synthesis pathway. Among the three tested concentrations 0.25mM, 0.10mM and 0.05mM, it was observed that 0.10mM was the most effective concentration and 0.05mM of the compound was not sufficient to downregulate the trichothecene pathway genes. The highest concentration, 0.25mM was also downregulating the genes effectively. When considered for field applications, primary results showed that the best concentration for use would be at 0.10mM of the compound.

3.4.2 Dry weight and LC-MS analysis

For the first group chemicals dry weight and LC-MS results are listed in Table 3.12 and for the second group of chemicals, results are shown in Table 3.13.

Table 3.12: Dry weight and toxin concentrations (3-ADON, DON, 15-ADON and total toxin content) in the presence of ferulic acid, ferulic acid dimer, compounds 1 or 2. (±: standard deviation; %: average where control is considered 100; Avg.: average values).

Sample Name		Dry Weight [mg]	Dry Weight (%)	3-ADON [ng/mL]	3-ADON (%)	DON [ng/mL]	DON (%)	15-ADON [ng/mL]	15-ADON Avg.	Total Toxin [ng/mL]	Total Toxin (%)
control	Set1	24	100±4.6	1220.02	100±20.4	53.66	100±16.3	0.00	0.00	1273.68	100±20.1
	Set2	22		977.07		37.15		0.00		1014.22	
	Set3	24		679.03		37.73		0.00		716.76	
	Set4	24		1069.26		44.38		0.00		1113.64	
	Set5	25		934.25		38.98		0.00		973.23	
ferulic acid	Set1	21	91.6±1.9	124.82	32.1±16.1	0.00	0.00	0.00	0.00	124.82	30.8±15.4
	Set2	22		166.36		0.00		0.00		166.36	
	Set3	22		380.95		0.00		0.00		380.95	
	Set4	22		464.21		0.00		0.00		464.21	
	Set5	22		432.06		0.00		0.00		432.06	
ferulic acid dimer	Set1	24	95.8±3.2	3195.33	378.5±131.1	240.11	488.1±103.8	66.03	55.8±16.6	3435.44	383.0±127.4
	Set2	23		4591.34		173.60		48.32		4764.94	
	Set3	22		5367.39		263.35		79.69		5630.74	
	Set4	22		2135.81		197.92		39.65		2333.73	
	Set5	23		3178.94		159.37		45.14		3338.31	
Compound 1	Set1	23	100.8±5.1	1239.14	121.5±97.4	174.66	313.1±226.7	64.05	84.3±24.6	1413.80	129.4±102.6
	Set2	23		306.80		27.06		84.47		333.86	
	Set3	24		2733.07		272.51		125.81		3005.58	
	Set4	24		1128.92		122.59		67.83		1251.51	
	Set5	26		519.13		66.69		79.17		585.82	
Compound 2	Set1	19	79.0±3.5	382.77	38.9±12.9	0.00	53.1± 25.3	0.00	0.00	382.77	39.5±13.1
	Set2	18		517.39		35.26		0.00		552.65	
	Set3	20		316.61		26.63		0.00		343.24	
	Set4	18		477.76		35.02		0.00		512.78	
	Set5	19		205.39		15.59		0.00		220.98	

Table 3.13: Dry weight and toxin concentrations (3-ADON, DON, 15-ADON and total toxin content) in the presence of 0.25mM of compound 3 and 0.25mM, 0.10mM and 0.05mM of compound 4 (±: standard deviation; %: average where control is considered 100).

Sample Name		Dry Weight [mg]	Dry Weight (%)	3-ADON [ng/mL]	3-ADON (%)	DON [ng/mL]	DON (%)	15-ADON [ng/mL]	15-ADON (%)	Total Toxin [ng/mL]	Total Toxin (%)
control	Set1	35	100±4.7	24555.30	100±8.2	3336.87	100±8.0	494.73	100±11.3	28386.90	100±7.7
	Set2	32		27151.80		3145.92		453.54		30751.26	
	Set3	31		26566.10		2926.82		384.13		29877.05	
	Set4	33		25015.22		2949.32		473.15		28437.69	
	Set5	32		21876.50		2701.25		390.48		24968.23	
Compound 3	Set1	25	83.6±5.2	16320.60	64.8±18.6	2423.33	76.3±25.6	335.27	67.5±21.39	19079.20	66.1±19.4
	Set2	27		18666.61		2784.03		329.92		21780.56	
	Set3	29		18797.16		2745.17		338.13		21880.46	
	Set4	28		8118.03		942.42		128.92		9189.37	
	Set5	17		19201.33		2597.55		349.98		22148.86	
Compound 4 (0.25mM)	Set1	27	82.2±2.6	10888.47	32.5±11.9	1444.25	36.6±13.7	216.03	38.9±13.79	12548.75	33.0±12.0
	Set2	26		3081.80		401.50		64.29		3547.59	
	Set3	26		8902.57		1091.09		188.90		10182.56	
	Set4	28		8483.46		1349.51		186.74		10019.71	
	Set5	27		9322.15		1224.38		197.41		10743.94	
Compound 4 (0.10mM)	Set1	28	93.9±6.0	10069.32	42.7±7.3	1208.65	46.6±10.8	211.47	46.5±8.5	11489.44	43.1±7.6
	Set2	29		9686.39		1305.03		187.68		11179.10	
	Set3	32		13921.75		1977.13		262.33		16161.21	
	Set4	32		9757.93		1189.60		197.15		11144.68	
	Set5	32		9979.17		1343.28		161.56		11484.01	
Compound 4 (0.05mM)	Set1	27	89.0±5.01	11899.32	44.5±4.5	2687.10	71.9± 18.0	311.45	63.7±11.2	14897.87	47.7±6.0
	Set2	29		9471.05		1428.60		206.87		11106.52	
	Set3	31		11733.12		2428.82		308.44		14470.38	
	Set4	29		11426.08		2118.51		293.19		13837.78	

Table 3.12 contains information on mycelial dry weight results and toxin content of each five biological replicate, individually, as well as average values displayed as percentages where dry weight or toxin content of control group was considered as 100%. Dry weight results suggested that the presence of compound 1 did not affect mycelial growth, whereas the presence of ferulic acid or its dimer decreased it slightly. It was observed that the presence of 0.25mM compound 2 decreased mycelial growth around 20%, acting as a mild fungicide at this concentration.

When toxin content was observed, it was found that 3-ADON, the main toxin produced by *F. culmorum,* was lowest in the presence of ferulic acid with 32.1%, followed by compound 2 with 38.9%. Compound 1 slightly increased the 3-ADON production, whereas ferulic acid dimer led to production of three times more 3-ADON than control, with 378.5% toxin content.

In the presence of ferulic acid, no DON was detected, whereas compound 1 and ferulic acid dimer increased production of this secondary toxin dramatically. In the presence of compound 2, production was reduced to half.

Toxin 15-ADON was not produced in the control group, as well as in the presence of ferulic acid and compound 2. However, the presence of compound 1 and ferulic acid dimer led to production of this toxin, 55.77 ng/mL and 84.26 ng/mL, respectively.*TRI8* gene product leads to formation of 15-ADON from 3,15-diADON, which is a branching point for toxin production and may be converted into either 3-ADON, or DON or 15-ADON (Alexander, *et al.,* 2009). *TRI8* expression was not tested in this thesis study, however, in the presence of compound 1 and ferulic acid dimer, upregulation of this gene was expected since 15-ADON was produced.

When total toxin content was investigated, it was observed that at 0.25mM concentration compound 1 and ferulic acid dimer led to higher toxin accumulation levels compared to control and the best inhibiting phenolic compound was ferulic acid, decreasing toxin production to 30.8%, followed by compound 2, decreasing it to 39.5%. The problem with compound 2 is that it inhibited mycelial growth as well, as shown in dry weight results, and from an ecological point of view, compounds acting as fungicides are not preferred (Scherm, *et al.*, 2013). In addition, when compared to gene expression data shown in Figure 3.12, the results are conflicting since trichothecene pathway genes were shown to be upregulated. Other genes in the

trichothecene pathway should be tested in further studies to investigate whether the pathway is blocked in further steps.

Previous results with ferulic acid showed that at 0.25mM concentration, this compound reduces toxin production to 57% in *F. graminearum* (Bily, *et al.* 2003) and here it was shown that toxin production was reduced to 30.8% in *F. culmorum.* Ferulic acid was shown again to be one of the most effective compounds for inhibiting toxin production in *Fusarium* species.

Table 3.13 shows the dry weight and toxin production in the presence of 0.25mM of compound 3 and 0.25mM, 0.10mM or 0.05mM of compound 4 in the same manner as in Table 3.12. It was observed that all of the tested compounds decreased the mycelial growth when compared to control group.

Analysis of toxin content showed that second group of compounds all led to less 3-ADON, DON and 15-ADON production. In general, compound 4 was observed as a more effective inhibitor of toxin production compared to compound 3. Presence of compound 3 was shown to reduce 3-ADON production to 64.8%, DON production to 76.31% and 15-ADON production to 67.5% in comparison to the control samples, leading to 66.06% total toxin producton. It was also shown that the toxin profile was in line with the gene expression values (Figure 3.13).

Different concentrations of compound 4 affected toxin production according to the concentration applied. It was found that not only compound 4 was an effective inhibitor of toxin production, but also the inhibition was directly relatedto the concentration of the compound applied to the mycelia. All three different toxins tested in this study showed the same pattern where inhibition was stronger when the concentration was higher. The main toxin, 3-ADON, was reduced to 32.5% in the presence of 0.25mM of compound 4, whereas it was reduced to 42.68% and 44.47% in the presence of 0.10mM and 0.05mM of the compound, respectively. The remaining two kinds of toxins, DON and 15-ADON also showed the same pattern as 3-ADON. Total toxin amounts were obtained as 33.03% in the presence of 0.25mM, 43.15% in the presence of 0.10mM and finally, 47.67% in the presence of 0.05mM of the compound. These results show that compound 4 affected both the gene expression and the toxin production levels in the same manner and it is an effective inhibitor of tested mycotoxins.

4. CONCLUSIONS

Throughout this thesis work, three genes with unknown function, *FGSG_08817*, *FGSG_16572* and *FGSG_16588* were investigated. Under the stress conditions tested, the absence of these genes did not lead to any advantages or disadvantages in *F. culmorum*. To understand the function of these genes, further analysis of the tested strains is necessary. Nevertheless, it was shown that these genes are not directly associated with the pathogenic behavior of *F. culmorum*. For the ectopic strain of *FGSG_16588* deletion group (8-E), future experiments were suggested in order to locate the randomly inserted DNA fragment and understand the increased pathogenicity of this strain.

Gene expression and toxin analysis once again proved that ferulic acid is an effective inhibitor of trichothecene pathway; both attranscriptional and toxin production levels. Even though at gene expression level, the presence of ferulic acid dimer downregulatedthe trichothecene pathway genes tested; toxin analysis showed that the production of toxins was increased. Compound 1 also increased toxin production, both at RNA and toxin production levels. Compound 2 was shown to be another powerful inhibitor of trichothecenes; however, it also acted as a mild fungicide at this concentration. Compound 3 downregulated trichothecene pathway genes at 0.25mM concentration. Another phenolic compound; compound 4, was tested at three concentrations (0.25mM, 010mM and 0.05mM). It was observed that when this chemical was applied at 0.25mM and 0.10mM concentrations, gene expression levels for tested genes decreased dramatically, whereas 0.05mM of the compound was notsufficient to alter the gene expression. It was also found that higher concentrations of the compound increased the inhibitory effect of the compound. It was stated that compound 4 is a highly effective inhibitor of mycotoxin production, even at the lowest concentration tested, which is 0.05mM.

Another important finding of this study was the fact that each biological repeat showed different results, leading to very high standard deviation and standard error values. For example, toxin results showed that in the presence of 0.25mM compound

1, total toxin contents were 333.86 ng/mL for Set 2 and 3005.58 ng/mL for Set 3, even though the experiments were carried out exactly under identical conditions for each biological repeat. These results showed that the experimental procedures should be improved for future studies to obtain more consistent results.

Future studies with these compounds were proposed and these chemicals will be tested *in planta* in the green house and in the field, to observe the effects after infection with *F. culmorum.*

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