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Deciphering the genome and secondary metabolome of the plant pathogen Fusarium culmorum

Schmidt, R.L.; Durling, M.; de Jager, V.C.L.; Menezes, R. C.; Nordkvist, E.; Svatos, A.; Dubey, Mohit; Lauterbach, L.; Dikschat, J.S.; Karlsson, M.; Garbeva, P.V.

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Deciphering the genome and secondary metabolome of the plant pathogen *Fusarium culmorum*

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Keywords:	Genome, Fusarium culmorum, Pathogenicity, Terpenes, secondary metabolites

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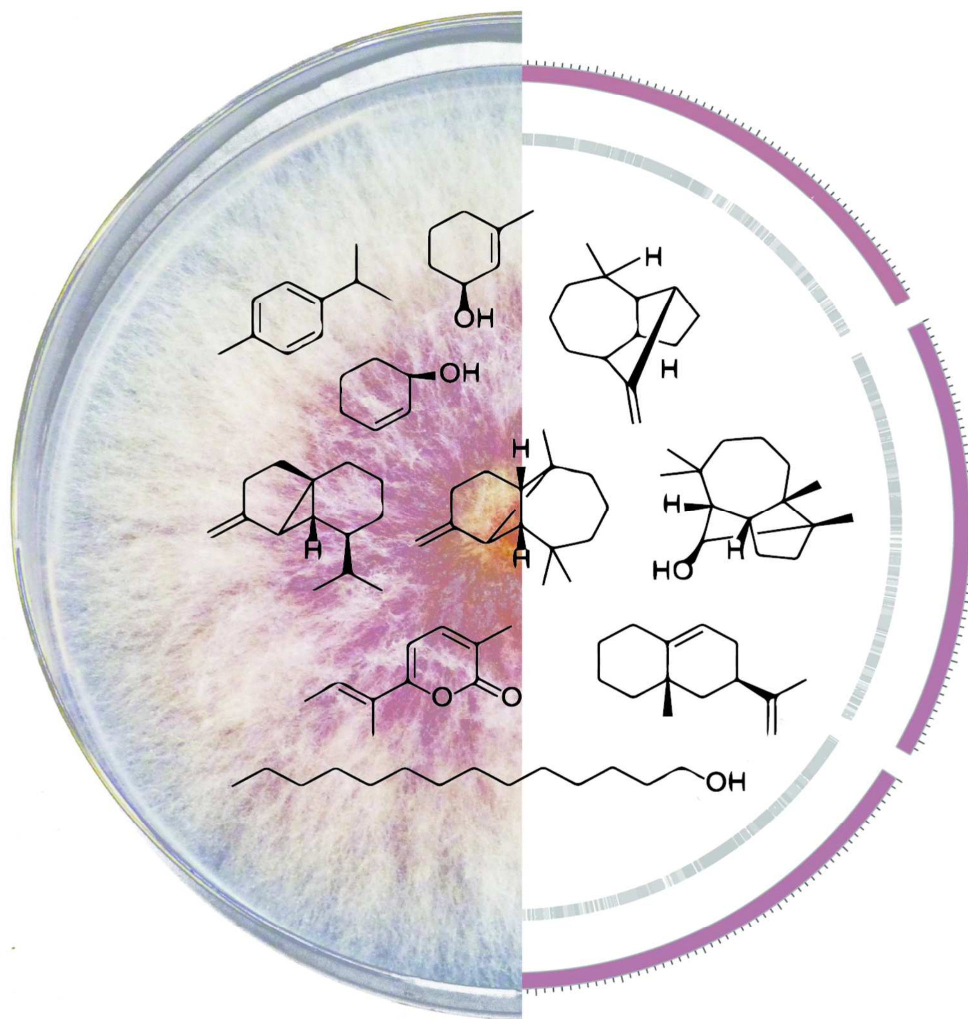

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1 # Deciphering the genome and secondary metabolome of the plant pathogen
2 # *Fusarium culmorum*

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Abstract

Fusarium culmorum is one of the most important fungal plant pathogens that causes diseases on a wide diversity of cereal and non-cereal crops. We report herein for the first time the genome sequence of *F. culmorum* strain PV and its associated secondary metabolome that plays a role in the interaction with other microorganisms and contributes to its pathogenicity on plants. The genome revealed the presence of two terpene synthases, trichodiene and longiborneol synthase, which generate an array of volatile terpenes. Furthermore, we identified two gene clusters, DON and ZEN, which encode for the production of mycotoxins. Linking the production of mycotoxins with *in vitro* bioassays, we found high virulence of *F. culmorum* PV on maize, barley and wheat. By using UPLC/MS, we confirmed several compounds important for the behaviour and lifestyle of *F. culmorum*. This research sets the basis for future studies in microbe-plant interactions.

Keywords: Genome; *Fusarium culmorum*; Pathogenicity; Secondary metabolites; Terpenes

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3 36 ## Introduction
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5 37 Fungi produce a plethora of structurally diverse secondary metabolites. Due to their broad
6
7 38 range of biological activities, secondary metabolites can provide an advantage to the
8 39 producing organism under different environmental conditions (Demain & Fang, 2000, Keller
9
10 40 *et al.*, 2005, Keller, 2015). Many fungi release both volatile and polar water-soluble
11 41 compounds (Keller *et al.*, 2005, Morath *et al.*, 2012, Keller, 2015, Dickschat, 2017), some of
12
13 42 which exhibit antagonistic bioactivity towards competing organisms in the same habitat while
14 43 others function as signalling chemicals.
15
16 44 Volatile organic compounds (VOCs) are recently discussed to not only act in the direct
17
18 45 surrounding of their producer, but also in long-distance microbial interactions (Effmert *et al.*,
19 46 2012, Schmidt *et al.*, 2015). Especially, terpenes were shown to play important roles in the
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21 47 long-range communication between microorganisms (Schmidt *et al.*, 2017, Schulz-Bohm *et*
22 48 *al.*, 2017). These molecules are generated via terpene synthases, a highly sophisticated class
23
24 49 of enzymes that turns simple linear and achiral precursor molecules into usually polycyclic
25
26 50 terpene hydrocarbons or alcohols (Degenhardt *et al.*, 2009, Quin *et al.*, 2014, Dickschat,
27 51 2016). The initial products can be further converted into highly functionalised and bioactive
28
29 52 molecules such as mycotoxins. In the past two decades, several fungal terpene synthases and
30
31 53 their related products have been characterised (Hohn & Beremand, 1989, Hohn & Plattner,
32 54 1989, Proctor & Hohn, 1993, McCormick *et al.*, 2010, Brock *et al.*, 2013, Burkhardt *et al.*,
33
34 55 2016). Although the principal processes of terpene biosynthesis are well understood, it
35 56 remains a challenging task to predict the structure of a terpene just from the amino acid
36
37 57 sequence of its corresponding synthase. With the advances in sequencing technology, whole
38
39 58 genome sequences have become available for identification and mining of unexplored
40 59 secondary metabolite genes.
41
42 60 Aside from VOCs, fungi are well-described producers of pigment metabolites as well
43
44 61 as mycotoxins, including trichothecenes, fumonisins, ochratoxins, and zearalenone that have
45 62 been identified for several *Fusarium* spp. and related fungi (Keller *et al.*, 2005). Mycotoxins
46
47 63 may accumulate in infected crops and cause toxicoses in humans or animals consuming
48
49 64 contaminated food, can induce apoptosis, and play an important role as virulence factors
50 65 (Scherm *et al.*, 2013). Trichothecenes in *Fusarium* sp. are sesquiterpenoids that can be
51
52 66 classified in two groups (Type A and B) based on the substitution pattern of their tricyclic
53 67 core structure (McCormick *et al.*, 2011). The most common Type B trichothecenes are
54
55 68 deoxynivalenol (DON), nivalenol (NIV), and acetylated derivatives, while Type A
56 69 trichothecenes include HT-2 and T-2 toxins. In *Fusarium*, trichothecene biosynthetic enzymes
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3 70 are encoded by genes at three loci: the single-gene *TRI101* locus, the two-gene *TRI1-TRI16*
4 71 locus, and the 12-gene core *TRI* cluster, of which the genes *Tri13* and *Tri7* have been shown
6 72 to determine DON and NIV producing chemotypes in *F. gramineaum* (Lee et al., 2002). In
8 73 contrast, fumonisin biosynthetic enzymes identified to date are all located at one locus, the
9 74 17-gene *FUM* cluster (Alexander *et al.*, 2009). Fumonisins are produced by *F. verticillioides*
11 75 and other related species of the *Gibberella fujikuroi* complex, with the two most important
13 76 classes belonging to B1 and B2. An important characteristic of *Fusarium* spp. is their ability
14 77 to synthesize the estrogenic polyketide zearalenone (ZEN) (Yazar & Omurtag, 2008). The
16 78 complete genome of *F. graminearum* allowed for genome-wide polyketide synthase genes
17 79 (PKS) functional studies that identified *PKS4* and *PKS13* as essential genes for its production
19 80 (Lysoe *et al.*, 2006, Lysoe *et al.*, 2009).

21 81 With its range of secondary metabolites produced, the genus *Fusarium* represents an
22 82 important group of fungal plant pathogens, causing various diseases on a broad spectrum of
24 83 crops worldwide including cereal and non-cereal crops (Scherm *et al.*, 2013, King *et al.*,
26 84 2015). For instance, *F. culmorum* causes foot and root rot (FRR) and fusarium head blight
27 85 (FHB) on different small-grain cereals, in particular wheat and barley. However, despite the
29 86 ecological and agricultural importance of *F. culmorum* its genome remains unknown. Our
30 87 previous research revealed that *F. culmorum* emits a specific blend of VOCs including
32 88 various terpenes (Schmidt *et al.*, 2016). Furthermore, we observed that *F. culmorum* VOCs
34 89 play an important role in the long-distance interaction with bacteria by inducing both motility
35 90 and production of secondary metabolites.

37 91 The aim of this study was to sequence the genome of *F. culmorum* and to explore and
38 92 identify genes associated with secondary metabolite production that potentially play important
40 93 roles in fungal pathogenicity and fungal-bacterial interactions. Furthermore, we aimed to
42 94 correlate the genomic data with metabolomic analyses of water-soluble and volatile
43 95 compounds.
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Materials and Methods

Fungal strains and growth conditions

The *F. culmorum* isolate PV, originally isolated from a sandy dune soil in The Netherlands (De Boer *et al.*, 1998), was used in this study. The isolate was grown on King's B agar, supplemented with 50 mg/mL rifampicin, kanamycin and ampicillin and subsequently transferred to Oatmeal agar OA (24 g/L Difco), supplemented with 50 mg/mL rifampicin, kanamycin and ampicillin. The spores were washed with 50 mg/mL rifampicin, kanamycin and ampicillin, collected over glass wool with sterile deionized water and stored at -80 °C.

Bioassay with *F. culmorum* on wheat and barley

Pathogenicity of *F. culmorum* was tested on wheat and barley in an *in vivo* bioassay using a sand seedling test. Per treatment, five biological replicates with 10 plants per replicate were used following the procedure as described previously (Knudsen *et al.*, 1995, Dubey *et al.*, 2014). The seeds were surface sterilised and sown in a 1- to 2-cm hole, 1 seed in each hole, in moistened, autoclaved sand in individual plastic pots (5 x 5 x 5 cm). Pathogen inoculation was carried out by placing a 5-mm PDA plug containing *F. culmorum* mycelia close to the seed. PDA plugs without *F. culmorum* were used as control. After inoculations, the holes were filled with moist sand. Pots were kept in a growth chamber with a photoperiod of 12 h light, with 150 µMol m^2 s^{-1} light intensity, and 12 h dark, 70% ±5 relative humidity, and 20°C ± 1 temperature. The shoot length was measured every 5 days. Seedlings were harvested 3 weeks post inoculation and root length and fresh biomass were determined. Disease symptoms were scored on a 0 to 4 scale as described previously (Knudsen *et al.*, 1995): 0 = healthy plants with no symptoms, 1 = slightly brown roots and coleoptiles, 2 = moderately brown roots and coleoptiles, 3 = severely brown roots and coleoptiles, and 4 = dead plants. Analysis of variance (ANOVA) was performed, and pairwise comparisons were made using the Tukey–Kramer method at the 95 % significance level ($P \leq 0.05$). Results are shown as mean ± standard deviation (S.D.).

Pairwise comparisons were made using the Tukey–Kramer method at the 95 % significance level ($P \leq 0.05$). Results are shown as mean ± standard deviation (S.D.).

Fungal DNA extraction and genome sequencing

Fungal plugs were grown on 0.5 strength PDA plates at 20 C for 5 days. The mycelium was removed from the surface of the agar with a sterile scalpel and transferred to 200 mL 1.75%

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128 malt extract + 0.25% peptone supplemented with 50 mg/mL rifampicin, kanamycin and
129 ampicillin and incubated for 10 days at 20°C, 120 rpm. The liquid was removed by vacuum
130 filtration and the mycelium was freeze-dried and stored at −20 C. 500 mg of freeze-dried
131 mycelium was ground to a fine powder in a sterile mortar in liquid nitrogen using a pre-
132 cooled pestle. The ground mycelium was immediately transferred to 10 mL G2 buffer of the
133 genomic DNA extraction kit (Qiagen), 10 µl RNase A (10 mg/mL) and 100 µl proteinase K
134 (20 mg/mL) and incubated at 50°C for one hour and mixed by inverting the tube several
135 times. The suspension was centrifuged twice at 5,000 rpm for 10 min at 4°C and the
136 supernatant was transferred to 4 ml QBT buffer (Qiagen) equilibrated Genomic-tip 100/G
137 (Qiagen) and fixed in a 50 mL Falcon tube. The next steps were performed according to the
138 midi-prep instructions of the Genomic-tip Protocol as part of the Genomic DNA Handbook
139 (Qiagen). After isopropanol precipitation, the DNA was harvested using a glass rod, washed
140 with 70% ethanol, dried and dissolved in 200 µL Nuclease-free water and stored at −20 C.
141 The quality and quantity of the DNA was checked on a 0.8% agarose gel with the
142 O'GeneRuler 1 kb DNA Ladder and the 23 kb DNA Molecular Weight Marker II (Sigma-
143 Aldrich). High-quality genomic DNA was then submitted to the Institute of Genome Sciences
144 (Baltimore, Maryland, USA) for generation of a 15-30 kb fragment library, followed by and
145 PacBio RS II sequencing. The genome was deposited at NCBI as Bioproject PRJNA375977.

146 **Genome assembly and annotation**

147 *F. culmorum* reads were extracted and assembled with the SMRT Portal software
148 (smrtanalysis_2.3.0.140936.p4.150482) using the HGAP3 (Chin *et al.*, 2013) assembly
149 protocol with an estimated assembly length of 39 Mb and default settings. The resulting
150 contigs were scaffolded using PBJelly v15.8.24 (publicly available at
151 https://sourceforge.net/projects/pb-jelly/) with the same PacBio reads as in the HGAP3
152 assembly. The scaffolds of the PBJelly analysis were used as reference for 2 rounds of Quiver
153 polishing using the BAM_RS_resequencing_Beta.1 protocol of the SMRT Portal software.
154 The resulting polished contigs were annotated using Maker 2.31 with the available *Fusarium*
155 gene models from Augustus and Ensembl predicted proteins of *F. graminearum* str. PH-1 and
156 *F. culmorum* UK99. The resulting polished contigs were matched against the reference
157 genome of *F. graminearum* str. PH-1 using CONTIguator (Galardini *et al.*, 2011) to get an
158 indication of which contigs belong to which chromosome. To filter potential bacterial
159 sequences out of the assembly, blobology (publicly available at
160 https://github.com/blaxterlab/blobology) was run on the unmapped contigs. Protein-coding

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161 genes in the *F. culmorum* genome were annotated using MAKER 2 (Holt & Yandell, 2011).
162 We configured MAKER 2 to use SNAP (Korf, 2004), Augustus (Stanke & Waack, 2003), and
163 GenemarkES (Ter-Hovhannisyan *et al.*, 2008) for ab initio gene calls. The **B**enchmarking
164 **U**niversal **S**ingle-**C**opy **O**rthologs (BUSCO) program v .3.0.2 (Simão *et al.*, 2015, Waterhouse
165 *et al.*, 2018) was used to assess the completeness of our genome assembly.

166 **Phylogenetic Analysis**

167 A custom pipeline was used for construction of a phylogenomic tree by using whole predicted
168 proteome sequences. Six species were included for the topology construction, including *F.*
169 *graminearum* (isolate PH-1), *F. oxysporum* forma specialis (f. sp.) *lycopersici* (isolate FOL
170 4287), *F. solani* (isolate 77-13-4), *F. verticillioides* (isolate 7600), and *F. fujikuroi* (isolate
171 IMI 58289), and *Clonostachys rosea* (isolate IK726) (Karlsson *et al.*, 2015) as outgroup.
172 Except for *C. rosea*, the proteome sequences of the included species were obtained from the
173 JGI Mycocosm portal (Grigoriev *et al.*, 2014). The predicted proteomes were clustered using
174 cd-hit and clusters were matched with the BUSCO fungal dataset (Simão *et al.*, 2015).
175 Predicted proteins occurring once in all six species were selected and aligned. A consensus
176 tree was built by concatenating the variation from the selected core proteins using RaxML
177 (Stamatakis, 2014). Phylogenetic trees were visualised using FigTree (publicly available at
178 http://tree.bio.ed.ac.uk/software/figtree/).

179 ***In silico* identification of secondary metabolite gene clusters**

180 For identification of secondary metabolite gene clusters antiSMASH 2.0 (Blin *et al.*, 2013)
181 was used. BLAST homology searches were performed with mycotoxin genes of the DON
182 (Deoxynivalenol) and ZEN (Zearalenone) gene clusters of the closely related strain *F.*
183 *graminearum* (Lee *et al.*, 2001, Kim *et al.*, 2005).

184 **Terpene production and phylogeny of terpene synthases**

185 An agar plate culture of *F. culmorum* was inoculated with small pieces of an agar plate
186 preculture and grown at 28°C for 3 days. The emitted volatile mono- and sesquiterpenes were
187 trapped on charcoal filters by use of a closed-loop stripping apparatus for 24 h, followed by
188 extraction of the traps with dichloromethane. The obtained extracts were analysed by GC/MS
189 and compounds were identified by comparison of the recorded mass spectra to library spectra,
190 and of retention indices determined from a homologous series of n-alkanes with published
191 reference data. Positive compound identification was assumed for mass spectral matches

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192 >900/1000 and measured retention indices deviating no more than 10 units from the published
193 value.
194 The terpene synthase encoded in the genome of *F. culmorum* were identified by a
195 sequence similarity search using BLAST with the amino acid sequence of trichodiene
196 synthase from *F. sporotrichioides* (Hohn & Beremand, 1989) as a probe, resulting in two
197 candidates with 90% (*F. culmorum* trichodiene synthase) and 24% (*F. culmorum* longiborneol
198 synthase) sequence identity. Both putative terpene synthases were inspected for the presence
199 of the highly conserved motifs required for functionality, including the aspartate-rich motif
200 (DDXXD) (Cane *et al.*, 1996), the NSE triad (ND(L,I,V)XSXXXE), the pyrophosphate
201 sensor (highly conserved arginine ca. 46 residues upstream of the NSE triad) (Baer *et al.*,
202 2014), and the RY dimer near the C-terminus. A phylogenetic tree containing the two terpene
203 synthases from *F. culmorum* and 2501 other terpene synthase homologs that were all checked
204 for the presence of the highly conserved motifs was constructed using the tree builder of
205 Geneious 7.0.6 (Biomatters).

206 **Mycotoxin production**

207 20 g of maize or wheat were weighted into a 150 ml Erlenmeyer flask and 15 ml distilled
208 water was added. The bottle was autoclaved for 1 h at 120 °C. After cooling, three agar plugs
209 of 6 mm in diameter were inoculated onto the substrate from 1-week-old *F. culmorum*
210 cultures. Sterilized maize and wheat grains without *F. culmorum* served as control. After the
211 incubation period, the grain cultures were freeze dried and finely ground to pass a 1.0-mm
212 sieve by an impact crusher (Retsch ZM 100, Nino Lab, Stockholm, Sweden). The samples
213 were analysed by LC-MS/MS according to Tevell Åberg *et al.* (2013).

214 **Analysis of secondary metabolites using UPLC-MS/MS**

215 *F. culmorum* agar plugs were used to inoculate 0.5 strength PDA plates and incubated at 28°C
216 for 4 days. The mycelium was scraped off the agar and homogenized with liquid nitrogen in a
217 glass vial, 2.5 ml ice-cold solvent of methanol and water (1:2) were added and the mixture
218 was ultrasonicated for 30 min. The sample was then centrifuged to separate the cell debris.
219 The clear supernatant was collected. To the pellet, 2 ml of water and chloroform (1:1) was
220 added. The sample was ultrasonicated and spun. Finally, the two liquid phases were separated.
221 The polar and non-polar fractions were analyzed separately using Ultra-performance liquid
222 chromatography-mass spectrometry (UPLC-MS). UPLC-MS analyses of *F. culmorum*
223 extracts were performed on a Q-Exactive Plus Hybrid Quadrupole-Orbitrap Mass

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224 Spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Ultimate 3000
225 series RSLC (Dionex, Sunnyvale, CA, USA) chromatograph. Chromatographic separation
226 was achieved on an Acclaim C18 column (150 × 2.1 mm, 2.2 µm particles with 120 Å pore
227 diameter, Dionex, Sunnyvale, CA, USA) with a flow rate of 300 µl min^{-1} in a binary solvent
228 system of water (Solvent A) and acetonitrile (Solvent B), both containing 0.1% (v/v) formic
229 acid. 5 µl each of extracts were loaded onto the column and eluted by using a gradient as
230 follows: linear increase from 0% B to 100% B within 15 min – 100% B constant for 5 min –
231 equilibration time at 0% B for 5 min. The mass spectrometer was operated in positive and
232 negative ionization modes using Heated-Electrospray Ionization (H-ESI) in the mass range of
233 m/z 100 to 1500 using 70,000 m/Δm resolving power in the Orbitrap mass analyzer. H-ESI
234 source parameters were set to 4 kV for spray voltage, 35 V for transfer capillary voltage at a
235 capillary temperature 300 °C. Data was evaluated and interpreted using Xcalibur v.3.0.63
236 software (Thermo Fisher Scientific, Waltham, MA, USA). Fragmentation was achieved with
237 data dependent acquisition using a resolution of 70,000 m/Δm and stepped normalized
238 collision energy of 10, 30 and 60.

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1 2 3 4

Results

Pathogenicity of *F. culmorum* on wheat and barley

First, we tested the pathogenicity of *F. culmorum* on barley and wheat. Our results showed a significant ($P \leq 0.05$) decrease in the shoot length of barley and wheat seedlings when infected with *F. culmorum* over a period of two weeks as compared to the control (Figure 1a). Disease symptoms were high with severely brown roots and coleoptiles in both barley and wheat (Figure 1b). Moreover, root length and fresh weight were significantly ($P \leq 0.05$) reduced in both barley and wheat when infected with *F. culmorum* as compared to the control (Figure 1c and d). These results show the high prevalence of *F. culmorum* as a pathogen in both crops. To link the pathogenicity to secondary metabolites produced by *F. culmorum*, such as mycotoxins, the next step was to sequence and investigate its genome.

General features of *F. culmorum*

The *F. culmorum* genome was deep sequenced using 5 PacBio SMRT cells to at least 115× coverage and assembled with HGAP3. The total assembly length was 38.24 Mb spread across 37 scaffolds with an N_{50} read length of 5215.4 kb (Table S1). Using BUSCO, we detected 274 (94.5%) out of 290 complete and single-copy orthologs (SCO) based on the Fungi-odb9 set of widely conserved fungal core genes. Six (2.1%) partial SCOs were detected and ten (3.4%) SCOs from the set were missing. No duplicate complete SCOs were detected (Table S2). The mapping of *F. culmorum* genome resulted in mapped contigs to 4 chromosomes, 1 mitochondrial chromosome and 12 unmapped contigs (Table S3). A total of 12,979 gene models coding for proteins were annotated using MAKER 2. All gene models were functionally annotated using InterProScan5 to assign Gene Ontology (GO) terms (Ashburner *et al.*, 2000) and Pfam domains (Table S1, Figure 2). Pfam domains were found in 8,688 gene models, whereas GO terms were assigned to 6,917 genes.

Phylogenetic placement of *F. culmorum*

In order to determine the phylogenetic relationship between *F. culmorum* and other sequenced *Fusarium* species, a whole-proteome analysis was conducted. The phylogenetic analysis showed that *F. culmorum* clustered together with *F. graminearum* with 100% bootstrap support (Figure 3). *F. fujikuroi* and *F. oxysporum* f. sp. *lycopersici* clustered as sister taxa with 100% bootstrap support.

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3 270 **Distribution of secondary metabolite gene clusters in *Fusarium***
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5 271 Generally, the biosynthesis genes for fungal secondary metabolites are located in clusters that
6 272 can span more than 10,000 bases (Brakhage, 2013). In most fungal genomes, the clusters
7 273 contain one or several central biosynthesis genes encoding extremely large multidomain,
8 274 multimodular enzymes belonging to the polyketide synthases (PKSs) or non-ribosomal
9 275 peptide synthetases (NRPSs). To link genes to secondary metabolites, we examined the
10 276 genomes of six *Fusarium* species *in silico*. The analysis revealed multiple genes clusters
11 277 across the six species, with the highest number encoding for NRPSs, type1-PKSs, terpenes
12 278 and other secondary metabolites (Table S4). The whole-genome analysis of *F. culmorum*
13 279 predicted a total of 41 gene clusters, comprising 9 NRPS, 2 hybrid NRPS-type 1 PKS, 6 type
14 280 1 PKS, 1 hybrid type 1 PKS-terpene, 1 type 3 PKS, 9 terpenes, 1 terpene-type 1 PKS, 2 type 1
15 281 PKS-NRPS, 3 fatty acids and 7 gene clusters classified as other (Table S4). In this study, we
16 282 focused mainly on predicted clusters that may play a role in pathogenicity and communication
17 283 with other microorganisms, including genes encoding for mycotoxins and terpenes.
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19 284 **Genes encoding for mycotoxins**
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21 285 The major mycotoxins produced by previously investigated *F. culmorum* strains are the
22 286 trichothecenes deoxynivalenol (DON), nivalenol (NIV) and the polyketide zearalenone (ZEN)
23 287 (Wagacha & Muthomi, 2007). Many of the trichothecene biosynthesis genes are located in a
24 288 gene cluster comprising at least 10 genes: trichodiene synthetase (*Tri5*), P450 oxygenases
25 289 (*Tri4* and *Tri11*), acetyltransferase (*Tri3*), a transcription factor (*Tri6*), a toxin efflux pump
26 290 (*Tri12*), and several unidentified hypothetical proteins (*Tri7*, *Tri8*, *Tri9*, and *Tri10*). We
27 291 identified all *Tri* genes in the genome of *F. culmorum* PV with identity levels from 77-98% at
28 292 the amino acid level, except for *Tri7, Tri9* and *Tri13*, which were only partially identified
29 293 (Figure 4, Table S5). The order and transcription directions of the open reading frames
30 294 (ORFs) were identical for the gene cluster described in *F. graminearum*.
31 295 The ZEN gene cluster was described in *F. graminearum*, showing that four genes are
32 296 essential for ZEN biosynthesis: the two polyketide synthases, *PKS13* and *PKS4,* the alcohol
33 297 oxidase *ZEB1,* and the transcription factor *ZEB2* (Kim *et al.*, 2005). We identified all four
34 298 genes in the genome of *F. culmorum*, including several other genes located upstream and
35 299 downstream with identity levels from 82-96% at the amino acid level (Figure 4, Table S5),
36 300 suggesting that *F. culmorum* strain PV is a ZEN producer.
37 301 To confirm the production of DON and ZEN, we determined the mycotoxin produced
38 302 by *F. culmorum* strain PV upon infection of maize and wheat grains.
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303 **Mycotoxin production during plant infection**

304 Mycotoxins play important roles in the pathogenesis of *Fusarium*. Thus, we measured the
305 mycotoxin levels in maize and wheat under infection with *F. culmorum*. Infection of maize
306 and wheat grains resulted in high levels of several mycotoxins (Figure 4, Table S6). Levels of
307 DON with up to 251 mg/kg in maize and up to 256 mg/kg in wheat, and ZEN with up to 150
308 mg/kg in maize and up to 230 mg/kg in wheat were detected (Figure 4, Table S6). Traces of
309 HT2-toxin, T2-toxin, Fumonisin B1, fumonisin B2 and ochratoxin A were present, but were
310 not detected in any significant amounts as compared to the control.

311 **Genes encoding for terpene synthases and terpene products**

312 Terpene synthases are a highly specialised group of enzymes that catalyse the formation of a
313 wide range of terpenes (Dickschat, 2016). In order to link genes encoding for terpenes, we
314 first looked at amino acid sequences of terpene synthases in the genome of *F. culmorum*. By
315 BLAST comparison with known terpene synthases form other fungi we identified homologs
316 of two previously characterised enzymes, trichodiene and longiborneol synthase (Hohn &
317 Plattner, 1989, McCormick *et al.*, 2010) (Figure 5). Using GC/MS, a range of terpenes was
318 identified (Figure 6a, b and c, Table 1), some of which were also detected in previous studies
319 and linked to changes in motility in the interacting bacteria (Schmidt *et al.*, 2016).

320 **Identification of secondary metabolites using UPLC-MS/MS**

321 To get an overview of water-soluble secondary metabolites produced *F. culmorum*, we
322 employed UPLC-MS/MS. In total, three metabolites were identified (Table 2, Figure S1).
323 Rubrofusarin, an orange polyketide pigment, is reported to be produced by various fungi,
324 including *F. graminearum*, *Aspergillus niger*, *A. parasiticus* and *Ustilaginoidea virens*, and
325 was shown to inhibit the human DNA topoisomerase II-α and to have general antibiotic
326 effects on *Mycobacterium tuberculosis* and various filamentous fungi (Rugbjerg *et al.*, 2013).
327 This pigment adds to the characteristic appearance of *F. culmorum* on the macroscopic scale
328 and possibly contributes to its protection to secondary metabolites produced by other
329 microorganisms in its surrounding. Further, two mycotoxins were identified, with the
330 trichothecenes DON and ZEN. The detected mycotoxins likely contribute to the high
331 pathogenicity of *F. culmorum* in several crops, including maize, barley and wheat.

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Discussion

Fusarium culmorum is an important fungal plant pathogen, whose genome provides insight into genes and secondary metabolites linked to its pathogenicity and interaction with plant and other microorganisms. Here we describe for the first time the genome of *F. culmorum* and its secondary metabolome. This *F. culmorum* isolate was shown to be highly virulent to maize, wheat and barley and to produce a wide range of secondary metabolites, including volatile terpenes and mycotoxins.

The whole-proteome analysis revealed that *F. culmorum* is phylogenetically related to the cereal pathogen *F. graminearum*. Both pathogens have a small number of chromosomes (four chromosomes) and a similar genome size (38.2Mb and 36.2Mb). The smaller number of chromosomes in *F. culmorum* and *F. graminearum* is the result of chromosome fusion relative to other *Fusarium* genomes (e.g. *F. oxysporum* 59.9Mb with 15 chromosome). The difference in genomes size may explain the differences in *Fusarium* host range. For example, while *F. culmorum* and *F. graminearum* have a narrow host range, infecting predominantly cereals, *F. oxysporum* has a remarkably broad host range, infecting both monocotyledonous and dicotyledonous plants and is an emerging pathogen of immunocompromised humans and other mammals (Ma *et al.*, 2010).

Fungal secondary metabolites play important role in fungus-plant interactions and pathogenicity. In general, fungal metabolites are divided into four main chemical classes: polyketides, terpenoids, shikimic acid derived compounds, and non-ribosomal peptides. Moreover, hybrid metabolites composed of moieties from different classes are common, such as meroterpenoids, which are fusions between terpenes and polyketides (Matsuda & Abe, 2016). While PKS gene clusters have long been suspected to play a role in pathogenicity of fungi, this is the first study that shows the repertoire of PKS genes from *F. culmorum*. Using publicly available genomes, we predicted seven PKS genes and six hybrid PKS-NRPS/terpene genes, which are in the range of predicted number of PKS gene clusters found in other related *Fusarium* spp. that produce known polyketides, such as zearalenone and fumonisin. The terpene gene cluster encoding for NIV/DON/3-ADON was detected in *F. culmorum* and in the closely related species *F. graminearum*, while being absent in other *Fusarium* spp. A recent study on the geographic distribution of *Fusarium* strains across Europe concluded that for *F. culmorum* the prevalent genotype was 3-ADON with 59.9%, while the NIV genotype accounted for 40.1% (Pasquali *et al.*, 2016). When linking gene cluster to product by measuring mycotoxins in wheat and barley infected grains, NIV was not detected, while DON and ZEN were present in high concentrations. These results show that

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3 367 DON and ZEN are the prevalent mycotoxins in *F. cumorum* PV, being amongst the most
4 368 concerning toxins for safety of wheat and barley products (Tittlemier *et al.*, 2013, Tima *et al.*,
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6 369 2016).
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8 370 Differences in chemical structure between DON and NIV (i.e., presence or absence of
9 371 hydroxyl group at the C4 position), was shown to originate from nucleotide variations in the
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11 372 two *TRI* genes (*TRI13* and *TRI7*) (Lee *et al.*, 2001, Lee *et al.*, 2002). In the trichothecene gene
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13 373 cluster, *Tri7, Tri9,* and *Tri13* were only partially identified, creating an impaired NIV gene
14 374 cluster. These results, together with the mycotoxin measurements confirm that *F. culmorum* is
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16 375 a DON chemotype. The partial presence of *Tri7* and *Tri13* suggests an evolutionary loss of
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18 376 the virulence-associated genes, probably due to fitness consequences, altering the bioactivity
19 377 and toxicity of trichothecenes (Ward *et al.*, 2002). Interestingly, trichothecene chemotype
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21 378 differences have been shown to not correlate well with evolutionary relationships within the
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23 379 *F. gramineaum* complex phylogeny, suggesting a discord between species and toxin evolution
24 380 (O'Donnell *et al.*, 2000). Ward *et al.* (2002) demonstrate a trans-species evolution for TRI-
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26 381 cluster genes, indicating that polymorphism within the TRI-cluster has been maintained
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28 382 through multiple speciation events by balancing selection acting directly on chemotype
29 383 differences. The close relatedness between *F. culmorum* and *F. graminearum* via
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31 384 phylogenetic analysis shows evidence that the presence and structure of the DON and ZEN
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33 385 biosynthetic gene clusters have been co-evolved in the two species. Thus, a similar
34 386 evolutionary relationship can be expected for *F. culmorum*. With more genome resources of
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36 387 *F. culmorum* becoming available, it remains to be a topic for future studies to investigate the
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38 388 evolution of *TRI* genes within species of the same clade, preferably with biogeographically
39 389 distinct species.
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41 390 Based on the hypothesis that volatile terpenes play an important role in the interaction with
42 391 other microorganisms and the pathogenicity of the fungus, we screened the genome for
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44 392 terpene clusters, which resulted in 9 terpene and 2 hybrid PKS-terpene clusters. Gas
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46 393 chromatography measurements showed that *F. culmorum* produces a wide array of terpenes
47 394 with a variety of different functions, including the monoterpene β-Phellandrene that was
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49 395 shown in our previous study to modulate the motility of bacteria (Schmidt *et al.*, 2015). Some
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51 396 identified terpenes are known to be involved in plant-insect interactions, including the floral
52 397 volatiles Gossonorol, β-Ylangene and Longifolene (Elzen *et al.*, 1984, Holopainen & Blande,
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54 398 2013). Furthermore, the pheromones Piperitol and β-Farnesene are involved in the attraction
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56 399 between bugs and in deterring aphids (Stowe *et al.*, 1995, Bhatia *et al.*, 2015). So far,
57 400 however, these compounds have not been shown to be produced by fungi. Only recently, the
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production of plant terpenes has been acknowledged for fungi too. For example, the sesquiterpene β-Barbatene was identified in amobae, and hypothesized to be involved in the attraction of insects for sporal dispersal (Chen *et al.*, 2016). This indicates that terpenes have a much wider role in the interkingdom microbial-plant network than presently assumed (Schulz-Bohm *et al.*, 2017).

Up till now, only few structures of microbial and plant sesquiterpens synthases are known and it remains an intense field of study to link terpene synthases to their respective products (Quin *et al.*, 2014). Here, sesquiterpenes acting as mycotoxins, longiborneol and trichodiene, could be identified with their respective synthases. However, predicting the structure based on the gene cluster proves to be a challenging task due to the peculiar feature of some terpene synthases to produce multiple, usually structurally related terpenes (Quin *et al.*, 2014, Dickschat, 2016). Additionally, only few terpenes synthases have been described in *Fusarium* strains, including the recently described longiborneol synthase (McCormick *et al.*, 2010). For example, gene deletion studies in *F. fujikuroi* identified the terpene synthase involved in the production of α-acorenol (Brock *et al.*, 2013), however the synthase could not be identified and linked to the production of the respective terpene in our study. It remains to be addressed in future studies, whether the identified trichodiene and longiborneol synthases are responsible for biosynthesis of all identified terpenes or whether there are yet unidentified enzymes with terpene synthase activity in the genome of *F. culmorum*.

In conclusion, here we describe for the first time the genome of *F. culmorum* and its secondary metabolome. This *F. culmorum* isolate was shown to be highly pathogenic to maize, wheat and barley and to produce a wide range of secondary metabolites, including volatile terpenes and mycotoxins. Sequencing of the *F. culmorum* genome and establishment of its predicted secondary metabolite clusters provides a starting point for future investigations regarding the ecology of this important species. Moreover, the role of terpenes as interacting molecules between fungi and plants as well as fungi and bacteria is a new and emerging research filed, thus not much is known yet about the kind of terpenes that regulate such interactions. Hence, unraveling the ecological role of terpenes in microbial-plant interactions remains to be a subject for future studies.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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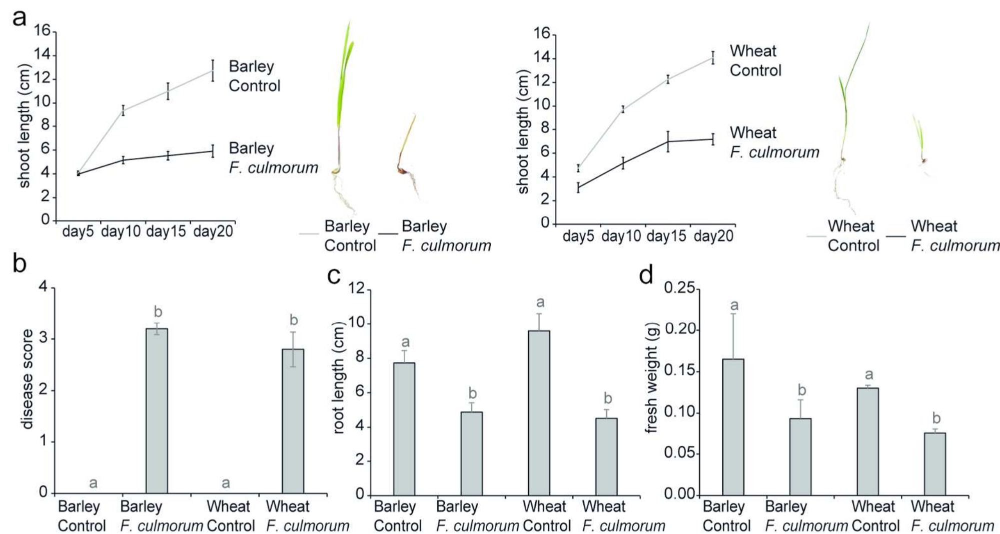

Figure 1: In vivo pathogenicity test of *F. culmorum* on wheat and barley. Seeds were planted in moist sand together with a *F. culmorum* agar plug. The shoot length was measured every 5 days (a). Seedlings were harvested 3 weeks post inoculation and disease symptoms were scored on a 0 to 4 scale (b) (no disease symptoms were observed for the controls), and root length (c) and fresh biomass (d) were determined. The experiment was performed in five biological replicates with 10 plants in each replicate. Error bars represent standard deviation based on five biological replicates. Different letters indicate statistically significant differences (P ≤ 0.05) within experiments based on the Tukey-Kramer test.

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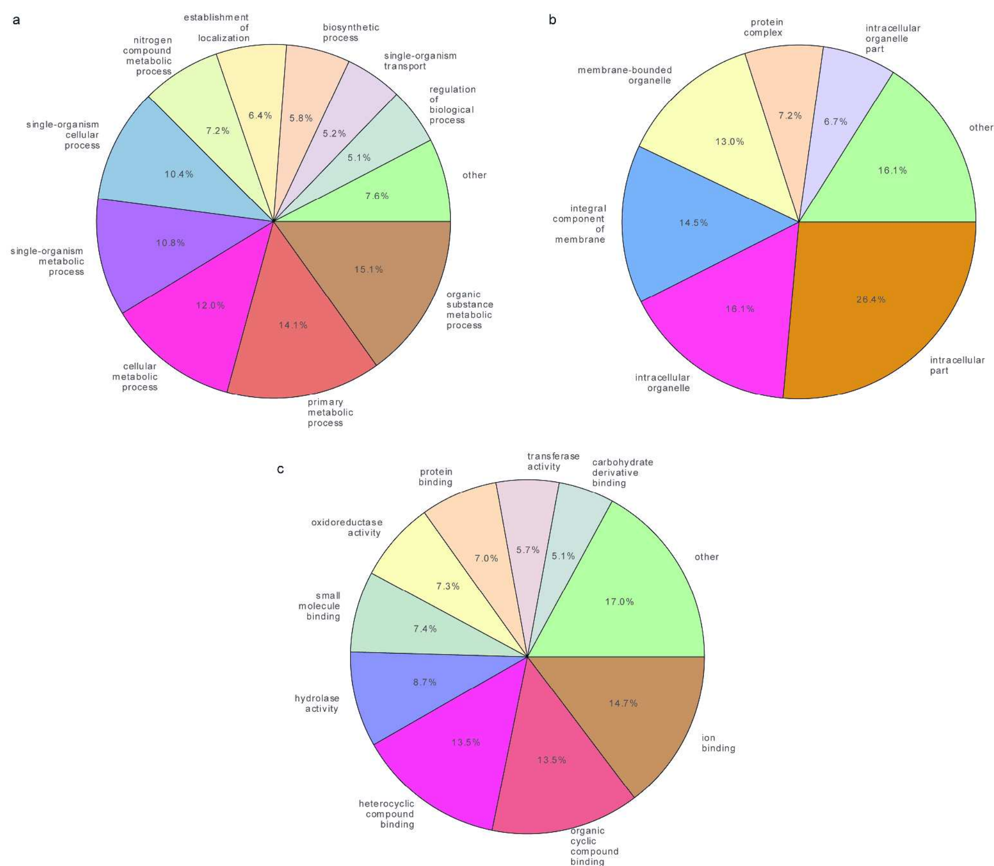

Figure 2: Functional annotations of *F. culmorum* genes. GO terms of the three ontologies biological process (a), cellular component (b) and molecular function (c) were assigned using InterProScan.

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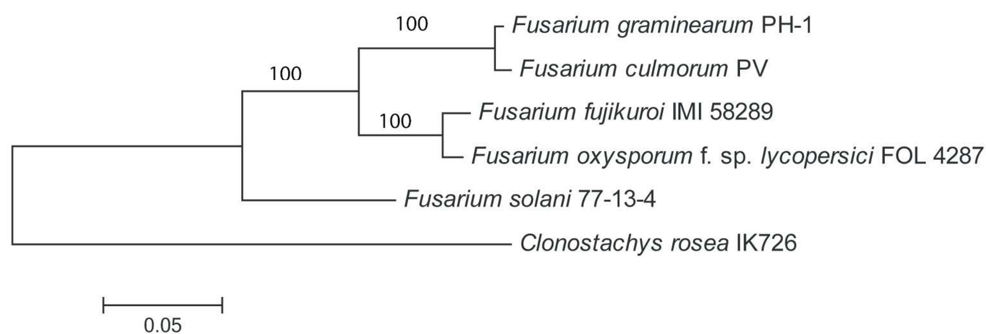

Figure 3: Phylogenetic placement of *F. culmorum*. The phylogenomic tree was constructed using whole predicted proteome sequences. Six species were included for the topology construction, including *F. graminearum, F. oxysporum f. sp. lycopersici, F. solani, F. verticillioides*, and *F. fujikuroi*, and *C. rosea* as outgroup. Bootstrap support values are associated with lineages. Strain identifications are given after species names.

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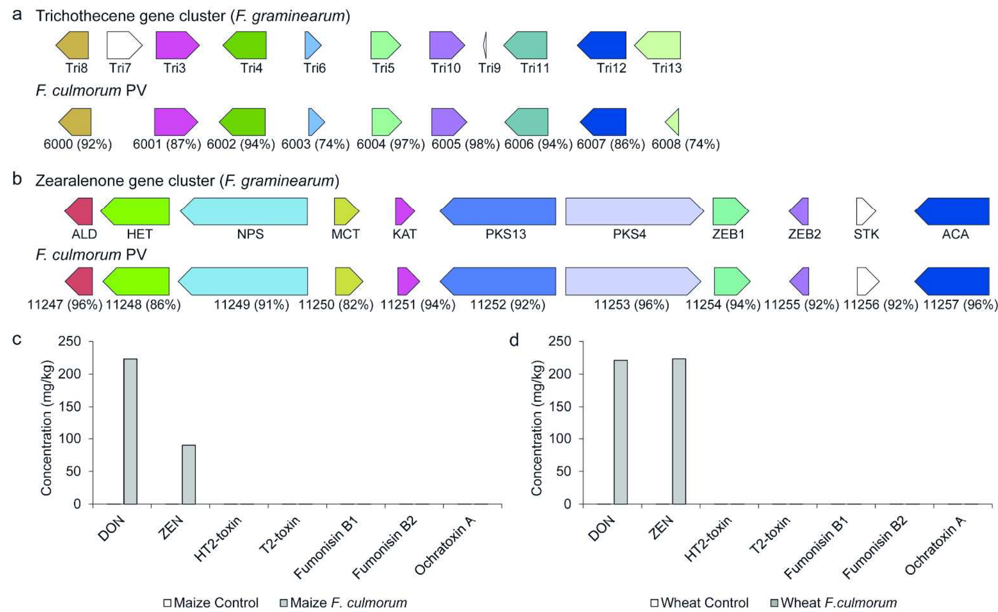

Figure 4: Mycotoxin gene clusters of DON (a) and ZEN (b) in *F. culmorum* and mean mycotoxin concentration. Locus tags are indicated for each gene along with the percentage of amino acid sequence identity to triochothecene and zearalenone gene clusters in *F. graminearum*. Concentrations (mg/kg) were measured after three weeks of infection with F. culmorum in wheat (c) and maize (d). DON: Deoxynivalenol, ZEN: Zearalenone.

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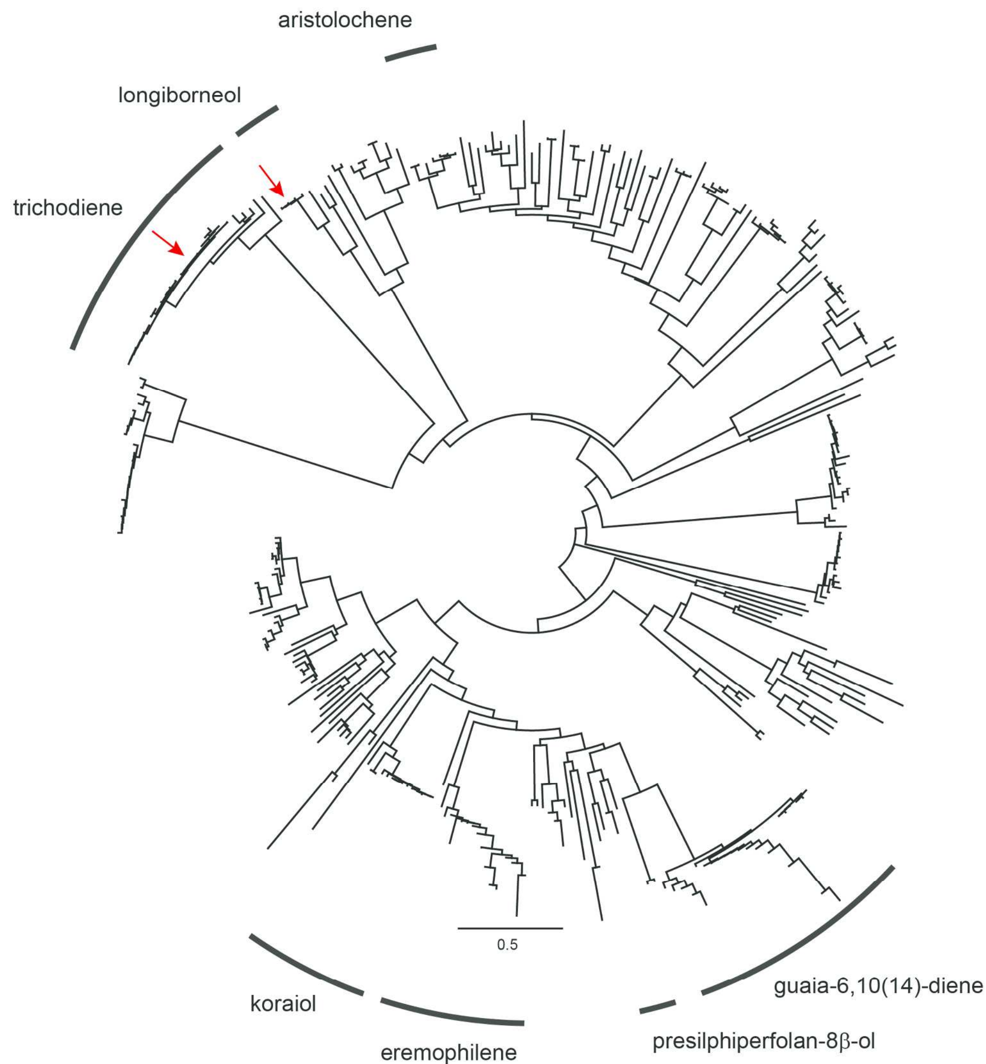

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Figure 5: Phylogenetic tree of fungal terpene synthases. The black bars indicate groups of highly similar enzymes from which at least one enzyme was functionally characterised. The red arrows point to the two enzymes from *F. culmorum* for trichodiene and longiborneol.

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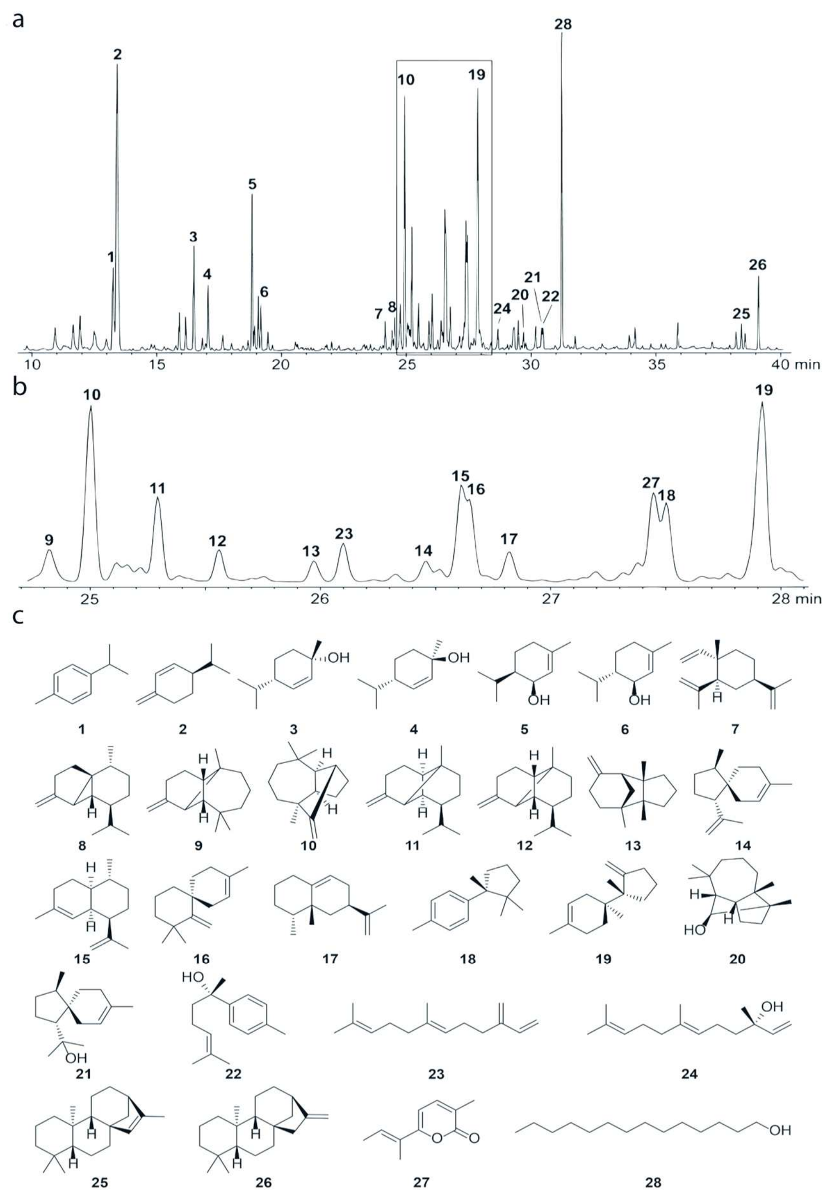

Figure 6: Terpenes produced by *F. culmorum*. (a) Chromatogram of all terpenes produced by *F. culmorum* according to numbers in Table 2 (b) Zoomed in chromatogram of major terpenes in *F. culmorum* (c) Structures of identified terpenes.

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Table 1: Identified terpenes in *F. culmorum.* I: Experimental retention indices, I (Lit.): Retention indices found in literature.

***F. culmorum* PV**	**RT [min]**	**I**	**I (Lit.)**	**sample 1**	**sample 2**	**sample 3**	**sample 4**
Benzaldehyde	10.95	959	952			x	
1-Octen-3-ol	11.67	979	974			x	x
3-Octanone	11.92	982	979			x	x
p-Cymene (**1**)	13.27	1024	1026	x	xx	xx	xx
β-Phellandrene (**2**)	13.46	1029	1026	x	xxx	xxx	xxx
n-Nonanal	15.96	1102	1100		x	x	
Phenylethyl alcohol	16.21	1110	1106		x	x	
cis-*p*-Menth-2-en-1-ol (**3**)	16.54	1120	1118		xx	xx	xx
trans-*p*-Menth-2-en-1-ol (**4**)	17.10	1138	1136		xx	x	xx
cis-Piperitol (**5**)	18.87	1194	1195	x	xx	xx	xx
trans-Piperitol (**6**)	19.22	1205	1207		xx	x	x
β-Elemene (**7**)	24.22	1383	1389			x	
β-Cubebene (**8**)	24.49	1393	1390	x		x	
β-Longipinene (**9**)	24.82	1405	1405	xx	x	x	x
Longifolene (**10**)	25.00	1412	1413	xxx	xx	xx	
β-Ylangene (**11**)	25.29	1423	1425	xx	x	xx	
β-Copaene (**12**)	25.55	1434	1430			x	
β-Barbatene (**13**)	25.98	1450	1445	x		x	
(*E*)-β-Farnesene (**23**)	26.10	1455	1454	x	x	x	
β-Acoradiene (**14**)	26.45	1469	1469		x	x	
Amorpha-4,11-diene (**15**)	26.61	1475	1472	xx		xx	x
Isochamigrene (**16**)	26.64	1476		xx	x	xx	
5-epi-Aristolochene (**17**)	26.82	1483	1477	xx	x	x	
Gibepyrone A (**27**)	27.45	1508	1509	x	xx	xx	

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Cuparene (**18**)	27.50	1510	1504	xx		xx	
Trichodiene (**19**)	27.93	1528	1533	xxx	xxx	xx	x
(*E*)-Nerolidol (**24**)	28.72	1561	1561			x	
Hexadecane	29.56	1596	1600			x	
Longiborneol (**20**)	29.76	1605	1607	x	x	x	
β-Acorenol (**21**)	30.50	1637	1636		x	x	
Gossonorol (**22**)	30.55	1639	1636			x	
Tetradecanol (**28**)	31.28	1671	1671			xxx	
Heptadecane	31.83	1695	1700			x	
Octadecane	34.00	1795	1800			x	
Kaur-15-ene (**24**)	38.29	2007	1997			x	
Kaur-16-ene (**26**)	39.19	2056	2049		x	xx	

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Table 2: Secondary metabolites of *F. culmorum* detected by UPLC-MS

#	Metabolite	Molecular Formula	$[M+H]^+$	Mass error (△ppm)	Chemical classification
1	Rubrofusarin	$C_{15}H_{12}O_5$	273.07586	0.659	Polyketide
2	Deoxynivalenol (DON)	$C_{15}H_{20}O_6$	297.13326	-0.016	Trichothecene B
3	Zearalenone (ZEN)	$C_{18}H_{22}O_5$	319.15411	0.344	Polyketide

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Supplementary Materials

Table S1: General features of the *Fusarium culmorum* genome: assembly and annotations

Genome size (Mb)	38.24
Number of scaffolds	37
Gap length	0
G+C content (%)	47
Scaffold N50 (kb)	5215.4
Gene count	12,979
Total coding length (Mb)	18.5
Genes with EST support	12,487
Average/median transcript length (bp)	1,598.04/1,324
Average/median exon count	3.01/3

Table S2: Statistics of the completeness of the *F. culmorum* genome assembly

C:94.5%[S:94.5%,D:0.0%],F:2.1%,M:3.4%,n:290	
274	Complete BUSCOs (C)
274	Complete and single-copy BUSCOs (S)
0	Complete and duplicated BUSCOs (D)
6	Fragmented BUSCOs (F)
10	Missing BUSCOs (M)
290	Total BUSCO groups searched

Table S3: Mapped contigs of *F. culmorum*

Chr1	Chr2	Chr3	Ch4	ChrMt	ChrUn
Contig7	Contig31	Contig26	Contig2	Contig20	Contig8
491364	342892	64407	5215404	131517	9941
Contig0	Contig23	Contig11	Contig28		Contig9
7279442	494602	7892604	2383920		43951
Contig1	Contig5	Contig3	Contig30		Contig13
1326286	5548199	1587654	61509		235940
Contig15	Contig21	Contig4	Contig22		Contig16
2657141	235338	411792	501601		407754
	Contig24	Contig6	Contig12		Contig18
	117598	588014	28010		5319
			Contig10		Contig19
			10111		17678
			Contig14		Contig25
			15100		21888
			Contig32		Contig27
			18503		58135
			Contig33		Contig29
			8960		5722
			Contig17		Contig34
			6263		5395
					Contig35

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Contig36
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Table S4: Distribution of secondary metabolite gene clusters among six *Fusarium* species

Gene cluster type	Compound with gene cluster of highest similarity	*F. verticilloides*	*F. oxysporum*	*F. fujikuroi*	*F. solani*	*F. graminearum*	*F. culmorum*
indole		3	3	2	1		
nrps	Gliotoxin_biosynthetic_gene_cluster	1					
	Beauvericin_biosynthetic_gene_cluster		1				
	Acetylaranotin_biosynthetic_gene_cluster			1			
	Apicidin_biosynthetic_gene_cluster			1			
	Zearalenone_biosynthetic_gene_cluster					1	
		10	10	9	9	9	9
Sum		**11**	**11**	**11**	**9**	**10**	**9**
nrps-t1pks	Equisetin_biosynthetic_gene_cluster	1	2	1			
	Fumonisin_biosynthetic_gene_cluster	1		1			
	NG-391_biosynthetic_gene_cluster	1		1			
	Fujikurins_biosynthetic_gene_cluster		1				
	NG-391_biosynthetic_gene_cluster				1		
	Zearalenone_biosynthetic_gene_cluster						1
	NG-391_biosynthetic_gene_cluster						1
		1		1			
Sum		**4**	**3**	**4**	**1**	**0**	**2**
other	Fusaridione_A_biosynthetic_gene_cluster	1	1	1			
	Fusaric_acid_biosynthetic_gene_cluster			1			1
		9	10	8	7	7	6
Sum		**10**	**11**	**10**	**7**	**7**	**7**
phosphonate					1		
siderophore						1	
t1pks	Bikaverin_biosynthetic_gene_cluster	1	1	1			
	Depudecin_biosynthetic_gene_cluster	1	1				
	Fujikurins_biosynthetic_gene_cluster	1		1			

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	Fusaric_acid_biosynthetic_gene_cluster	1	1	1			
	Fusarubin_biosynthetic_gene_cluster	1	1	1	1		1
		5	6	6	10	6	5
Sum		**10**	**10**	**10**	**11**	**6**	**6**
t1pks-terpene	Pyripyropene_biosynthetic_gene_cluster					1	
	Paxilline_biosynthetic_gene_cluster						1
		1		1			
Sum		**1**	**0**	**1**	**0**	**1**	**1**
t1pks-t3pks					1		
t3pks		1	1	1	1	1	1
terpene	Aspyridone_biosynthetic_gene_cluster		1				
	Nivalenol_/_deoxynivalenol_/_3-acetyldeoxynivalenol_/					1	1
		8	9	10	6	7	8
Sum		**8**	**10**	**10**	**6**	**8**	**9**
terpene-t1pks		1	1			1	1
t1pks-nrps						1	2
Cf_fatty_acid							3

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Table S5: Blast comparison DON (Deoxynivalenol) and ZEN (Zearalenone) gene clusters in *F. culmorum*

Gene	Annotation (*F. graminearum*)	Locus	Annotation (*F. culmorum*)	Identity (%)	E-value	Query Coverage (%)
Deoxynivalenol (AN: AF336365)						
Tri8	trichothecene C-3 esterase	FCULT00006000_1	Similar to ARB_00790 Lipase A (Arthroderma benhamiae (strain ATCC MYA-4681 / CBS 112371))	79	0	85
Tri7		no hit				
Tri3	putative 15-O-acetyltransferase	FCULT00006001_1	Protein of unknown function	87	0	91

Tri4	putative cytochrome P450	FCULT00006002_1	Similar to TRI4 Trichodiene oxygenase (Fusarium sporotrichioides)	94	0	96
Tri6	positive regulatory protein	FCULT00006003_1	Protein of unknown function	74	2.00E-69	79
Tri5	trichodiene synthase	FCULT00006004_1	Similar to TRI5 Trichodiene synthase (Fusarium culmorum)	97	0	98
Tri10		FCULT00006005_1	Similar to ustR ustiloxin B cluster transcription factor ustR (Aspergillus flavus (strain ATCC 200026 / FGSC A1120 / NRRL 3357 / JCM 12722 / SRRC 167))	98	0	99
Tri9		no hit				
Tri11	putative trichothecene C-15 hydroxylase	FCULT00006006_1	Similar to TRI11 Isotrichodermin C-15 hydroxylase (Fusarium sporotrichioides)	94	0	98
Tri12	trichothecene efflux pump	FCULT00006007_1	Protein of unknown function	86	0	92
Tri13	putative cytochrome p450 monooxygenase	FCULT00006008_1	Protein of unknown function	74	4.00E-40	81
Zearalenone (AN: DQ019316)						
ALD	aldehyde dehydrogenase	FCULT00011247_1	Similar to ALDH2B4 Aldehyde dehydrogenase family 2 member B4, mitochondrial (Arabidopsis thaliana)	96	0	97
HET	heterokaryon incompatibility protein	FCULT00011248_1	Similar to het-6 Heterokaryon incompatibility protein 6, OR allele (Neurospora crassa (strain ATCC 24698 / 74-OR23-1A / CBS 708.71 / DSM 1257 / FGSC 987))	86	0	92
NPS	non-ribosomal peptide synthetase	FCULT00011249_1	Similar to dtxS1 Nonribosomal peptide synthetase dtxS1 (Metarhizium robertsii (strain ARSEF 23/ ATCC MYA-3075))	91	0	95

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MCT	monocarboxylate transporter like-protein	FCULT00011250_1	Protein of unknown function	82	0	87
KAT	K+ channel protein	FCULT00011251_1	Similar to KAB1 Probable voltage-gated potassium channel subunit beta (Arabidopsis thaliana)	94	0	95
PKS13	polyketide synthase	FCULT00011252_1	Similar to cla3 Non-reducing polyketide synthase cla3 (Cladosporium cladosporioides)	92	0	94
PKS4	polyketide synthase	FCULT00011253_1	Similar to cla2 Highly reducing polyketide synthase cla2 (Cladosporium cladosporioides)	96	0	97
ZEB1	isoamyl alcohol oxidase	FCULT00011254_1	Similar to patO FAD-linked oxidoreductase patO (Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1))	94	0	96
ZEB2	bZIP domain-containing transcription factor	FCULT00011255_1	Protein of unknown function	92	0	95
STK	protein kinase Eg2-like protein	FCULT00011256_1	Similar to ark1 Serine/threonine-protein kinase ark1 (Schizosaccharomyces pombe (strain 972 / ATCC 24843))	92	0	95
ACA	Ca2+ ATPase	FCULT00011257_1	Similar to pmc1 Calcium-transporting ATPase 2 (Schizosaccharomyces pombe (strain 972 / ATCC 24843))	96	0	96

Table S6: Mycotoxin concentration (mg/kg) after three weeks of infection with *F. culmorum* in maize and wheat. DON: Deoxynivalenol, ZEN: Zearalenone

Sample	DON	ZEN	HT2-toxin	T2-toxin	Fumonisin B1	Fumonisin B2	Ochratoxin A
Control Maize	<0.3	<0.3	<0.04	<0.04	0.226	<0.1	<0.052
Maize 1	251	150	<0.04	<0.04	0.226	<0.1	<0.052
Maize 2	226	57	<0.04	<0.04	0.123	-	-
Maize 3	192	63	<0.04	<0.04	>0.1	<0.1	<0.052
Control Wheat	<0.1	<0.01	<0.01	<0.01	<0.05	<0.1	<0.015
Wheat 1	207	220	<0.01	<0.01	<0.05	<0.1	<0.015
Wheat 2	200	220	<0.01	<0.01	<0.05	<0.1	<0.015
Wheat 3	256	230	<0.01	<0.01	<0.05	<0.1	<0.015

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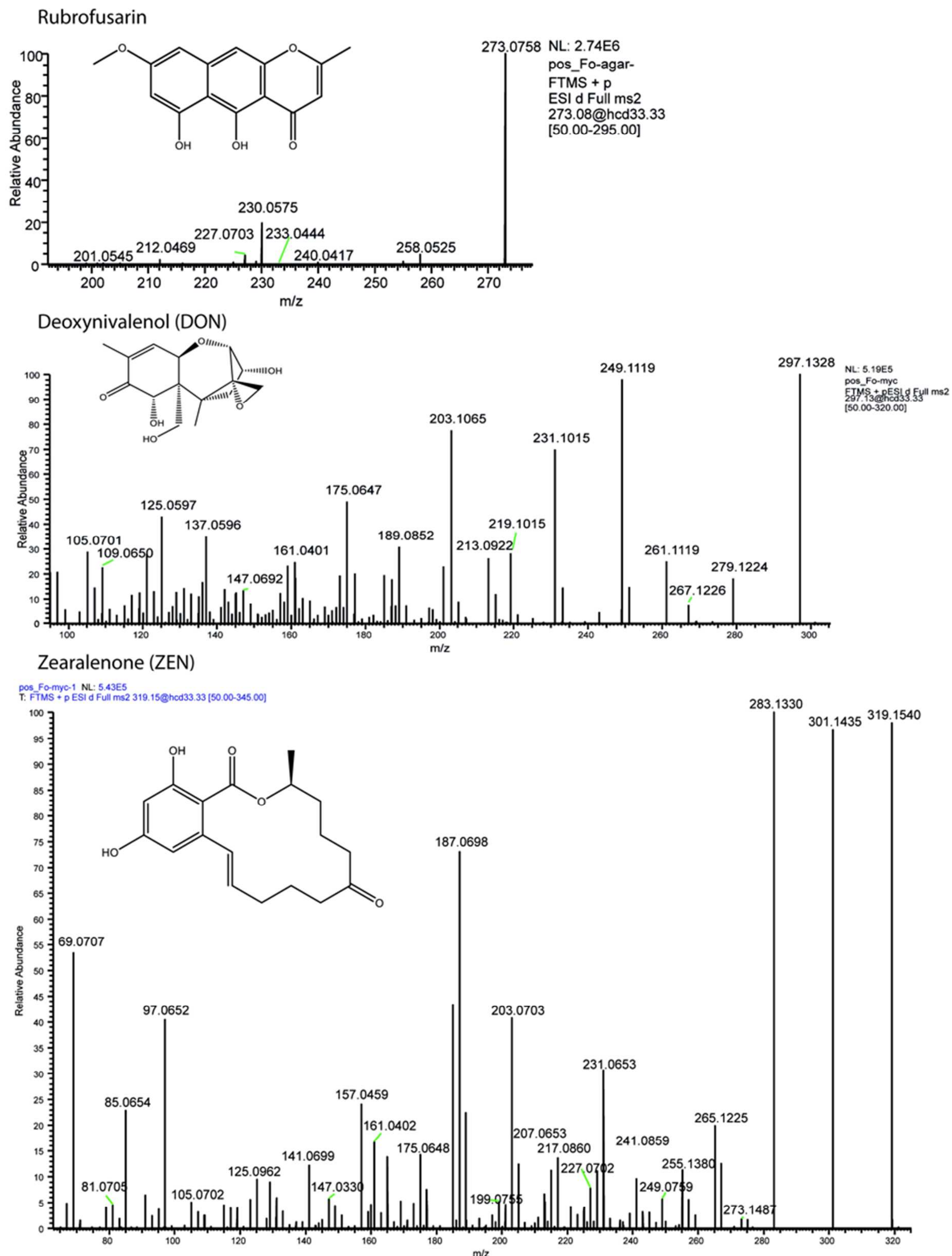

52 **Figure S1. Data-dependent MS/MS (DDMS2) spectra of mycotoxins from *F culmorum*.**
53 Included in the DDMS2 spectra are experimental masses of fragments with the spectra from
54 fungal extracts. Fragmentation patterns were compared to those available in literature
55 (References 1-6). Experimental conditions are provided in Material and Methods.
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1. Liao CD, Wong JW, Zhang K, Hayward DG, Lee NS & Trucksess MW (2013) Multi-mycotoxin analysis of finished grain and nut products using high-performance liquid chromatography-triple-quadrupole mass spectrometry. *Journal of agricultural and food chemistry* **61**: 4771-4782.

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3. Neumann NK, Lehner SM, Kluger B, Bueschl C, Sedelmaier K, Lemmens M, Krska R & Schuhmacher R (2014) Automated LC-HRMS(/MS) approach for the annotation of fragment ions derived from stable isotope labeling-assisted untargeted metabolomics. *Analytical chemistry* **86**: 7320-7327.

4. Renaud JB & Sumarah MW (2016) Data independent acquisition-digital archiving mass spectrometry: application to single kernel mycotoxin analysis of Fusarium graminearum infected maize. *Analytical and bioanalytical chemistry* **408**: 3083-3091.

5. Sagawa N, Takino T & Kurogochi S (2006) A simple method with liquid chromatography/tandem mass spectrometry for the determination of the six trichothecene mycotoxins in rice medium. *Bioscience, biotechnology, and biochemistry* **70**: 230-236.

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