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Molecular and phenotypic diversity in populations of *Fusarium culmorum* on cereal hosts

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Contents

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[2] Castiblanco, V., Marulanda, J. J., Würschum, T., & Miedaner, T. (2017). *Candidate gene based association mapping in Fusarium culmorum for field quantitative pathogenicity and mycotoxin production in wheat.* BMC genetics, 18(1), 1–12.

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Abbreviations

15-ADON	15-acetyl-deoxynivalenol
3-ADON	3-acetyl-deoxynivalenol
AMMI	Additive main effects and multiplicative interactions
Avr	Avirulence gen
BH-MADR	Bonferroni-Holm with re-scaled MAD standardized
BLUEs	Best linear unbiased estimators
BW	Bread wheat
CUT	Cutinase gene
DON	Deoxynivalenol
DU	Durum wheat
F. avenaceum	*Fusarium avenaceum*
F. culmorum	*Fusarium culmorum*
F. graminearum	*Fusarium graminearum*
F. poae	*Fusarium poae*
F. pseudograminearum	*Fusarium pseudograminearum*
FC	*Fusarium culmorum*
FDR	False discovery rate
FG	*Fusarium graminearum*
FHB	Fusarium Head Blight
GWAS	Genome-wide association study
h^2	Heritability
HOG	High osmolarity glycerol
HR	Hypersensitive response
LD	Linkage disequilibrium

MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
NIV	Nivalenol
nsLTPs	nonspecific lipid transfer proteins
OLI	Oberer Lindenhof
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
QDR	Quantitative disease resistance
QTL	Quantitative trait loci
QTNs	Quantitative trait nucleotides
REML	Restricted maximum likelihood
RY	Rye
SNA	Synthetic nutrient-poor agar
SNPs	Single nucleotide polymorphisms
SVD	Singular value decomposition
TC	Triticale
VdCUT11	*Verticillium dahliae* extracellular cutinase
ZEA	Zearalenone
ρG	Genotypic variance

1 General Introduction

Fusarium head blight (FHB) is one of the most devastating diseases of small-grain cereals, including bread wheat (*Triticum aestivum L.*) and rye (*Secale cereale L.*), which can be caused by several species of the genus Fusarium (McMullen et al., 1997). The most frequent species are those belonging to the *Fusarium graminearum* (FG) (teleomorph: *Gibberella zeae* (Schw.) Petch) species complex and *Fusarium culmorum* (FC) (W.G. Smith) Sacc. (teleomorph: unknown). These pathogens can affect the plant in different physiological stages and plant parts, yet result in different symptomatology. Examples are foot and root rot in young plants and the characteristic symptomatology of FHB which occurs from flowering to grain maturity (Scherm et al., 2013). Economic losses are difficult to estimate given the complexity of the symptoms and the different types of crops affected. In order to understand the impact of the disease most studies divide the losses as direct and indirect. Direct losses are considered mainly the reduction in grain yield and indirect losses are comprised of increases in production costs related to efforts to control the disease and the reduction in the quality of the final product. As reviewed by McMullen et al., (1997), only in the United States, FHB was found in 31 of 40 states surveyed in 1917, with losses estimated at 288,000 metric tons (10.6 million bushels), primarily in Ohio, Indiana, and Illinois (Atanasoff, 1920). Fusarium head blight caused an estimated loss of 2.18 million metric tons (80 million bushels) of winter and spring wheat throughout the United States in 1919 (Dickson, 1929). Due to the major outbreaks reported during the decade of

1990, the disease is considered as re-emerging. Since 1991, FHB outbreaks of varying intensity have been common and widespread across much of the eastern half of the United States, resulting in a drop in production by approximately 25% or 2.72 million metric tons across 11 states concerning 6.1 million hectares yearly (Kephart, 1991).

The main effect on quality of wheat originates from the contamination of grain by mycotoxins, more specifically deoxynivalenol (DON), nivalenol (NIV), and zearalenon (ZON) (Hellin et al., 2016). Recent compelling evidence link the Fusarium mycotoxins with several livestock and human disorders and syndromes, including feed anorexia, emesis and refusal, digestive tract lesions, reproductive and endocrine system dysfunction (D'Mello et al., 1999). Due to the drastic effects of mycotoxins on animal and human health, strict regulatory limits have been stablished in many countries. The maximum allowable DON content in the EU is 1250 μg/kg for unprocessed cereals and 1750 μg/kg for unprocessed durum wheat, oats and maize when used for human food (COMMISSION OF THE EUROPEAN COMMUNITIES, 2007). On the other hand, mycotoxins have been suggested to play an important role in the aggressiveness of phytopathogenic Fusarium species towards plant hosts (Proctor et al., 1995).

Management strategies consist of (1) chemical treatment (fungicides) (2) crop rotation, (3) soil tillage by ploughing, and (4) breeding of resistant varieties. Given the constantly increasing cost of management strategies and the emergence of resistant pathogen populations to chemical control, reduced profitability and logistic complexity of alternative crop rotations and the high ecologic impact of soil management, plant breeding emerges as a reasonable and long-lasting method of disease management. The success of breeding for disease resistance has found challenges in two specific scenarios: First, for some pathosystems, where it is relatively fast to breed completely resistant varieties, the pathogen populations are equally fast

or even faster at developing new and stronger pathotypes (Palloix et al., 2009). This scenario evidenced the need of more research to understand how pathogenicity (meaning the ability of the pathogen to infect a susceptible host) acts and what determines its dynamics and strength. Second, for other pathosystems, it is not possible to find completely resistant varieties. Instead, there are varieties expressing partial resistance, which are harder to develop, but more stable in time.

The theoretical framework to understand complete resistance (also known as qualitative, vertical, race-specific, oligogenic) and partial resistance (also known as quantitative, horizontal or polygenic) date back to Vanderplank's studies in epidemiology (Zadoks & Schein, 1988). Two terms exist referring to intensity or strength of the pathogen's ability to attack a plant: Virulence and aggressiveness. Virulence is used when talking about monogenic/qualitative interactions and aggressiveness is used for polygenic/quantitative interactions. When addressed from the perspective of the plant, the quantitative interaction is called "Quantitative disease resistance (QDR)", but when addressed from the perspective of the pathogen it is known as "aggressiveness". FHB-causing species are considered to follow a quantitative interaction with their hosts and it has been proven that resistant cereal cultivars remained resistant against different Fusarium isolates and even Fusarium species (Tóth et al., 2008), specific interactions between cultivars and isolates have not been observed (Van Eeuwijk et al. 1995, Voss et al. 2010). Now, considering the wide host range of FHB, research is still pending to test whether any type of interaction exist when comparing different host species rather than different cultivars within species.

The understanding of the molecular mechanisms controlling pathogeniticy, either virulence or aggressivenness, is key for the design of durable management strategies to include in plant breeding. Flor (1971) proposed the gene-for-gene theory, an explanation from the genetic perspective of

the scenario of qualitative resistance. In this theory, a specific resistance (R) gene in the plant interacts with its corresponding avirulence gen (Avr) in the pathogen unleashing the plant defense responses resulting in resistance. Most of the known resistance genes codify for membrane proteins responsible of the recognition of secreted pathogen associated molecular patterns (Dangl & Jones, 2001; Schulze-Lefert et al., 2011). A comprehensive study of quantitative plant-pathogen interactions, on the other hand, has taken much longer (Corwin & Kliebenstein, 2017; Poland et al., 2009). Quantitative interactions are defined as the coordinated action of many plant and pathogen genes leading to a disease reaction highly influenced by the environment. Current studies point out a gray area between the extremes represented by the gene-for-gene model and the quantitative interaction. For example, several authors have questioned whether the loci controlling the two types of resistance are distinct (Jones & Dangl, 2006), suggesting that quantitative and qualitative resistance could be controlled by the same genetic mechanisms. As a result, a model called "the zigzag model of plant immune system" has been proposed to indicate that quantitative interaction could be the shadow effect of several broken gen-for-gen interactions accumulated during a coevolutionary process between plant and pathogen (Jones & Dangl, 2006). It is therefore key to study the role of pathogen secreted proteins in quantitative interactions to test this hypothesis.

It is most likely that due to the high impact of FHB disease, *F. graminearum* has been considered as a model system for the study of quantitative plant pathogen interactions. A wide variety of approaches have been used to unveil the molecular components involved in the signaling pathway of aggressiveness. Case in point, secretomics (Yang et al., 2012), mutational analysis (Beliën et al., 2007), transcriptomics (Lysøe et al., 2011), genome wide association (Talas et al., 2016) and network-based data integration (Lysenko et al., 2013) are just some examples to name a few. All approaches revealed a large number of molecular features related with aggressiveness.

Even if all those studies have been useful to evince the complexity of the aggressiveness regulation, it is still unclear which are the key factors leading to different levels of aggressiveness. While knock-out based studies declared major regulators as being related with aggressiveness, this methodological approach did not allow to differentiate between i) a pleiotropic effect in both the basic metabolism and aggressiveness; ii) or a unique key-regulating role in aggressiveness. As an example, a transcription factor involved in respiration would definitively show an association with aggressiveness in a knockout strain, as an organism defective in this metabolic pathway could not be a successful pathogen. An approach exploiting the natural diversity found within the pool of alleles of specified genes could dismantle the pleiotropic effect and explain the quantitative differences among isolates. Genome wide association mapping uses the diversity within natural populations to dissect the genetic architecture of quantitative traits, but its elevated costs for genotyping limits the implementation at present.

Candidate gene association mapping is a low cost variation of association mapping, and a powerful tool that can confirm real functional polymorphisms associated with aggressiveness and therefore, polymorphisms that distinguish different aggressive haplotypes. This methodology allows the identification of (1) genes not belonging to the basic metabolism ("house-keeping genes"), (2) not affecting pathogenicity itself, but only the amount of pathogenicity (=aggressiveness) and explaining quantitative differences in aggressiveness. From the molecular genetics perspective, the implementation of this approach requires some previous information on putative functionality to select the candidate genes to be tested and on genomics, in order to construct the primers to amplify the selected genomic regions to study. The first genome sequence published for a pathogen associated with FHB was for FG (Cuomo et al., 2007). Currently, two isolates have been sequenced for FC: the Australian CS7071 (PRJEB1738) ("About - Wheat Pathogens Genomes - Organizations - data.bioplatforms.com,") and UK99, an isolate from the United Kindom belonging to the chemotype DON/3-ADON

("Ensembl Genomes"; Urban et al., 2016). FC was reported to possess four chromosomes similar to those found in FG (Urban et al., 2016). For the specific case of FHB caused by FC, only few components associated with aggressiveness have been identified (Baldwin et al., 2010; Urban et al., 2010; Pasquali et al., 2013; Skov et al., 2004; Spanu et al., 2018), and it remains totally unclear, which might be the differences in terms of regulation of aggressiveness between FG and FC. Candidate gene association mapping has proven its utility in FG, where association of aggressiveness and single nucleotide polymorphisms (SNPs) in the genes *TRI1*, *MetAP1*, *Erf2* were identified (Talas et al., 2012). The genes coding for biosynthesis of DON are located in at least two genomic regions. *TRI1* is located in one cluster and has a confirmed role in the production of DON. The function of *MetAP1* is still not described in *Fusarium* spp., however a deletion in *Saccharomyces cerevisiae* reduces hyphae growth (Li & Chang, 1995). *Erf2* gene is a component of the RAS pathway. *RAS2* plays a role in hyphal growth regulation and production of hydrolytic enzymes, suggesting a direct effect in pathogenesis (Bluhm et al., 2007).

The success of the candidate gene approach highly depends on the accurate selection of the genes to be tested. This is a task of major complexity in FHB, as the number of genes proposed to play a role in aggressiveness is large (Geng et al., 2014). Understanding pathogens' host range, life style and speciation pattern would facilitate the selection of proper candidate genes. It has been proposed that several biotrophic pathogens evolved from symbiotic relationships with the host and tend to develop a monogenic type of resistance/virulence (Lundberg et al., 2012; Ortega et al., 2016), while necrotrophic pathogens which could have evolved from saprophytes, have a much wider host range and tend to develop quantitative interactions (Kohler et al., 2015). FC is a hemibiotroph whose natural host range expands to several species of *Gramineae*, although has been occasionally reported in other plants like sugar beet, potato (Scherm et al., 2013) and Arabidopsis (Cuzick et al., 2009). Hemibiotrophs establish a biotrophic interaction at

the very beginning of the colonization, but once they settle within the host tissue they behave as necrotrophs. Therefore, a comprehensive selection of candidate genes for aggressiveness in FC should include proteins involved in the first line of communication with the host (for the possible role during the biotrophic phase), as well as degradation enzymes (for the possible role during the necrotrophic phase), and some other master regulators at different layers within the signaling pathway.

The implementation of any association mapping approach relies heavily in the use and identification of the proper phenotyping tools and data analysis methodologies. Most of the research in the field of molecular pathology focused on monogenic resistances, especially for cereal rusts and powdery mildews. This trend could be partially explained by the relative easiness to phenotype when only two categories for symptomatology exists, e.g, resistant and susceptible. Advances in the field of phenotyping and data analysis promote the shift from a qualitative to a quantitative perspective in plant pathology. Currently, multi-isolate field experimental designs and proper statistical tools (mixed-model approaches) for the analysis of quantitative data have been generated (Malosetti et al., 2017). Mixed model analyses allow to disentangle the relative contribution of different sources of variation, which is useful in the understanding of isolate-by-host and isolate-by-environment interactions. Similar approaches have already been used in the field of quantitative pathogenicity analyzing a multi-factorial experiment using a collection of *Botrytis cinerea* isolates (Corwin et al., 2016).

Objectives

The main objective of this study was to enhance our understanding of the aggressiveness in FC, in terms of the molecular factors involved in the control of the aggressive reaction and the interaction with the environment. The specific objectives of our study were:

1. Understand the isolate by host and isolate by environment interactions on the expression of aggressiveness using a mixed-model approach in data generated by a multi-environment field trial with a subpopulation of 28 FC isolates inoculated in four different cereal host species (bread wheat, durum wheat, rye, triticale) (Castiblanco et al., 2020).

2. Implement the candidate gene association mapping approach in a population of 100 naturally occurring isolates of FC, in order to identify and validate 17 previously suggested candidate genes for their association with field aggressiveness and mycotoxin production in wheat (Castiblanco et al., 2017).

3. Compare the outcome of the candidate gene association mapping approach in natural occurring isolates of FC evaluated for aggressiveness in bread wheat with the results of the same isolates in rye (Castiblanco et al, 2018).

2 Be flexible and adapt easily – The great role of plasticity relative to genetic variation for aggressiveness and deoxynivalenol (DON) production of *Fusarium culmorum* isolates

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Be flexible and adapt easily—The great role of plasticity relative to genetic variation for aggressiveness of *Fusarium culmorum* isolates

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Abstract

The phenotypic variation in an array of pathogen isolates in natural environments can be partitioned into genotypic variation and environmental plasticity. The present study uses a mixed-model approach to partition the relative contribution of both factors among isolates of *Fusarium culmorum* from natural field populations in various environments. Twenty-eight and 38 isolates from an international collection were phenotyped for aggressiveness and deoxynivalenol (DON) accumulation across two locations during the years 2015 and 2016, respectively, on four winter type cereals as hosts: bread wheat, durum wheat, triticale and rye, thus providing 16 environments. Aggressiveness, measured as Fusarium head blight (FHB) severity, was assessed by visually rating the symptoms of all isolates on infected hosts, and for 10 isolates, additionally the mycotoxin deoxynivalenol (DON) was measured in the grain after harvest. Despite significant genotypic variation among the isolates, the interactions with years and locations explained the largest proportion of variance which disentangled the overwhelming role of plasticity. Host-by-isolate interaction was not significant and no significant ($p < .001$) change in the ranking of isolates from one host to another was detected. As the main factor of plasticity was isolate-by-year interaction, this implies that seasonal changes might be an important evolutionary driver in *F. culmorum* populations.

KEYWORDS

bread wheat, durum wheat, evolutionary aspects, mycotoxins, rye, symptoms, triticale

1 | INTRODUCTION

Fusarium head blight (FHB), also referred to as head scab or ear blight, is one of the most destructive diseases that affects small-grain cereal cultivation worldwide (Goswami & Kistler, 2004). Pathogen survey studies have indicated that in Europe *Fusarium graminearum*, *F. culmorum*, *F. poae* and *F. avenaceum* are among the most common species causing FHB (Parry, Jenkinson, & McLeod, 1995; Pasquali et al., 2016). The economic losses associated with this fungal disease are reduced grain yield, a decrease in grain quality and mycotoxin contamination, therefore rendering the grain inappropriate for food or feed (Scherm et al., 2013). *Fusarium culmorum* (W.G. Smith) Sacc.

is a generalist infecting all plant parts with a wide host range which also includes dicotyledonous plants, occurring worldwide as parasite and saprophyte (Becher, Miedaner, & Wirsel, 2013) producing several mycotoxins during infection, such as zearalenone and the B-type trichothecenes deoxynivalenol (DON) or nivalenol (NIV, Scherm et al., 2013). These mycotoxins represent a significant risk to food safety and the health of humans and animals due to different toxicological effects (Yazar & Omurtag, 2008). *Fusarium culmorum* is categorized as a hemibiotrophic pathogen. Hemibiotrophs present a short biotrophic phase throughout the primary phase of infection and then switch to necrotrophy and secretion of mycotoxins and enzymes to degrade host cell walls (Goswami & Kistler, 2004; Kazan, Gardiner, & Manners, 2012). *Fusarium culmorum* survives on cereal debris or in the soil and infects the roots or stem bases of crops causing in Europe a symptom known as "brown foot rot" (Becher et al., 2013). The production of perithecia and sexual spores is not known from *F. culmorum*. The asexual spores (conidia) can subsequently infect cereal heads through the florets under sufficient humidity (Scherm et al., 2013). Therefore, cereal plants are most vulnerable to FHB infection during the flowering stage. The disease is most severe in years with frequent precipitation around the flowering period (Cowger, Patton-Özkurt, Brown-Guedira, & Perugini, 2009; De Wolf, Madden, & Lipps, 2003).

In the cereal–*Fusarium* pathosystem, a key factor that determines the parasitic fitness of an isolate is aggressiveness, which is a quantitative measurement of the level of disease caused by the pathogen (Pariaud et al., 2009; van der Plank, 1984). Aggressiveness is frequently evaluated by directly assessing symptom development at different time points (=epidemic rate) (Castiblanco, Castillo, & Miedaner, 2018; Miedaner & Schilling, 2004; Miedaner, Gang, & Geiger, 1996; Pariaud et al., 2009). It reflects several basic quantitative traits of the fungal life cycle, such as infection efficiency, spore production rate, sizes of the lesion and for some cases, also toxin accumulation (Cumagun & Miedaner, 2004; Lannou, 2012).

Aggressiveness is of complex inheritance with many genes, each contributing only to a small part of the total variation. Recently, a candidate gene-based association study reported an association of mitogen-activated protein kinase (MAPK) HOG1 gene with aggressiveness and deoxynivalenol (DON) accumulation, explaining 10.29 and 6.05% of the genotypic variance in wheat, respectively, and of a cutinase gene explaining 16.05% of genotypic variance in rye (Castiblanco et al., 2018; Castiblanco, Marulanda, Würschum, & Miedaner, 2017). The role of DON in the different phases of the Fusarium life cycle (e.g. saprophytic and pathogenic), as well as abiotic factors influencing DON biosynthesis, has been condensed recently in a review (Audenaert, Vanheule, Höfte, & Haesaert, 2013). *Fusarium culmorum* isolates largely differ in their DON accumulation (Burlakoti et al., 2007; Gang, Miedaner, Schuhmacher, Schollenberger, & Geiger, 1998). However, the association between FHB symptoms and DON accumulation at harvest might be lower when DON accumulation is normalized by fungal biomass (Miedaner, Reinbrecht, & Schilling, 2000; Voss, Bowden, Leslie, & Miedaner, 2010). DON is known to allow fungal spread beyond initial infection (Bai, Desjardins, & Plattner, 2002; Maier et al., 2006) and might even induce the switch from biotrophy to necrotrophy (Bönnighausen, Schauer, Schäfer, & Bormann, 2019). This illustrates that DON is an aggressiveness factor rather than a pathogenicity factor (Proctor, Hohn, & McCormick, 1995). Accordingly, Desjardins et al. (1996) found by using a trichothecene-deficient isolate a reduced aggressiveness rather than non-pathogenicity in field tests.

Pathogens have to respond to a variety of stresses to be successful invaders. They are caused by seasonal changes (abiotic) and by the host (biotic) resulting in evolutionary pressures that can lead to the development of plasticity, defined as the ability of a pathogen to detect changes in its environment and the use of signal transduction pathways to alter its phenotype in response to environmental changes (Price, Qvarnström, & Irwin, 2003). This allows a generalist pathogen to optimize its growth and aggressiveness across an array of environments (locations, years and host species). To get more insight into the dynamics of pathogens, it is necessary to analyse the relative contribution of plasticity and genetic variation for aggressiveness. As aggressiveness is a quantitative trait, proper statistical tools of quantitative genetics and statistics should be applied in the analysis (Malosetti, Ribaut, & Eeuwijk, 2013). Plasticity and genetic variation can be estimated applying the mixed-model approach in the analysis of a multi-factorial experiment using a collection of isolates (Corwin, Subedy, Eshbaugh, & Kliebenstein, 2016). This allows to partition phenotypic variation into genotypic (isolate) variation and plasticity caused by environmental variation and all interactions of isolate with locations, years and hosts (Scheiner & Lyman, 1989). Cereal-associated *Fusarium* species are particularly suitable for investigating plasticity effects: (a) they occur in the temperate climate zones over a wide geographical range from Japan, Siberia and Europe until the Canadian Prairies and the Western U.S. states, (b) they have a wide host range, *F. graminearum* and *F. culmorum* for example, can infect all small-grain cereals, maize and an array of other crops (Goswami & Kistler, 2004), (c) they can infect living hosts effectively, but are also able to survive over years in the soil and debris saprophytically and change regularly between these life styles depending on the availability of a host, (d) concerning host specificity, no consistent host genotype × isolate interaction was reported, although these studies used only a few isolates (Van Eeuwijk et al., 1995; Voss et al., 2010), and (e) high molecular diversity was identified in *F. culmorum* populations by microsatellites using 186 isolates (Miedaner, Caixeta, & Talas, 2013).

The following hypotheses were addressed in this case study by partitioning the phenotypic variation in *Fusarium* isolates into its genotypic and plasticity components by a mixed-model approach: (a) plasticity of aggressiveness plays an important role in pathogenic variation; and (b) isolates affect the four winter cereal host species bread wheat, durum wheat, triticale and rye in a similar way. In order to prove these hypotheses, 28 and 38 *F. culmorum* single-spore isolates, respectively, were phenotyped in terms of aggressiveness and DON accumulation under 16 environments that is the combination of four host crops, 2 years and 2 locations.

2 | MATERIALS AND METHODS

2.1 | Fungal materials and inoculum production

Isolates from the international collection of *F. culmorum* at the State Plant Breeding Institute, University of Hohenheim, were randomly chosen for field tests. All isolates belonging to this international collection were derived from single-spore cultures to avoid mixture of genotypes and were always treated independently to preserve their identity. In addition, all isolates from the collection were genetically different due to clone correction based on the information obtained with 10 microsatellite markers from a previous study of genotype diversity (Miedaner et al., 2013). A multiplex PCR (polymerase chain reaction) assay verified the identification of the species and the chemotype of the collection (Miedaner et al., 2013). In 2015, 28 *F. culmorum* isolates were used for our analysis. During 2016, the sample was extended by an additional 10 isolates. The isolates used differ mainly in year of isolation and site of origin where they were collected and all but two correspond to the 3-ADON chemotype (Table 1). The German isolates from Entringen and Nufringen as well as the Russian isolates from Novgorod were each collected from a single field and preselected for their molecular diversity (Miedaner et al., 2013). The Syrian isolates were collected on a transect between Tal-Kalakh and Aleppo (ca. 250 km). All other isolates were collected from heads with visual symptoms of premature bleaching on individual fields that were naturally infected with one isolate per collection site. Inoculum of each isolate was produced using a modified form of Bilay's liquid medium as described by Reid, Mather, Hamilton, and Bolton (1992). A 1 cm^2 plug of potato dextrose agar culture from each single isolate was added to an independent 500-ml Erlenmeyer flask with 300 ml of autoclaved medium, with special care taken in order to avoid mixture and contamination of isolates. The flasks were placed on a shaker under natural light supplemented with cool white fluorescent lights and shaken for 8 days, and stored in centrifuge tubes of 15 ml or 50 ml under −80°C until usage. According to Marc Lemmens, IFA Tulln, Austria (pers. commun.), this deep freezing preserves the level of aggressiveness of the isolate.

2.2 | Design of field trials and inoculation

Field experiments were conducted during 2015 and 2016, each across two locations: Hohenheim (HOH, longitude 9°11′23″E, latitude 48°42′54″N, altitude 403 m) and Oberer Lindenhof (OLI, longitude 9°18′17″E, latitude 48°28′25″N, altitude 702 m). The experiments followed a split-plot experiment with two replications, in which the main-plot factor "host" is laid out in randomized complete blocks, while the subplot factor "isolate" is assigned as an incomplete block design (α-design) within main plots. Four winter cereal crops were used as hosts: (a) bread wheat (*Triticum aestivum* L.), cultivar Inspiration (KWS LOCHOW GMBH, Bergen, Germany),

TABLE 1 Origin of the *Fusarium culmorum* isolates under study: all isolates are of the 3-ADON chemotype derived from bread wheat (*Triticum aestivum* L.) except those otherwise noted

Isolate ID	Origin	Year of sampling	Contributor
7D23	Entringen, Germany	2008	Talas, Miedaner
7D26	Entringen, Germany	2008	Talas, Miedaner
7D28	Entringen, Germany	2008	Talas, Miedaner
8D33	Herrenberg 1, Germany	2008	Talas, Miedaner
9D1	Nufringen, Germany	2008	Talas, Miedaner
9D34	Nufringen, Germany	2008	Talas, Miedaner
9D38	Nufringen, Germany	2008	Talas, Miedaner
9D40	Nufringen, Germany	2008	Talas, Miedaner
FC104	Fundulea, Romania	1996	Ittu
FC33[a]	Chewendowa, Poland	1991	Miedaner
FC40	Szeged, Hungary	1991	Mesterhazy
FC50[b]	Wageningen, Netherlands	1987	Snijders
FC60[b]	CBS 251.52, Netherlands	1952	Unknown
FC65[a]	Svalöf, Sweden	1992	Miedaner
FC74	Crookston, Minnesota, USA	1992	Univ. of Minnesota
FC75	Zürich–Reckenholz, Switzerland	1992	Miedaner
FC90[c]	Luz, Portugal	1993	Miedaner
Russ111	Novgorod, Russia	1994	Levitin
Russ1411	Novgorod, Russia	1994	Levitin
Russ1611	Novgorod, Russia	1994	Levitin
Russ1911	Novgorod, Russia	1994	Levitin
Russ411	Novgorod, Russia	1994	Levitin
Russ711	Novgorod, Russia	1994	Levitin
Russ811	Novgorod, Russia	1994	Levitin
S022	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S060	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S109	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S129	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S222	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S256	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S259	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S264	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S265	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S275	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S276	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S280	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S289	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner
S299	Tal-Kalakh—Aleppo, Syria	2007	Talas, Miedaner

[a]Collected from rye (*Secale cereale* L.).

[b]NIV chemotype.

[c]Collected from durum wheat (*T. turgidum* var. *durum*).

(b) durum wheat (*Triticum turgidum* ssp. *durum*) cultivar Lupidur (Saatzucht Donau, Probstdorf, Austria), (c) triticale (X *Triticosecale* Wittm.) cultivar Cando (Lantmännen SW Seed B.V., Emmeloord, the Netherlands) and (d) rye (*Secale cereale* L.), genotype L2177-PxL2184-N (HYBRO Saatzucht GMBH&Co.KG, Germany). As all commercial cultivars of rye are either open-pollinated populations or three-way cross hybrids, both with a certain degree of heterogeneity, we used a specially produced F_1 single cross of the two mentioned inbred lines being highly homogeneous. The crossing was done on the basis of cytoplasmic male sterility to achieve enough seed. All host genotypes were moderately susceptible to FHB as known from previous experiments. Field plots were arranged as such that each inoculated entry plot of the host under study was surrounded by four border plots of a tall triticale cultivar with a plant height of 1.10 m (X *Triticosecale*, cultivar Aveo, KWS LOCHOW GMBH) to minimize plot-by-plot interference as described earlier (Talas, Kalih, Miedaner, & McDonald, 2016; Talas, Würschum, Reif, Parzies, & Miedaner, 2012). Experimental units were three-row plots (1.0 m long, 0.625 m wide), which were machine sown with 220 kernels per m^2, a seeding rate that results in a uniform stand.

For inoculation, each plot was inoculated at mid-flowering with 62.5 ml of a suspension diluted to a constant concentration of 2×10^5 conidiospores/ml. Mid-flowering was considered as the stage in which more than 50% of all spikes of a plot extruded anthers. Inoculum for each isolate was sprayed using a hand-held atomizer (Model "Multispray" with 0.7 L flask, ewo, H.Holzapfel GmbH&Co.KG) connected with a tractor to achieve constant air pressure of three bar to ensure full coverage of all heads of the plot with exactly the same dosage. All plots of the same host genotype could be inoculated at the same date per location. Due to flowering differences, however, each host species required a different inoculation day; for exact dates, please refer to Figure 1 and Figure S1.

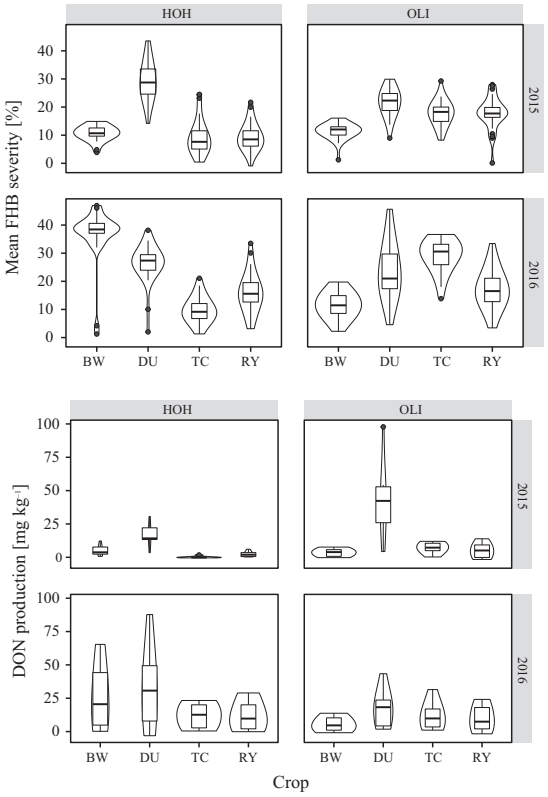

FIGURE 1 Violin boxplots of (a) Best Linear Unbiased Estimators (BLUEs) of mean Fusarium head blight (FHB) severity for 28 (2015) and 38 (2016) isolates of *Fusarium culmorum* and (b) BLUEs of deoxynivalenol (DON) accumulation for 10 isolates evaluated under field conditions on four host species (bread wheat [BW], durum wheat [DU], triticale [TC], rye [RY]) across 2 years (2015 and 2016) and two locations (OLI = Oberer Lindenhof, HOH = Hohenheim); the boxes indicate 25 and 75 percentiles, respectively, of the distribution, the horizontal line within boxes indicate the median

2.3 | Assessment of aggressiveness

To assess aggressiveness of isolates, FHB symptoms were visually rated in each plot at least three times starting with the onset of symptom development on each host, about 14 days after inoculation (Stage 75 on the cereal growth scale: fruit formation, medium milk ripening) and was continued at 3- to 5-day intervals until the beginning of the yellow ripening stage (Stage 87: Ripening, hard dough). Rating was performed as a percentage of infected spikelets per plot (0%–100%). This reflects both the percentage of infected spikes per plot (type I resistance) and the percentage of infected spikelets per spike (type II resistance) in a single rating as earlier described by Miedaner et al. (1996). This rating is equivalent to the FHB index used in North America, which is the product of incidence and severity divided by 100 (von der Ohe et al., 2010). The arithmetic mean of at least three ratings is called mean FHB severity throughout the paper and is used a measure of aggressiveness. This procedure was followed to take into account the quantitative nature of aggressiveness. When only one unique rating is used, the data behave as categorical data instead as quantitative data. By repeated measurements of the same plot, categorical data behave as quantitative and become suitable for a quantitative genetic analysis. Extensive studies have been developed in order to identify the proper strategy to quantify Fusarium symptoms on cereals (Emrich, Wilde, Miedaner, & Piepho, 2008; Miedaner et al., 2004, 2006; Simko & Piepho, 2012). This standard procedure is widely used in Europe and also part of the official trials for cultivar registration.

2.4 | Quantification of DON accumulation

To measure DON accumulation, plots were harvested at full ripening (Stage 89 on the cereal growth scale: hard grain) by hand at both

locations in 2015 and 2016. As precipitation and relative humidity at HOH and OLI during May and June 2016 were higher than the last 30-year average (Landwirtschaftliches Technologiezentrum (LTZ)-Agrarmeteorologie Baden-Württemberg 2019), FHB severity reached 100% for most of the plots before the ripening stage (stage 89) and in these plots grain was aborted or too small to be harvested. For only 10 isolates, it was possible to obtain enough grains (>50 g) to proceed with milling and DON extraction: 7D23; 7D26; 8D33; FC33; FC50; FC60; FC65; Russ1611; S60 and S129 (Table S1). Harvested grains for those ten isolates in both years and both locations were threshed in a single-head thresher (Walter-Wintersteiger) and cleaned with reduced wind speed. The remaining fragments of glumes and rachis were manually picked out to retain even the very small kernels in the sample. Cleaned grain was ground in a commercial laboratory mill (Foss Cyclotec™ 1093, Foss GmbH) with a sieve size of 1 mm. Later, the coarse meal was analysed to quantify the amount of DON by the commercially available immunotest RIDASCREEN® DON according to the instruction of the manufacturer (R-Biopharm AG). We analysed all field plots of the respective isolates, including the replicates. Five standard solutions of known DON concentration (0, 3.7, 11.1, 33.3 and 100 ppb) provided by the manufacturer were located in each plate with two replicates as controls of the quantification process.

2.5 | Data analyses

A mixed-model approach and additive main effects and multiplicative interactions (AMMI) analysis were used. For the description of the models, the syntax suggested by Patterson (1997) was followed, where fixed effects appear before the colon and random effects after the colon. The FHB data were analysed based on the following linear mixed model:

$$I + H + H \times I + Y + L + Y \times L + Y \times L \times R + I \times Y + I \times L + I \times Y \times L + H \times Y + H \times L$$
$$: H \times Y \times L + H \times I \times L + H \times I \times Y + I \times Y \times L \times H + Y \times L \times R \times H + Y \times L \times R \times H \times B,$$

and the following AMMI model:

$$I + E + M \times N \times H + P$$

where the abbreviated letters mean isolate (I), host (H), year (Y), location (L), replication (R), incomplete block (B) and environment (E) or their interactions, respectively. The term environment (E) in the AMMI analysis is regarded as the combination of host, location and year, while M, N, H and P correspond to the singular value for SVD (singular value decomposition) axis m, isolate singular vector value for SVD axis n, environment singular vector value for SVD axis h and the AMMI residuals, respectively. DON analyses were carried out in a randomized complete block design instead of the α-design. Given that only ten entries with two replicates were processed for each trial in all environments, the incomplete block (α-design) structure was lost. The linear mixed model used for DON analyses was the same used for FHB data, with factor B missing.

According to Scheiner and Lyman (1989) phenotypic plasticity is the change in phenotype caused by a change in the environment and is equivalent to the sum of the environmental variance (σ^2_E) and genotype–environment interaction variance ($\sigma^2_{G\times E}$). Plasticity variance was calculated as a ratio of plastic variance (σ^2_{PI}) and phenotypic variance. The ratio is equivalent to the percentage of total variance according to plasticity.

Variance components and best linear unbiased estimators (BLUE) of each isolate were determined by the restricted maximum likelihood (REML) method. Significance of variance components was tested by model comparisons with likelihood ratio tests (Stram & Lee, 1994). Heritability (H^2) was estimated as heritability on an entry-mean basis following the approach suggested by Piepho and Möhring (2007). Heritability in the broad sense, also known as entry-mean heritability, is defined as the proportion of genotypic to phenotypic variance where the number of replicates, locations and years are taken into account (Falconer & Mackay, 1996). AMMI analyses were performed using the function AMMI in the R package AGRICOLAE, version 1.2-8 (Mendiburu, 2017). All mixed-model analyses were performed using ASREML 3.0 (Gilmour, Gogel, Cullis, & Thompson, 2009) and R (R Core Team, 2016) combined with the graphical user interface RStudio. Genotypic correlation as a method for extracting genotypic effects from environmental interaction and error variances (Fisher, 1918) and phenotypic correlations were calculated with R package META-R, version 6.0 (Alvarado et al., 2015), for the dataset of aggressiveness.

3 | RESULTS

3.1 | Means and distributions

Isolates produced characteristic symptoms of FHB on inoculated cereals and differences among isolates were observed at all hosts and all environments, which provided enough phenotypic variance to be studied (Table 2). Contamination with natural inoculum was negligible as verified by the border plots which always had a FHB severity of <5%. Additionally, the least aggressive isolate had a FHB rating of 1.23% in bread wheat and 2.02% in durum wheat, the two most susceptible hosts, illustrating only a very low background infection, if any.

TABLE 2 Means and ranges (in brackets) for mean Fusarium head blight (FHB) severity and deoxynivalenol (DON) accumulation in four host species across 28 and 38 (FHB) isolates, respectively, and 10 (DON) isolates across two locations in 2 years

Crop species	Mean FHB severity (%)	DON accumulation (mg/kg)
Bread wheat	17.55 (1.23–46.93)	9.99 (0.00[a]–65.36)
Durum wheat	25.32 (2.02–45.63)	26.51 (0.00[a]–97.80)
Triticale	16.56 (0.44–36.66)	8.11 (0.00[a]–31.48)
Rye	15.04 (0.00–33.49)	7.24 (0.00[a]–28.95)

[a]Below detection limit of 0.2 mg/kg.

Among hosts, winter rye flowered first, followed by triticale, bread wheat and lastly durum wheat. Distributions of the best linear unbiased estimators (BLUEs) for mean FHB severity and DON accumulation for each environment (combination of cereal host, location and year) are plotted in Figure 1. Mean FHB severity ranged from 0.0 for isolate FC60 with rye as host in OLI-2015 to 46.93 for isolate S264 with bread wheat as host in HOH-2016. Values of DON accumulation ranged from 0 mg/kg for isolate FC60 in TC-HOH-2015 and WW-HOH-2015, to 97.80 mg/kg for isolate 8D33 with durum wheat as host in OLI-2016 (Table S1).

3.2 | Mixed-model approach

The mixed-model approach applied to the mean FHB severity revealed that out of all fixed effects only isolate (I) and the isolate-year interaction (I × Y) variances were highly significant ($p < .001$) for both traits (Table 3). In order to reveal variance components to compare the different sources of variation, the factor isolate and all its interactions were treated as random in a subsequent step. Isolate–location (I × L) and host–isolate–location (H × I × L) interactions were not significant at all and were, therefore, removed from the model. All variance components were calculated against the isolate variance (I) that was set as 1 (Table 3). The highest component was the interaction host–year–location (H × Y × L), which reflects the factor environment (8.98), after that, the factors isolate (1), isolate–year (0.88), isolate–year–host–location (0.45) and isolate–year–host (0.26) were also significant ($p < .001$).

The mixed-model analysis of DON accumulation revealed the fixed factors isolate, host and the interaction year–location (Y × L) as highly significant ($p < .001$). The differences in terms of isolate ranged between 0 mg/kg (displayed by 7D26, FC60 and S60 in triticale 2015) and 54.61 mg/kg (displayed by 7D23 in bread wheat; $p < .001$); in terms of host species, the values were overall higher in durum wheat and lower in triticale ($p < .001$). Similar to mean FHB severity, the isolate–location (I × L) and host–isolate–location (H × I × L) interactions were not significant and, therefore, removed. The highest variance component was again found for the host–year–location (H × Y × L) interaction, reflecting the environment (1.69), followed by isolate–year (1.09), isolate (1), isolate–year–host–location (0.93) and isolate–year–location (0.38) interactions, all of them significant ($p < .001$).

Interestingly, the isolate–host interaction (I × H) was not significantly different from zero for any dataset. The overall entry-mean heritability was 0.83 for mean FHB severity and 0.88 for DON accumulation, illustrating a high relevance of genotypic variance. Plasticity variance accounted for 73% of the FHB severity and 49% of DON accumulation (Table 3).

3.3 | Meteorological data and disease progress curves

As the interaction isolate–year (I × Y) was revealed as significant by mixed models for both aggressiveness and DON accumulation, we present a comparison of meteorological conditions between years, which can explain the reason why the year played such an important role (Figure 2, Figure S1). All meteorological values were expressed as daily average across the disease progress period from the 21 May to the 20 July. Compared to 2016, the year 2015 was characterized by lower rainfall (HOH: 1.2 mm; OLH: 2.0 mm), lower relative humidity (HOH: 68.8%, OLI: 72.4%) and higher temperatures (HOH: 19.6°C, OLI: 17.6°C) during the flowering/infection period (almost 10 days). In contrast, the year 2016 presented constant rainfall (HOH: 2.6 mm, OLI: 4.4 mm), high relative humidity (HOH: 76.9%, OLI: 83.4%) and lower temperatures (HOH: 18.2°C, OLI: 15.9°C) during the same disease period of time (flowering/infection period).

The disease progress curves calculated from mean FHB severity across all isolates in each environment show an earlier expression of symptoms as well as significantly higher final FHB severity scores in 2016 than in 2015, although not all isolates reacted in the same magnitude as indicated by the significance of isolate–year interaction ($p < .001$). Since the inoculation in each host was made at its respective day of mid-flowering, conclusions cannot be made by visual comparison but only by looking at the mixed model. As the host–isolate interaction was not significant, it is concluded that hosts, as well as flowering dates, had only a minor influence.

TABLE 3 Variance components calculated relative to the isolate variance (σ_I^2) for Fusarium head blight (FHB) severity and deoxynivalenol (DON) accumulation in four host species across 28 and 38 (FHB) isolates, respectively, and 10 (DON) isolates across two locations in 2 years

Parameter[a]	Mean FHB severity (%)	DON accumulation (mg/kg)
Variance components:		
σ_I^2	1.00***	1.00***
$\sigma_{I\times H}^2$	0.10	0.00
$\sigma_{I\times Y}^2$	0.88***	1.09***
$\sigma_{I\times Y\times L}^2$	0.05	0.38***
$\sigma_{H\times Y\times L}^2$	8.98***	1.69***
$\sigma_{I\times Y\times H}^2$	0.26**	0.13*
$\sigma_{I\times Y\times H\times L}^2$	0.45***	0.93***
σ_e^2	2.60[b]	3.39[b]
Plasticity[c]	0.73	0.49
Heritability	0.83	0.88

[a]Variance components for isolate (σ_I^2), isolate × host interaction ($\sigma_{I\times H}^2$), isolate × year interaction ($\sigma_{I\times Y}^2$), isolate × year × location interaction ($\sigma_{I\times Y\times L}^2$), host × year × location interaction ($\sigma_{H\times Y\times L}^2$), isolate × year × host interaction ($\sigma_{I\times Y\times H}^2$), isolate × year × host × location interaction ($\sigma_{I\times Y\times H\times L}^2$), pooled error ($\sigma_e^2$).

[b]As heterogeneous variance for error was assumed the reported value is the mean value of the individuals' errors.

[c]Plasticity as defined by Scheiner and Lyman (1989).

***Significance at $p < .001$.

**Significance at $p < .01$.

*Significance at $p < .05$.

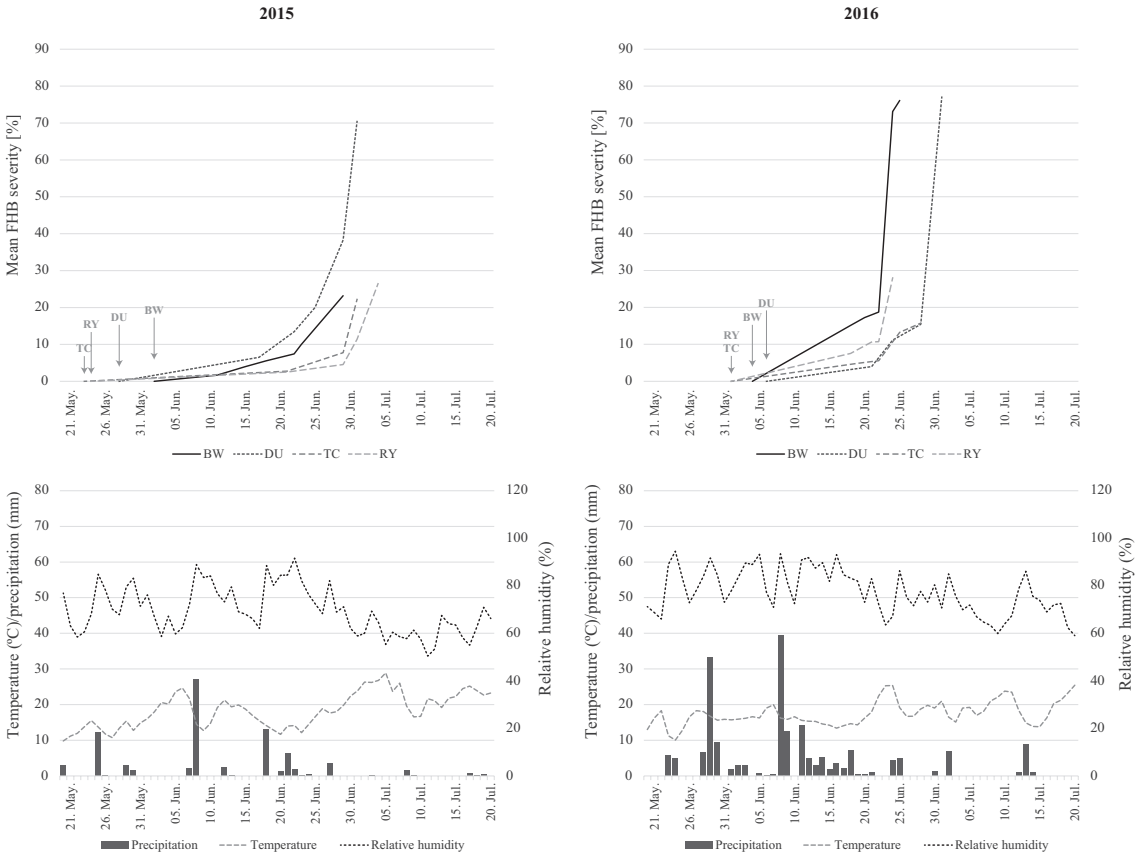

FIGURE 2 Average curves of disease progress of four host species (bread wheat [BW], durum wheat [DU], triticale [TC] and rye [RY]) in function of meteorological conditions at HOH during 2015 and 2016. Arrows indicate inoculation dates for each crop (for OLI see Figure S1)

3.4 | Effects of environment and isolate × environment interaction as revealed by AMMI analysis

The multiplicative terms from the AMMI analysis can be visualized with the aid of a biplot (Figure 3), where isolates and environments are depicted as points on a two-dimensional plane. Distances from the origin are indicative of the amount of interaction that was exhibited by either isolates over environments or environments over isolates (Voltas et al., 2002). Biplots facilitate the exploration of relationships between genotypes and/or environments. Genotypes that are more similar to each other are closer to each other in the plot than genotypes that are less similar. The same is true for environments. Genotypes/environments that are alike tend to cluster together (Malosetti et al., 2013). The closer to the origin, the more stability is exhibited. For instance, isolates as 9D38, Rus1911, FC60, S259, 7D26 and 9D1 were the most stable isolates across the different environments, which meant that those isolates exhibited a similar behaviour in all environments tested. Likewise, durum wheat in OLI-2015 was the most stable environment across isolates. In contrast, bread wheat in HOH-2016 and rye in OLI-2016 were the environments that showed the strongest interactions. Most environments from 2016 are located in the upper left corner and 2015 in the lower right, showing the high effect of the year on interaction and differentiation of the aggressiveness, which is consistent with the analysis of variance.

3.5 | Phenotypic versus genetic correlations among hosts

Consistently with the fact that isolate–host interaction was not significant, as revealed by the analysis of variance using the mixed-model approach, the phenotypic and genotypic correlations calculated from FHB severity data were always positive and significant ($p < .001$, Table 4). Coefficients of genetic correlation ranged from 0.46 to 0.99 in 2015 and from 0.71 to 0.83 in 2016. They were higher than coefficients of phenotypic correlation throughout, as they are separate from environmental interactions and error variances.

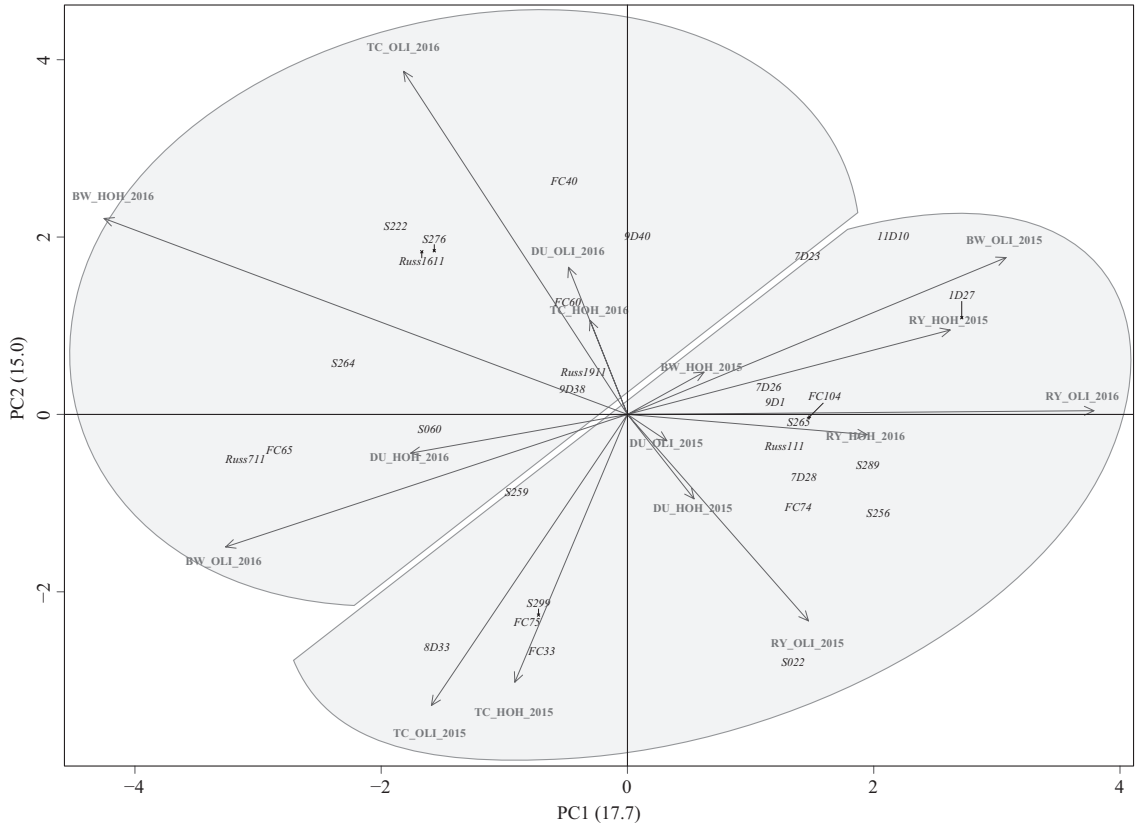

FIGURE 3 Biplot representation of AMMI analysis for mean Fusarium head blight (FHB) severity of 28 (2015) and 38 (2016) isolates of *Fusarium culmorum* tested at two locations (HOH = Hohenheim, OLI = Oberer Lindenhof) on four host species (bread wheat [BW], durum wheat [DU], triticale [TC] and rye [RY]); for the isolate ID, please refer to Table 1; the values in brackets are the % variance explained by this principal coordinate (PC)

Year	2015				2016			
Host	BW	DU	TC	RY	BW	DU	TC	RY
BW		0.738[a]	0.421	0.605		0.769	0.797	0.706
DU	0.999		0.524	0.662	0.770		0.770	0.732
TC	0.463	0.644		0.447	0.797	0.770		0.824
RY	0.871	0.999	0.711		0.705	0.732	0.827	

Abbreviations: BW, bread wheat; DU, durum wheat; RY, rye; TC, triticale.

[a]All phenotypic coefficients of correlations were significant at $p < .001$.

TABLE 4 Coefficients of phenotypic Pearson product-moment correlation (above diagonal) and genotypic correlation (below diagonal) of aggressiveness of 28 (2015) and 38 (2016) isolates of *Fusarium culmorum* in four hosts across 2 locations in 2 years

4 | DISCUSSION

The adaptive response of plant pathogens to natural environments may involve individual plasticity and genotypic variation among isolates or both. Plasticity, understood as the ability to produce different phenotypes by a single isolate under different environmental conditions, is considered an important component of phenotypic change in nature and its identification entails measuring the trait on several isolates across different environments (Corwin et al., 2016). Genotypic variation within a pathogen species is the working substrate for evolution affecting the evolutionary potential of a pathogen (McDonald & Linde, 2002). Assessing the variation in the phenotype of pathogen isolates across environments is required in order to partition the phenotypic variance into genotypic and plasticity variances. From the perspective of the pathogen, an environment is defined as the integration of a specific host under certain climatic conditions over the studied time frame at a particular location. Therefore, for the purpose of the present study, plasticity is

defined as the interactions of isolate with host, location and year. In our study, we exposed 28 (2015) and 38 (2016) single-spore isolates to 16 environments (combination of two locations, two years and four host species) to determine their plasticity and genetic variation with regard to aggressiveness, measured as mean FHB severity, and DON accumulation. High genetic diversity was identified in this *F. culmorum* population by 10 microsatellite markers after correction for genetically identical clones (Miedaner et al., 2013). While there are several studies that have analysed the plasticity or genetic variation in individual traits (for example Aamot et al., 2015), only few have investigated the combination of genotypic and environmental variation on aggressiveness in a hemibiotrophic pathogen (Corwin et al., 2016; Miedaner et al., 1996; Miedaner, Schilling, & Geiger, 2001). Our approach was to utilize the power of the mixed model to disentangle the relative contribution of plasticity and genetic variation in a diversity panel of *F. culmorum*.

4.1 | Relative contribution of plasticity and genotypic variation

Genotypic variation among isolates was significant and explained an important part of the total variance (Table 3). This is in accordance to earlier results, where *F. culmorum* isolates always displayed great differences in terms of aggressiveness and DON accumulation (Gang et al., 1998; Miedaner et al., 1996, 2001). The combined effects of the calculated interaction variances with isolates, illustrating the role of plasticity (Table 3), were much higher than the effect of genotypic variance among isolates. The isolate–year interaction variance especially accounted for an amount of similar variation than isolate variance for aggressiveness and even exceeded the isolate variance for DON accumulation. Additionally, a high and significant variance component for isolate–year interaction was observed, illustrating that isolates reacted differently with seasonal changes. This might have been triggered by the different weather conditions in both years. Accordingly, isolate–host–environment ($\sigma^2_{I\times Y\times H\times L}$) interaction variance was significantly different from zero illustrating a dynamic interplay of the factors involved in the disease triangle. However, it should be mentioned that the four host species flowered at different dates. Because they all belonged to the winter type, a coordination of flowering times by different seeding dates is not possible. The effect of different environmental conditions at flowering/inoculation/penetration dates was accounted for by the interactions host–year–location (H × Y × L) and isolate–year–host–location (I × Y × H × L), which where both significant. The data revealed that the environment (H × Y × L) itself played a determining role in the disease progress, but not all isolates reacted equally at different environments (isolate–year–host–location). In our study, the year–location interaction was not significant while host–year–location was significant meaning that meteorological conditions affected the development of the disease differently depending on the host species.

From these results, we conclude that despite ample genotypic variation among isolates, plasticity including interactions with host species played the most important role in disease development. Accordingly, plasticity reached values of (Table 3) 73% and 49% of the total phenotypic variance for FHB severity and DON accumulation, respectively, illustrating its great importance. This is supported by previous studies on environmental effects in *Fusarium* (Harris, Balcerzak, Johnston, Schneiderman, & Ouellet, 2016; Voss et al., 2010; Walter, Nicholson, & Doohan, 2010).

4.2 | High plasticity also observed for colonization of different host species

Plant pathogens must adapt to changes in their local environment, which represent evolutionary pressures. In a pathogen with a broad host range like *F. culmorum*, the evolutionary pressures from changing hosts during rotation afford a high plasticity required to counteract diverse host resistance reactions (Corwin et al., 2016). To the best of our knowledge, this is the first time that a randomized and replicated experimental design was used to study the role of different small-grain cereal species as hosts to understand the phenotype of the hemibiotrophic pathogen *F. culmorum*. Host species–isolate interaction was found to be negligible illustrating that the ranking of isolates did not differ according to host species. Most isolates were able to infect all hosts in all environments, which might imply triggering different mechanisms of infection by the fungus to overcome the anatomic and biochemical differences between hosts. Although DON accumulation was clearly explained by isolate effects and the host factor was significant, again no interaction with the host (crop) was detected. Differences in DON accumulation when infecting different hosts were observed, for example most of the isolates accumulated more DON on mature grain of durum wheat than on other hosts. But this is not a sign of host specificity because the ranking of the isolates for DON accumulation did not significantly change according to the host.

All four host species (bread wheat, durum wheat, triticale, rye) belonged to the *Poaceae* family. *Fusarium culmorum* is a well-known cereal pathogen but has also been identified infecting dicotyledonous plant species like sugar beet, potato (Scherm et al., 2013) or even *Arabidopsis thaliana* (Cuzick, Maguire, & Hammond-Kosack, 2009; Urban, Daniels, Mott, & Hammond-Kosack, 2002). Although in our study the best and worst isolate in terms of aggressiveness and DON accumulation were not the same for all hosts, the isolate–host interaction was not significant. It has been proposed that basal defence, such as the battery of enzymes evolved by generalists and hemibiotrophs, plays a key role in the resistance of the host and all plant species within the same family share similar defence strategies (Schulze-Lefert & Panstruga, 2011). It would be interesting to study whether the absence of host–isolate interaction is also consistent when the study involves host species other than the *Poaceae* family (Burlakoti et al., 2007).

Previous studies showed that most resistant genotypes of bread wheat remain resistant when challenged against different isolates of *F. culmorum* or even different *Fusarium* species (Tóth, Kaszonyi, Bartok, Varga, & Mesterhazy, 2008; Van Eeuwijk et al., 1995). Therefore, the interaction cereal–*Fusarium* has always been considered as non-race specific following a quantitative resistance according to Van der Plank (1984). However, studies searching for resistance QTL in wheat (Anderson et al., 2001; Buerstmayr et al., 2002) and aggressiveness in *Fusarium* spp. (Castiblanco et al., 2017; Talas et al., 2012) involved only one pathogen isolate (in the search of resistance) or one host genotype (in the search of aggressiveness). Here, we could show that the same isolates are able to infect all four host species without significant rank changes, thus demonstrating the broad-spectrum expectation for this quantitative host–pathogen interaction. This, however, measures only the net effect of isolates on hosts. Still, individual genes involved in the host–pathogen interaction might display a host-specific action as shown in maize (Harris et al., 2016). A recent study comparing candidate genes associated with aggressiveness in wheat and rye revealed indeed two different host-specific genes contributing to aggressiveness (Castiblanco et al., 2018).

In conclusion, the effect of the host species on aggressiveness and DON accumulation was of minor importance. Accordingly, correlations among hosts were always positive and significantly different from zero suggesting no significant change in the rank order of the isolates from one host to another (Table 4). This is reported here for the first time by using four different host species of small-grain cereals in a factorial experiment. Previously, this has only been analysed as host genotype–isolate interaction within individual host species, mainly bread wheat and maize (Kuhnem, Ponte, Dong, & Bergstrom, 2015; Mesterhazy, 1988; Snijders & Van Eeuwijk, 1991).

4.3 | Potential of seasonal changes as evolutionary driver in *Fusarium culmorum* populations

In our study, both years experienced very different weather conditions. The year 2015 had, in our locations, a dry spring with a relative humidity of 68.8% and daily average rainfall of 1.2 mm (Figure 2, Figure S1). In contrast, inoculation period in the year 2016 had 10% more relative humidity and daily rainfall had more than doubled (2.64 mm). Once disease symptoms were visible, the disease reached extreme values in <1 week in 2016. Therefore, the highly and moderately aggressive isolates did not allow enough seed set in most cases to be analysed for DON accumulation. As a consequence, isolate × year interaction was significant for both traits and of similar high importance than isolate variance illustrating that isolates reacted differently to weather conditions of subsequent years. These results confirm that the environmental conditions during the penetration phase are among the most important factors in the development of FHB epidemics (Rossi, Ravanetti, Pattori, & Giosue, 2001; Shah et al., 2013). The effect of temperature and humidity on the germination and infection of ascospores of *F. graminearum* in wheat has been studied in detail using a single isolate (Manstretta, Morcia, Terzi, & Rossi, 2016). Unfortunately, such studies using macroconidia and involving multiple isolates are not available. Manstretta et al. (2016) showed that the germination of ascospores was lower at 65.5% than 76.0% relative humidity.

Caused by a strong environmental change from 1 year to another, higher disease rates were observed in 2016 than in 2015. However, those isolates which performed well under dry conditions of 2015 did not necessarily perform well in humid conditions of 2016, not even in the same host, as shown by the significance of the isolate–year and isolate–host–year interaction variances. This effect is also corroborated by the AMMI analysis, which groups together most environments according to the year. Under constant selection pressure, seasonal changes could drive the evolution in *F. culmorum* populations as previously reported for other pathosystems (Suffert, Ravigné, & Sache, 2015). We observed that the seasonal change is a much stronger force than the host species and might drive the evolution of *F. culmorum* populations, especially in view of the global climate change. However, the rate at which such adaptation at seasonal changes develops will depend on the selective pressure (Voss et al., 2010). We found higher correlations across different hosts in 2016, when the environmental conditions were more conducive for the disease development than in 2015. Similarly, Voss et al. (2010) reported that the large differences between high and low aggressive isolates detected under non-conducive environments almost vanished under conducive conditions. Weather is, of course, unpredictable and stresses the importance of plasticity for the fungus. In reality, this means that selection acts highly variable due to seasonal changes and the *Fusarium* population might thus not be able to continuously increase in aggressiveness.

5 | SUMMARY

Despite significant genotypic variation among isolates, (a) a large portion of the total variance was explained by plasticity; (b) the high contribution of the host–year–location interaction highlights the potential of seasonal changes as an evolutionary driver in *F. culmorum* populations; (c) no sign of host specialization was found given the lack of host–isolate interaction and the positive and significant genetic correlations among four cereal hosts. Because *F. culmorum* can thus be considered as a generalist, a rapid erosion of host resistance seems unlikely (McDonald & Linde, 2002; Pariaud et al., 2009). The rapid changes of environments as well as between the parasitic and saprophytic habits in *F. culmorum*, however, illustrate a high genetic flexibility and plasticity of the pathogen. This may be its secret for success in invading an array of hosts over a wide geographical range under differing weather conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

TM and VC designed the study. VC developed inoculum production and inoculations on the field, data collection and statistical analyses. HEC assisted with inoculations and data collection in one year. VC wrote figures and tables and prepared the manuscript. HEC and TM edited the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary material

Table 1.1: Means of all tested isolates in two years across two locations for FHB severity.

	Mean FHB severity (%)								
	2015					2016			
Isolate	Bread wheat	Durum wheat	Triticale	Rye		Bread wheat	Durum wheat	Triticale	Rye
7D23	11.33	27.44	8.05	6.06		36.51	23.11	11.85	15.92
7D26	9.46	31.42	6.55	4.41		33.26	21.56	4.44	14.91
8D33	10.57	28.70	12.06	8.44		36.73	22.89	6.31	12.99
FC33[a]	12.44	32.84	17.19	12.60		40.53	22.81	12.28	16.63
FC50[b]						28.47	20.56	6.14	12.88
FC60[b]	5.29	17.63	3.38	-0.21		22.86	15.94	2.37	7.49
FC65[a]	11.63	25.58	14.12	9.37		22.25	11.51	1.48	7.15
Russ1611	10.06	26.71	7.72	7.90		33.42	25.49	8.26	19.32
S060	11.00	23.72	4.76	5.06		35.88	27.88	6.05	14.06
S129						29.93	15.50	3.13	8.67
7D28	12.00	33.16	8.69	9.65		39.06	27.27	8.39	14.42
9D1	9.71	25.11	4.01	8.64		37.45	27.06	8.29	15.75
9D34						37.05	26.26	11.25	20.45
9D38	9.27	25.01	10.13	9.48		35.49	26.64	9.86	14.56
9D40	13.30	27.99	8.42	8.36		37.37	23.90	7.68	18.90
FC104	13.07	31.75	7.47	14.54		38.00	26.74	11.80	16.04
FC40	11.01	30.76	8.93	9.38		40.52	24.28	11.99	16.61
FC74	8.08	21.77	11.37	7.35		39.25	26.45	9.81	15.26
FC75	10.37	30.45	18.28	9.05		38.57	32.56	11.20	17.80
FC90[c]						42.63	28.08	9.90	16.41
Russ111	12.24	28.91	10.33	11.11		38.49	27.19	10.71	16.65
Russ1411						38.21	26.45	14.91	18.97
Russ1911	10.29	30.60	12.12	11.57		37.96	22.66	9.50	22.90
Russ411						40.94	29.98	13.64	22.69
Russ711	11.49	35.17	12.13	12.27		38.23	29.99	10.69	18.69
Russ811						40.72	30.90	14.70	25.31
S022	13.39	36.03	21.50	10.85		36.81	23.01	11.79	17.32
S109						37.47	27.03	10.37	22.21
S222	11.00	29.47	9.18	12.71		38.51	30.94	12.20	21.26
S256	10.39	28.21	13.92	6.96		35.49	25.06	9.67	14.19
S259	11.75	29.89	7.44	7.23		36.78	29.08	7.69	15.93
S264	11.00	30.22	11.78	7.66		38.56	31.76	15.48	29.09
S265	12.15	26.80	9.22	6.76		36.30	30.47	9.21	17.84
S275						40.02	31.02	14.51	24.57
S276	12.19	31.22	9.87	8.26		39.61	33.83	14.26	23.61
S280						30.08	25.03	7.75	11.58
S289	11.04	35.15	10.64	10.45		39.49	29.65	10.24	17.66
S299	11.50	31.01	11.75	8.19		40.44	32.54	13.67	18.82

a Collected from rye (*Secale cereale* L.)
b NIV chemotype.
c Collected from durum wheat (*T. turgidum* var. *durum*)

Table 1.2: Means of all tested isolates in two years across two locations for DON concentration.

	DON concentration (mg/kg)								
	2015					2016			
Isolate	Bread wheat	Durum wheat	Triticale	Rye		Bread wheat	Durum wheat	Triticale	Rye
7D23	3.805163	16.92047	0.12978	6.06		29.92153	54.61	19.05	7.93
7D26	2.365815	15.71994	-0.07796	4.41		22.18197	26.11	8.18	10.33
8D33	5.294865	23.9178	0.548692	8.44		33.09514	36.32	16.40	20.75
FC33[a]	4.113104	18.94123	0.398324	12.60		37.8162	49.81	24.73	20.68
FC50[b]	1.132084			7.13		17.74701	21.99	2.07	1.99
FC60[b]	1.280181	15.19416	-0.36	-0.21		17.61772	14.17	2.79	1.65
FC65[a]	4.774182	18.28381	0.25	9.37		18.92379	14.75	3.58	4.59
Russ1611	3.247936	16.49704	0.36	7.90		27.02101	34.86	17.38	26.58
S060	4.007143	16.88455	0.09	5.06		34.61667	39.62	17.51	23.34
S129	1.648495			1.28		20.59215	24.85	5.18	7.24

a Collected from rye (*Secale cereale* L.)
b NIV chemotype.

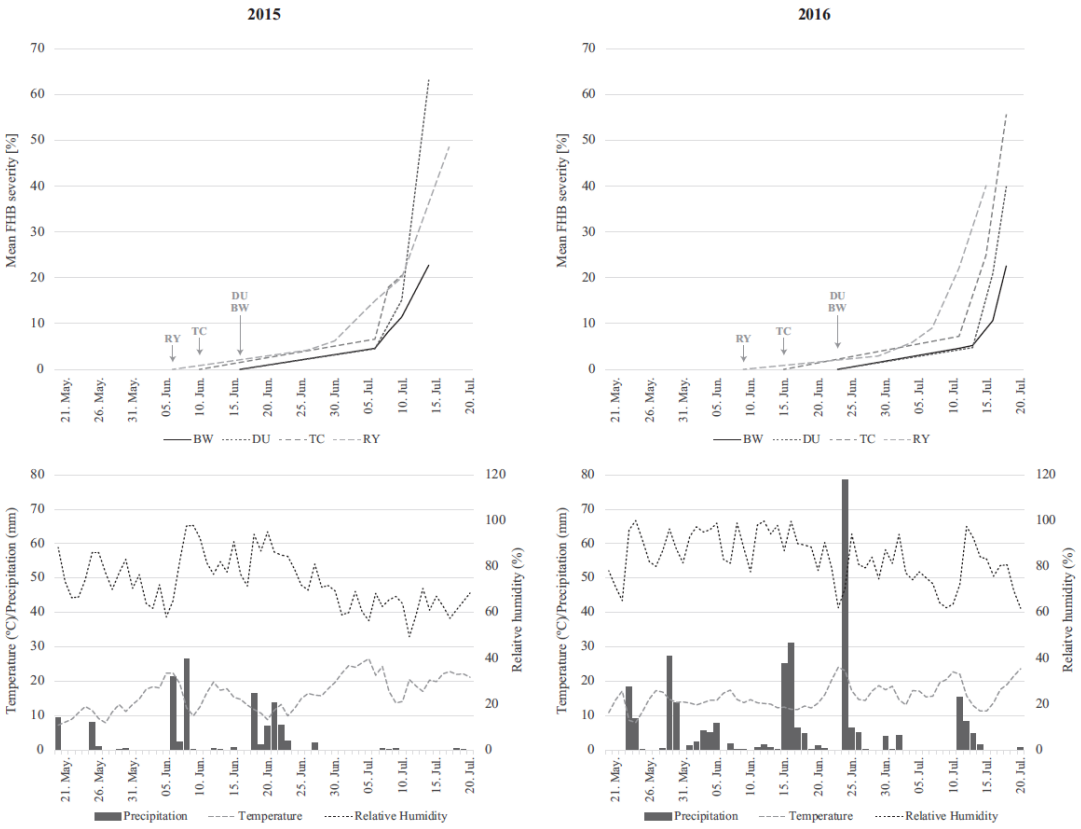

Figure S1: Average curves of disease progress of four host species (bread wheat [BW], durum wheat [DU], triticale [TC] and rye [RY]) in function of meteorological conditions at OLI during 2015 and 2016. Arrows indicate inoculation dates for each crop

3 Candidate gene based association mapping in *Fusarium culmorum* for field quantitative pathogenicity and mycotoxin production in wheat

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Candidate gene based association mapping in *Fusarium culmorum* for field quantitative pathogenicity and mycotoxin production in wheat

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Abstract

Background: Quantitative traits are common in nature, but quantitative pathogenicity has received only little attention in phytopathology. In this study, we used 100 *Fusarium culmorum* isolates collected from natural field environments to assess their variation for two quantitative traits, aggressiveness and deoxynivalenol (DON) production on wheat plants grown in four different field environments (location-year combinations). Seventeen *Fusarium graminearum* pathogenicity candidate genes were assessed for their effect on the aggressiveness and DON production of *F. culmorum* under field conditions.

Results: For both traits, genotypic variance among isolates was high and significant while the isolate-by-environment interaction was also significant, amounting to approximately half of the genotypic variance. Among the studied candidate genes, the mitogen-activated protein kinase (MAPK) *HOG1* was found to be significantly associated with aggressiveness and deoxynivalenol (DON) production, explaining 10.29 and 6.05% of the genotypic variance, respectively.

Conclusions: To the best of our knowledge, this is the first report of a protein kinase regulator explaining differences in field aggressiveness and mycotoxin production among individuals from natural populations of a plant pathogen.

Keywords: Aggressiveness, Quantitative pathogenicity, Fusarium head blight, Association mapping, Candidate genes

Background

Quantitative traits are a key feature in nature [1]. They are controlled by many genes, each contributing with a small effect to the overall phenotypic expression of a trait. Surprisingly, quantitative traits of pathogenicity have received only little attention in fungal biology [2, 3]. The expression of quantitative pathogenicity is not only controlled by the pathogen and the host, but also by the environment and their interaction [3]. Association mapping employing mixed models is a common method to dissect the genetic architecture of quantitative traits. Originally designed for the analysis of human diseases, association mapping is now extensively used in plant genetic research [4] either as genome-wide association study (GWAS) using anonymous molecular markers distributed across the whole genome or as candidate gene association by studying single nucleotide polymorphisms within candidate genes. The genetic basis of both methods is a linkage disequilibrium (LD) between molecular polymorphisms and phenotypic traits [4]. Therefore, a sound phenotypic trait evaluation is urgently required. Moreover, proper assessment of quantitative pathogenicity in natural field habitats, which accounts for host-by-environment and pathogen-by-environment-interaction, is indispensable for predicting disease risk and design effective breeding programs for durable resistance.

While qualitative plant-pathogen molecular interactions, especially gene-for-gene interactions, have been widely studied, quantitative realtionships have received far less attention [3, 5]. It is known that genes associated with qualitative pathogenicity frequently encode secreted effectors, which disassemble the host defense response [6, 7]. From the point of view of the host, some

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hypotheses for explaining the molecular mechanisms that control quantitative disease resistance have been suggested, although the authors remark that this is a poorly understood field [5]. From the point of view of the pathogen, the current understanding of the molecular mechanisms that control quantitative disease pathogenicity is even more scarce. Plant-pathogen qualitative interactions are frequent in biotrophic and obligate pathogens, while hemibiotrophic/necrotrophic pathogens more often show quantitative interactions. It has been proposed that host range, life style and speciation of pathogens are shaped by the type of molecular communication with the host, e.g. the ability to rapidly evolve pathogen effector repertoires [8]. In this context, attempts to answer the following questions are highly relevant: Which are the key molecular players in the quantitative disease pathogenicity? Are they located in the first line of molecular communication with the host, as effectors which are secreted, or are they masters of the molecular regulatory cascades, as transcription factors? Furthermore, are there different allelic variants of those "key players" and what is their role in aggressiveness? *Fusarium culmorum* is a hemibiotroph, with a wide host range encompassing most of the cereals [9], which makes it a perfect model for the study of quantitative interactions.

Fusarium head blight (FHB) is a devastating disease of bread wheat (*Triticum aestivum* L.) and other small-grain cereals worldwide [10]. It leads to significant losses not only in terms of yield but also quality, because of the contamination of kernels by mycotoxins, which pose a significant risk to human and animal health [11]. Isolates causing FHB can be classified by their production of type B trichothecenes, including nivalenol (NIV), deoxynivalenol (DON) and its acetylated forms 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), as well as estrogen analogues like zearalenone (ZEA) and other mycotoxins [12]. DON is the predominant and economically most important trichothecene detected in cereals in Europe [12]. Therefore, the European Union has set a limit for DON in unprocessed bread wheat for human consumption of 1.25 mg/kg [13]. Various *Fusarium* species have been reported as causal agents of FHB, among them the haploid ascomycetes *Fusarium graminearum* and *Fusarium culmorum* are two of the most important. *F. culmorum* has been traditionally related with FHB epidemics in the Mediterranean region [14–16] as well as northern, central and western Europe [17–20]. In contrast to *F. graminearum*, which has been extensively studied and whose full genome sequence has been published [21], a draft of *F. culmorum* genome was just recently released [22].

Aggressiveness, i.e. the quantitative pathogenicity as described by Van der Plank [23], and DON production are important determinants of parasitic fitness in *Fusarium* species [24, 25]. There is no generally recognized specific interaction between wheat genotypes and FHB causal agents [26, 27]. Resistant cultivars stay resistant even when challenged by highly aggressive isolates [28]. Aggressiveness is usually evaluated by directly measuring epidemic rates in this monocyclic disease. Higher aggressiveness is associated with faster symptom development and a larger amount of mycelium within the host tissue [29]. DON plays a key role in *F. graminearum* aggressiveness [30, 31] enabling faster pathogen spread from infected florets into the wheat rachis [32]. A distribution indicative of a quantitative inheritance was demonstrated in *F. graminearum* segregating progenies of three biparental crosses of genetically diverse isolates for both, aggressiveness and DON production [28, 33]. In contrast, the perfect stage (teleomorph) of *F. culmorum* is unknown, and therefore such studies could to date not be performed [34]. In order to understand the inheritance and the genetic control of aggressiveness in *F. culmorum*, the historically accumulated variation in natural populations can be exploited.

Thousands of genes have been characterized affecting host-fungus interaction in agricultural pathosystems, and many of them were found to have a pleiotropic effect [35, 36]. These experiments mainly worked with knock-out or deletion mutants, which are not adequate for explaining quantitative differences among isolates, because frequently the expression of aggressiveness is confounded with basic survival functions of the fungi in these studies. Candidate-gene association mapping appears as a promising and powerful tool to detect functional polymorphisms associated with differences in aggressiveness and DON production in *F. culmorum* populations.

To enhance our understanding of quantitative pathogenicity, we performed a candidate gene association mapping based on pathogenicity-related candidate genes that firstly, have been reported or predicted as related with quantitative variations in pathogenicity, but do not affect the survival of the fungus and secondly, play a role in the pathogenicity signaling cascade. Based on the sequenced *F. graminearum* genome, *F. culmorum* homologues for four transcription factors, eight signal transducers, two membrane receptors and three secreted proteins were sequenced. Using a natural population of 100 isolates of *F. culmorum* of diverse origin, we aimed to (i) determine the phenotypic variance for aggressiveness and DON production in replicated field experiments across two locations and 2 years with a moderately susceptible bread wheat cultivar as host, (ii) study sequences of the 17 candidate genes to identify nucleotide diversity within selected gene regions, (iii) test these quantitative trait nucleotides (QTNs) for their association with aggressiveness and DON production and estimate the proportion of genotypic variance explained by them.

Methods

Fungal materials and field trials

One hundred *F. culmorum* isolates from a previous diversity study [18] were phenotyped under field conditions for aggressiveness and deoxynivalenol (DON) production following a chessboard design described previously [37, 38]. In short, field plots were arranged such that each inoculated entry plot of bread wheat was surrounded by four border plots of a tall triticale cultivar (*x Triticosecale*) to minimize plot-by-plot interference. Fungal material consisted of four field populations (one from Russia and three from Germany), one transect population from Syria and one international collection (Additional file 1). The field populations were randomly collected isolates from symptom-bearing FHB infected heads from individual commercial wheat fields as described earlier [18]. All isolates were used as single-spore derived isolates.

Field experiments with these isolates were conducted during 2014 and 2015, and across two locations: Hohenheim (HOH, longitude 9° 11′ 23″ E, latitude 48° 42′ 54″ N, altitude 403 m) and Oberer Lindenhof (OLI, longitude 9° 18′ 17″, latitude 48° 28′ 25″ N, altitude 702 m) in Southwestern Germany. The experiments followed an incomplete block design (α design) with two replications. Experimental units were three-row plots (1.0 m long, 0.625 m wide), which were machine sown with 220 kernels per m^2, a seeding rate that results in a homogeneous wheat stand. A moderately susceptible winter wheat cultivar was used as host ("Inspiration", KWS LOCHOW GMBH, Bergen, Germany) with a FHB rating of 6 on the 1–9 scale, where 1 = without disease and 9 = fully infected.

Inoculum production was done in shaking cultures according to an existing procedure [39]. For inoculation, a dose of 100 ml suspension per square meter in a concentration of 2×10^5 conidiospores ml^{-1} was sprayed onto wheat heads during full flowering. Inoculum for each isolate was sprayed on its corresponding plot, according to the randomization of the experiment design, using a hand atomizer with constant air pressure of 3 bar from a tractor to ensure full coverage of all heads of the plot with the same dosage. All plots flowered simultaneously, because only one homogeneous wheat cultivar was used. This allowed inoculation and ratings for all plots at the same dates per location. Contamination with natural inoculum was negligible as verified by the border plots which had always a FHB rating < 5%.

Phenotyping aggressiveness and DON production

To assess aggressiveness of isolates, FHB symptoms were visually rated in each plot at least three times starting with the onset of symptom development, about 14 days after inoculation and was continued at 3- to 5-day intervals until the beginning of the yellow ripening stage. Rating was performed as percentage of infected spikelets per plot (0–100%). This reflects both the percentage of infected spikes per plot and the percentage of infected spikelets per spike in a single rating. The arithmetic mean of at least three ratings (i.e. the mean FHB ratings was used as aggressiveness trait).

To measure DON production, wheat plots were harvested by hand at full ripening, carefully threshed in a single-head thresher (Walter-Wintersteiger, Austria) and cleaned with reduced wind speed. The remaining fragments of glumes and rachis were manually picked out to retain highly shrunken kernels in the sample. Cleaned wheat grain was ground in a commercial laboratory mill with a sieve size of 1 mm. Later, the coarse meal was analyzed to quantify the amount of DON by a commercially available immunotest (R-Biopharm AG, Darmstadt, Germany).

Phenotypic data analyses

Phenotypic data of aggressiveness and DON production were obtained from one experiment performed during 2 years and across two locations, yielding four test environments. Aggressiveness data and DON production were arcsin transformed to meet the required normal distribution and subjected to outlier detection with the method BH-MADR (Bonferroni–Holm with re-scaled MAD standardized residuals) suggested by Bernal-Vasquez et al. [40]. For the description of the model, the syntax suggested by Patterson [41] was followed, where fixed effects (E, R, I) appear before the colon and random effects (I × E, B) after the colon. In order to obtain best linear unbiased estimators (BLUE) of each isolate genotype, the phenotypic data were analyzed based on the following linear mixed model:

$$E + R + I : I \times E + B,$$

where E, R, I, and B denote environment, replication, isolate, and block or their interaction, respectively. Variance components were determined by the restricted maximum likelihood (REML) method. Significance of variance components was tested by model comparisons with likelihood ratio tests [42]. Heritability (h^2) was estimated as heritability on an entry-mean basis following the approach suggested by Piepho and Möhring [43]. All statistical analyses were performed using ASReml 3.0 [44] and R [45] combined with the graphical user interface RStudio.

Gene selection and sequencing

Seventeen candidate genes with a confirmed or predicted role in pathogenesis of *Fusarium* spp., aggressiveness and/or trichothecene biosynthesis were used in our study (Table 1). Based on previous studies suggesting that genes associated with aggressiveness are involved in regulation or transport activities [37, 38], we focused mainly on those

Table 1 Identity of candidate genes under study

Rresv4.0 annotation[a]	Gene ID	SNPs	Predicted/confirmed function
Genes encoding transcription factors			
FGRRES_12164	*FGP1*	0	Regulates pathogenicity, toxin synthesis and reproduction in *F. graminearum* [86]
FGRRES_00472	*SCH*	0	Regulates conidium size, stress responses and pathogenesis in *F. graminearum* [89]
FGRRES_06874	*TOP1*	0	Pathogenesis and sporulation in *F. graminearum* and *F. culmorum* [90]
FGRRES_08811	*EFTU*	1	Elongation factor 1α elicit an immune response in the host (Pathogen Associated Molecular Pattern, PAMP) and was identified as differentially secreted in the study of Rampitsch [61]
Genes encoding proteins involved in signal transduction			
FGRRES_06878	*CMK1*	1	Predicted virulence associated protein by Lysenko et al. [107], probable Cmk1/2 protein kinase type I [62]
FGRRES_16491	*STE11*	1	Belongs to MAPK module that regulates fungal development and pathogenicity in *F. graminearum* [93]
FGRRES_08531	*ERF2*	1	Associated with aggressiveness in the study of Talas et al. [37]
FGRRES_09612	*HOG1*	3	Regulates hyphal growth, stress responses and plant infection in *F. graminearum*. [92]
FGRRES_16251	*TRI6*	2	Global transcription regulator in *F. graminearum* associated with affected severity in *F. culmorum* [108, 109]
FGRRES_15765	*LAEA1*	0	Involved in control of secondary metabolism, sexual development and virulence in *F. graminearum* [87]
FGRRES_16620	*FLBA*	0	Involved in conidia production, sexual development, spore germination, mycotoxin production and virulence [88]
FGRRES_09614	*GPA*	0	Required for pathogenicity and normal growth [110]
Genes encoding membrane proteins			
FGRRES_09435	*SHO1*	0	Fungal development and pathogenicity [93]
FGRRES_05633	*MSB2*	3	Transmembrane sensor that regulates invasive growth and plant infection in fungi [93, 111, 112]
Genes encoding secreted proteins			
FGRRES_02342_M	*CUT*	17	Predicted cutinase, required to penetrate the host cuticle [61]
FGRRES_05906	*FGL1*	4	Secreted fungal effector lipase [113–115]
FGRRES_00838	*HSP70*	1	Belong to the family Hsp70 involved in heat-shock response and was found to be secreted differentially under pathogenicity conditions in *F. graminearum* by Rampitsch et al. [61]

For each selected gene, the number of single nucleotide polymorphisms (SNPs) detected with minor allele frequencies (MAF) >5% and function is reported
[a]The given ID (FGSG) is the entry number of the Rres v4.0 annotation *F. graminearum* genome database [47]

groups of genes, although few genes codifying for proteins located in membrane and secreted proteins were also included. The most variable regions of the selected genes were identified using BLAST analysis. Specific primers (Additional file 2) for amplification of those regions in each gene were designed using the software Primer3 - version 0.4.0 [46]. DNA extracted from each of the 100 isolates was used in a polymerase chain reaction (PCR) with the designed primers for each gene, following a standard protocol. Amplicons for each isolate and gene combination were sequenced once using the Sanger method. The sequences were aligned against the reference sequence of *F. graminearum* in the revised complete genome of the strain PH-1, RRes v4.0 [47], available within ENSEMBL fungi (http://fungi.ensembl.org) using the program MEGA6 [48] to identify single nucleotide polymorphisms (SNPs) among the 100 isolates. Polymorphisms that had more than 20% missing values or a minor allele frequency (MAF) of <5% were not considered for further analyses.

Association mapping

Modified Rogers genetic distances (RD; Rogers [49]) based on 10 SSR markers were computed among all isolates under study, using the software *Plabsoft* [50]. Principal coordinate analysis (PCoA) was conducted on the Modified Rogers's distances. Additionally, pairwise kinship coefficients [51] among individuals in the collection were estimated as 1 minus the modified Rogers' distance [52]. A mixed linear model incorporating the BLUES obtained from the phenotypic analysis (Additional file 3), SNP's information (Additional file 4), the three main principal coordinates as fixed effect and a kinship matrix for the random genotypic isolate effect was used to identify marker-trait associations [53]. The p values obtained from the one-degree-of-freedom score test were corrected for possible inflation [53]. The proportion of genotypic variance (ρ_G) explained by each SNP was derived from the sums of squares of the SNP in a linear model using aggressiveness or DON production as dependent variables. As we found high multi-colinearity among SNPs within the candidate genes, only one SNP out of a group of highly linked SNPs (*HOG1*–380) was used in the linear model to assess the proportion of explained genotypic variance [54]. All calculations were done with the open-source statistical software R [45] including packages GenABEL [53] and APE [55]. The squared correlation coefficient (r^2) was used to

estimate linkage disequilibrium (LD) between each pair of marker loci [56] using TASSEL [57].

Results

Phenotypic data

All *F. culmorum* isolates successfully produced symptoms on the inoculated wheat spikes and differences among isolates were observed at all environments. The overall mean of aggressiveness was 15.97%, varying from a minimum of 4.2% for isolate FC60 and a maximum of 19.8% for the isolate S109. The overall mean for DON production was 12.98 mg kg^{-1} ranging from 0.7 mg kg^{-1} to 22.95 mg kg^{-1} for FC50 and 9D22, respectively. Nivalenol (NIV) chemotypes displayed always the lowest DON production values as well as lowest aggressiveness values. Significant correlation between aggressiveness and DON production was observed (r = 0.67, p < 0.001, Fig. 1). The overall entry-mean heritability was 0.87 for aggressiveness and 0.90 for DON production, illustrating a high relevance of genotypic variance. Accordingly, genotypic variances across all environments were significantly (p < 0.001) different from zero for both traits. Isolate-by-environment interaction was also significantly different from zero and about half that of the genotypic isolate variance (Table 2). The frequency distribution of aggressiveness and DON production was continuous and followed a normal distribution for both traits (Additional file 5). Variability within and among field populations, for aggressiveness and DON production, were observed. While the population from Russia produced on average the highest values for aggressiveness and DON production, the population 7D from Entringen, Germany, as well as the international collection had the lowest values for both traits (Fig. 2). Transect population from Syria displayed a large range for both trait values, almost the same range as displayed by the international collection (Fig. 2).

Table 2 Means, ranges and variance components of aggressiveness and deoxynivalenol (DON) production in two locations and 2 years

Parameter	Aggressiveness (%)	DON production (mg kg^{-1})
Means and ranges		
2014-HOH	28.80 (6.80–44.16)	14.75 (0.05–34.27)
2014-OLI	14.26 (3.50–30.50)	21.50 (0.19–55.99)
2015-HOH	8.98 (2.33–23.83)	9.05 (0.09–29.20)
2015-OLI	11.78 (1.00–25.00)	6.46 (0.11–24.96)
Combined	15.97 (1.0–44.16)	12.98 (0.05–55.99)
Variance components and heritabilities[a]		
σ_I^2	1.15×10^{-3} ***	4.80×10^{-3} ***
$\sigma_{I\times E}^2$	8.29×10^{-4}***	1.84×10^{-3} ***
σ_e^2	1.32×10^{-3}	4.17×10^{-3}
h^2	0.87	0.90

Ranges (in brackets), variance components for isolate (σ_I^2), isolate × environment interaction ($\sigma_{I\times E}^2$), error (σ_e^2), and entry-mean heritabilities (h^2)
HOH Hohenheim, *OLI* Oberer Lindenhof
*** Significant at p < 0.001
[a]Variance components and heritabilities calculated with arcsin transformed data

Genotypic and molecular analysis

A principal coordinate analysis based on the modified Rogers' distances among the 100 entries as revealed by SSR markers was performed [18]. The first three main coordinates explained 17, 10 and 10% of the molecular variance and were used to correct for population stratification in the subsequent association analysis (Additional

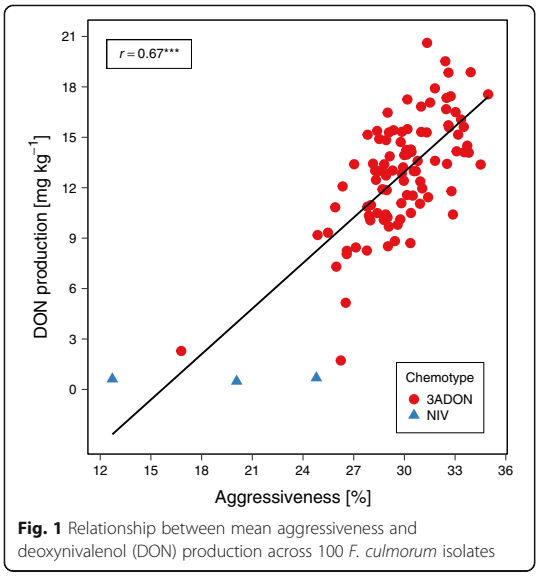

Fig. 1 Relationship between mean aggressiveness and deoxynivalenol (DON) production across 100 *F. culmorum* isolates

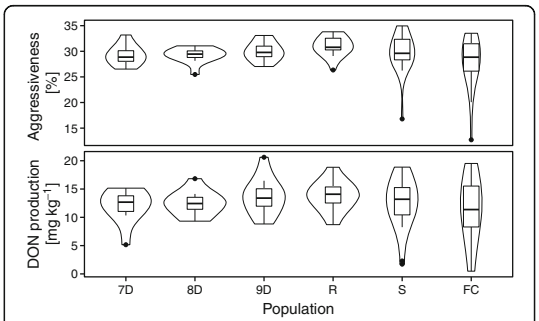

Fig. 2 Violin boxplots of mean aggressiveness and deoxynivalenol (DON) production for populations under study after inoculation on bread wheat across four environments. Field populations consisted in Entringen [7D], Herrenberg [8D], Nufringen [9D] and Novgorod [R], one transect population from Syria [S], and the collection of 22 isolates of *Fusarium culmorum* [FC]. Horizontal line within boxes = median, • = outliers

file 6). In accordance with previous results, we observed two clusters setting Syrian samples apart from the German isolates, but not from the international collection.

Analysis of the sequences of 17 genes for 100 *F. culmorum* isolates included in our study revealed 34 SNPs (Additional file 7) with a minor allele frequency higher than 5%, distributed in ten out of the 17 analyzed genes (Table 1). The gene displaying the highest number of SNPs was *CUT* with 17 SNPs, followed by *FGL1* with four SNPs. Different numbers of SNPs were found in genes with different function and cellular location of the encoded proteins. Genes encoding secreted proteins displayed the majority of SNPs, while no SNPs were found in any of the genes encoding transcription factors included in this study (*FGP1*, *SCH9*, *TOP1*). High LD was detected among SNPs within individual genes (Fig. 3) and within SNPs from two genes located on the same chromosome (*MSB2-FGL1*). Additionally, some SNPs in genes on different chromosomes showed high LD, for instance, the genes *TRI6/MSB2*, *TRI6/CUT*, *MSB2/CUT*. *HOG1* displayed the lowest level of LD with any of the other studied genes.

Association analysis for aggressiveness and DON production

Three SNPs closely linked to each other and located in *HOG1* were found to be significantly associated ($p < 0.05$) with aggressiveness and DON production. Given high collinearity, all three SNPs were analyzed as only one haplotype, which explains 10.29 and 6.05% of the genotypic variance of aggressiveness and DON production, respectively. Any haplotype was restricted to a single population, so no bias by population structure can be expected. Additionally, we used principal coordinate and kinship matrix analysis to correct for population structure. Associated polymorphisms are located within the gene at positions 380, 382 (intron 3) and 724 (intron 5) relative to the start codon (Fig. 4) and correspond to changes in base pairs C/T, C/T and G/A, respectively. Five more polymorphisms in introns and exons were found in *HOG1*, but were excluded from the analysis due to the minor allele frequency limit of 5%. Isolates having the most frequent *HOG1* haplotype expressed on average highest values for aggressiveness and DON production (Fig. 4). An association close to the significant threshold was also detected for one SNP in *TRI6* (TRI147, $p = 0.057$) with DON production and one insertion in *CUT* (CUT536 + 3, $p = 0.07$) with aggressiveness (Additional file 7). Both were excluded from further analysis because the p values were slightly higher than the threshold 0.05.

Discussion

Given the economic and public health importance of Fusarium head blight [10, 35], the causal fungi have been under intensive investigation and became a model to study quantitative plant-microbe interactions. The release of the *F. graminearum* genome sequence in 2007 [21] motivated research on this pathogen using a wide variety of approaches to better understand the basis of pathogen biology. As a result, genes related to

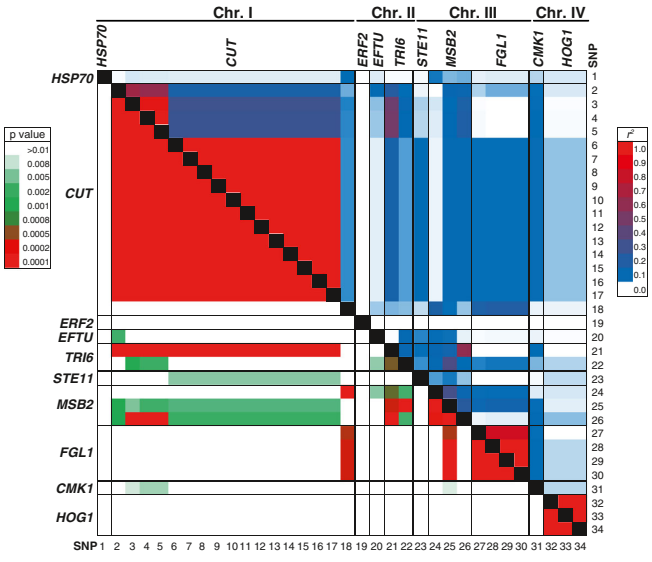

Fig. 3 Pairwise linkage disequilibrium (LD) within and among 10 candidate genes based on 100 *Fusarium culmorum* isolates. LD measured as r^2 between all pairs of selected SNP loci (above diagonal) and significance (below diagonal). The horizontal and vertical lines separate the candidate genes and chromosomes

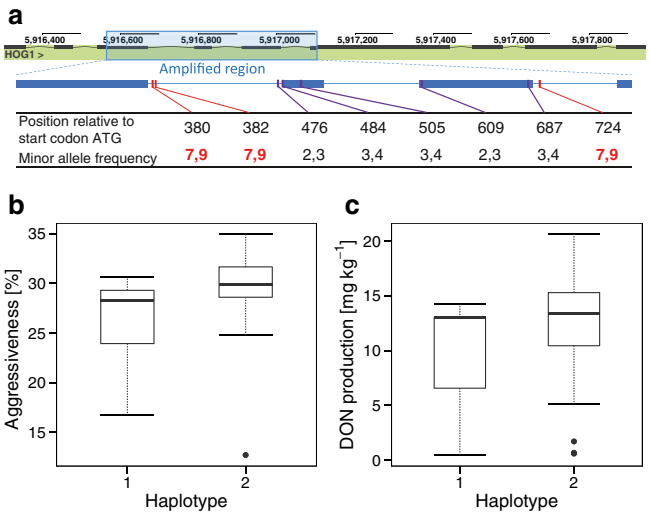

Fig. 4 *HOG1* sequenced region, SNPs found and genetic effects of the two haplotypes. **a** Gene structure taken from ENSEMBL fungi database [116] modified to show *HOG1* sequenced region on chromosome four and all SNPs found. The location of the gene in *F. graminearum* genome is expressed in base pairs. Minor allele frequency expressed in percentage. Boxplots diagrams depicting the genetic effects of two *HOG1* haplotypes with significant associations to (**b**) aggressiveness and (**c**) DON production

pathogenesis and other responses have been identified. Special attention has been given to genes encoding proteins of the secretome [58–61], kinome [62] and phosphatome [63]. However, little is known about which of those identified genes play an active role in explaining quantitative differences in aggressiveness among isolates occurring in natural populations, as the vast majority of studies have used only one *Fusarium* isolate. Furthermore, aggressiveness is a quantitative trait that is highly influenced by the environment. Thus, it is not surprising that candidate genes identified under controlled environments may not be relevant under field conditions [64–66]. In this study, we used 100 *F. culmorum* isolates collected from natural environments to evaluate the effect of previously identified candidate genes on aggressiveness and DON production across four field environments.

Large genetic variation of isolates from individual fields for aggressiveness and DON production

Quantitative variation within natural field populations has previously only been reported for a few plant pathogens like *Rhynchosporium commune* [67], *Zymoseptoria tritici* [68–70] and the close relative of *Fusarium culmorum*, *F. graminearum* [71]. To our knowledge, this is the first study reporting variability in aggressiveness and DON production within *F. culmorum* isolates from natural field populations.

Both traits under study followed a continuous distribution, as expected for quantitative traits (Additional file 5). We observed a high correlation between DON production and aggressiveness (r = 0.67, p < 0.001, Fig. 1), which is consistent with a previous study using 42 *F. culmorum* isolates [72] and *F. graminearum* [71]. Even if the correlation cannot conclude causality of DON in aggressiveness [72], this evidence confirms an important role of DON in aggressiveness. Moreover, high infestation on the field is represented by high levels of DON contamination on harvested grain, despite the fact that highly aggressive isolates do not necessarily produce more DON per mycelium unit than less aggressive ones [72].

Large isolate-by-environment interaction highlights the importance to study FHB using multi-environmental field trials

The genotypic variance is the component of phenotypic variability that could be exploited by selective forces [73, 74]. The observed genotypic variance, which corresponds to the isolate variance component in this study, was high and significant for both traits. In the light of evolution, our results suggest, that if aggressiveness increases the fitness of the population even during the saprophytic phases of the life cycle, the most aggressive genotypes will, with time and under a constant selection pressure, predominate in the population. However, the role of aggressiveness during the saprophytic phases of the life cycle are still to be studied.

Conducting experiments as multi-environment trials, using plots rather than single plants as experimental

units and applying mixed models in the statistical analysis are standard practices in the study of quantitative traits of plants [75]. These approaches have only recently been applied to study quantitative plant-pathogen relationships [37, 38]. Here we report an isolate-by-environment interaction significantly different from zero and amounting to about half that of the isolate genotypic variance. These results are in accordance with previous studies [38, 76] and illustrate the relevance of methods which account for the isolate-by-environment interaction in the study of quantitative disease pathogenicity.

High nucleotide variation found in candidate genes encoding secreted proteins

The *F. culmorum* draft genome sequence is currently under study, but gene annotation has not been completed yet [22]. Therefore, a wide array of information has been inferred from the well annotated reference genome sequence of its close relative *F. graminerum* [17, 77]. We designed primers for known genes in *F. graminearum* and used them successfully for amplification of all homologous genes in *F. culmorum* isolates. This further supports a high synteny and sequence homology among *F. culmorum* and *F. graminearum* genomes [78, 79].

The fact that the majority of SNPs were found in genes encoding secreted or membrane proteins, could be explained by their role in the molecular communication with the host and signal perception from the environment, which might be subject to positive selection pressure as widely reported [80–85]. By contrast, no informative SNPs were found in any of three out of four transcription factors included in this study (*FGP1*, *SCH9*, *TOP1*). This result and previous information concerning the function of those genes, suggested that they might be involved in the pleiotropic control of several basic physiological functions, among them pathogenicity [86–93].

High and significant LD was found among genes from different chromosomes, as for example *TRI6* and *CUT* (Fig. 3). Similar results were found in a study on *F. graminearum* reporting high levels of LD between genes of the TRI cluster and pathogenicity related candidate genes located on different chromosomes e.g. *TRI5/MetAP1*, *TRI10/MetAP1* [37]. Several hypotheses could explain these results: (i) simultaneous selection on the linked genes as a result of a common role in aggressiveness or a coordinated involvement in the same response pathway or (ii) low physical distance with other genes, which are responsible for the LD between different chromosomes.

HOG1, a gene involved in osmotic and oxidative stress is associated with quantitative pathogenicity

HOG1 was significantly associated with aggressiveness and DON production in our study. The mitogen-activated protein kinase (MAPK) *HOG1* is a core component of the high osmolarity glycerol (HOG) pathway and has been well characterized in *S. cerevisiae* [94]. MAPK pathways are three-tiered protein kinase modules that are present in all eukaryotic organisms and function in succession to transmit a variety of cellular signals [95]. Most fungal pathogens contain three MAPKs that are orthologues of the *S. cerevisiae* Fus3/Kss1, Slt2, and Hog1 MAPKs, and function in separate signaling cascades to regulate infection-related morphogenesis, cell wall remodeling, and high osmolarity stress response, respectively [96–99]. The first functional characterization of the kinome in a plant pathogenic fungi was developed using *Fusarium graminearum* as a model and generated deletion mutants for 96 protein kinase genes, out of them 42 kinase mutants were significantly reduced in virulence or non-pathogenic [62]. The *FgHOG1* is a core component of the HOG pathway in *F. graminearum*, which has been involved in the response to various environmental stresses [92]. Based on our results, we hypothesize that changes in *HOG1* regulation confer advantages in the response of *F. culmorum* to multiple stresses, especially to the osmotic and oxidative stresses resulting from the plant defense mechanisms. Our study further supports the observation of Talas et al. [38], suggesting that most of the genes associated with aggressiveness are involved in regulation or transport activities.

Quantitative traits are controlled by complex interactions of genes, and therefore single or few genes explaining a large percentage of genetic variation are not expected [1]. Consistently, the observed percentage of genetic variance explained by *HOG1* was 10.29 and 6.05% for aggressiveness and DON respectively. Similar results have been reported for *F. graminearum*, where genome-wide association reveled quantitative trait nucleotides explaining from 9% up to a maximum of 24% of genotypic variance [38]. The positive results of our study validate the application of candidate gene association mapping strategies to validate factors associated with pathogen aggressiveness under field conditions.

Isolates having the most frequent *HOG1* haplotype expressed on average the highest values for aggressiveness and DON production, which could be an effect of selection favoring these most aggressive genotypes. This fact highlights the importance of integrated plant disease management strategies to prevent undesired selection of the most aggressive genotypes of *F. culmorum*. Consistently, plant breeding must then steadily increase the level of resistance in cultivars, e.g. by pyramiding effective quantitative disease resistance loci.

A suggested role of intronic regions in the expression of aggressiveness

The three SNPs found in *HOG1* are located in introns three and five. The fact that SNPs located within a

noncoding region of *HOG1* are associated with the variation in aggressiveness and DON production could be explained by two hypotheses. Firstly, the detected SNPs are in linkage disequilibrium (LD) with causal polymorphisms in nearby genes or regulatory sequences that are responsible for the trait variance. Secondly, the nucleotide change within the intron is involved in post-transcriptional regulation, like alternative splicing, associated with the response to multiple stresses. Alternative splicing has been reported in fungi, including *F. verticillioides* [100] and *F. graminearum* [100–103]. In *F. graminearum*, grown under just one stress condition, 231 genes undergoing alternative splicing were found, but more genes are expected to follow post-transcriptional regulation if tested under different stresses [103], as shown for *Arabidopsis thaliana* [104]. Zhao et al. [103] demonstrated that for some genes in *F. graminearum* the alternative splicing events took place at different vegetative growth stages and suggested they might be important in adaptation of *F. graminearum* to changing environmental conditions. Furthermore, active functionality of intronic polymorphisms has been found in other organisms e.g. humans [105] and pigs [106].

Several SNPs in our study were discarded because of low calling rate, including some SNPs within *HOG1* located both in exons (positions 505, 609 and 686) and introns (positions 476, 484 and 730). On the other hand, some other genes had polymorphic SNPs with MAF > 5% but showed no association or had *p* values just slightly higher than the significance threshold (TRI147 and CUT563 + 3; Additional file 7). It must be noted, that this does not rule out a contribution of these genes to the variation in aggressiveness or DON production, as the population size of 100 isolates used in this study may have not allowed a statistically significant association.

Conclusions

We phenotyped 100 *F. culmorum* isolates under field conditions and analyzed aggressiveness and DON production. Our results further support a quantitative pathogenicity model for the bread wheat - *F. culmorum* pathosystem. Variation in aggressiveness was largely explained by isolate genotype although the isolate-by-environment interaction was also significant, as expected for quantitative interactions. Of the 17 candidate genes ten showed polymorphisms that were tested for their association with the two traits. *HOG1* was identified as a component of the quantitative pathogenicity of *F. culmorum*. To the best of our knowledge, this is the first report of a protein kinase regulator explaining differences in field aggressiveness and mycotoxin production in a natural population of a plant pathogen.

Additional files

Additional file 1: Number of isolates, sampling scheme, origin, and host characterizing the *F. culmorum* populations under study. (XLS 33 kb)

Additional file 2: Primer sequences, PCR conditions, and the full amplified sequences for the candidate genes used in this study. (XLS 53 kb)

Additional file 3: Best linear unbiased estimates (BLUEs) for mean aggressiveness and DON production calculated across four environments (location × year combinations) for 100 F. culmorum isolates. (XLSX 15 kb)

Additional file 4: Genotyping information of 100 isolates of F. culmorum. (XLSX 21 kb)

Additional file 5: Histograms for mean aggressiveness and DON production. Histograms of best linear unbiased estimates (BLUEs) for mean aggressiveness (top) and DON production (bottom) calculated across four environments (location × year combinations) for 100 *F. culmorum* isolates. (PDF 10 kb)

Additional file 6: Principal coordinate analysis for 100 *F. culmorum* isolates. Population structure and familial relatedness based on 10 SSR markers. Principal coordinate analysis for 100 *F. culmorum* isolates, based on modified Rogers' distance. Number in parentheses refer to the proportion of variance explained by the principal coordinate. (PDF 17 kb)

Additional file 7: Characteristics of identified single nucleotide polymorphisms (SNPs) in the candidate genes and *p* values for association tests against mean aggressiveness and DON production. (LOG 402 bytes) (XLS 34 kb)

Abbreviations

15-ADON: 15-acetyl-deoxynivalenol; 3-ADON: 3-acetyl-deoxynivalenol; BH-MADR: Bonferroni–Holm with re-scaled MAD standardized residuals; BLUE: Best linear unbiased estimators; DON: Deoxynivalenol; FHB: Fusarium head blight; h^2: Heritability; HOH: Hohenheim; LD: Linkage disequilibrium; MAF: Minor allele frequency; MAPK: Mitogen-activated protein kinase; NIV: Nivalenol; OLI: Oberer Lindenhof; PCoA: Principal coordinate analysis; REML: Restricted maximum likelihood; SNPs: Single nucleotide polymorphisms; SSRs: Single sequence repeats; ZEA: Zearalenone

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

TM designed the study, helped with analyses and interpretation of data, revised the manuscript and finally approved it. VC selected candidate genes, extracted DNA, designed primers, amplified gene regions and performed sequence analyses. VC developed inoculum production and inoculations on the field, data collection and biometrical analyses. JM assisted in data collection, biometrical analyses and improved the manuscript. VC wrote figures and tables and prepared the manuscript. TW assisted with association mapping, double checked the results, and improved the quality of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
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Ethics approval and consent to participate
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Supplementary material

Additional file 1: Number of isolates, sampling scheme, origin, and host characterizing the *F. culmorum* populations under study

No. of isolates	Name	Sampling scheme	Origin	Host	Year of sampling	Chemotype	Contributor
10	7D	Field population	Entringen, Germany	Winter Wheat	2008	3ADON	Talas, Miedaner
12	8D	Field population	Herrenberg, Germany	Winter Wheat	2008	3ADON	Talas, Miedaner
11	9D	Field population	Nufringen, Germany	Winter Wheat	2008	3ADON	Talas, Miedaner
19	R	Field population	Novgorod, Russia	Winter Wheat	1994	3ADON	Levitin
26	S	Transect population	Coastal mountains, from Tal-Kalakh till Aleppo, Syria	Spring wheat	2007	3ADON	Talas, Miedaner
22	FC	Individual isolates	International collection	Spikes, Crown, Soil	1952-1995	3ADON	
Individual isolates:							
	FC2		Finnland	Barley spike	1982	3ADON	BBA Berlin
	FC3		Norway	Soil wheat field	1982	3ADON	BBA Berlin
	FC7		Rothampsted, UK	Winter wheat seeds	1983	3ADON	Rothamsted
	FC33		Chewendowa, PL	Winter rye crown	1991	3ADON	Miedaner
	FC37		Flevoland, NL	Winter wheat seeds	1966	3ADON	Snijders
	FC40		Szeged, H	Winter wheat seeds	1991	3ADON	Mesterhazy
	FC46		Wageningen, NL	Wheat seeds	1966	3ADON	Snijders
	FC50		Wageningen, NL	Crown	1987	NIV	Snijders
	FC60		CBS 251.52, NL	*Wheat*, seeds	1952	NIV	
	FC65		Svalöf, S	Winter rye, seeds	1992	3ADON	Miedaner
	FC68		Oldambt (Groningen), NL	Wheat cv. Kanzler, spikes	1988	*NIV*	Snijders

Additional file 1 continued.

No. of isolates	Name	Sampling scheme	Origin	Host	Year of sampling	Chemotype	Contributor
	FC69		Zelder (Limburg), NL	Wheat cv. Beatrix, spikes	1988	3ADON	Snijders
	FC70		Ellighausen, NL	Spikes	1989	3ADON	Snijders
	FC72		Queensland, AUS	Soil debris	1992	3ADON	Wing, Sydney
	FC74		Crookston, Minnesota, USA		1992	3ADON	Dept. Plant Pathology St. Paul
	FC75		Zürich–Reckenholz, CH	Wheat, stems		3ADON	Miedaner
	FC89		Nemchinowka, RUS	Winter rye, Spikes	1993	3ADON	Miedaner
	FC90		Luz, P	Durum wheat, crown	1993	3ADON	Miedaner
	FC95		Messines, P	Oat, crown.	1995	3ADON	Miedaner
	FC98		Barriga, P	Wheat	1995	3ADON	Miedaner
	FC104		Romania	Wheat		3ADON	Ittu
	FC106		CPRO, NL	Grass Species		3ADON	Klap

UK, United Kindom; PL, Poland; NL, Netherlands; H, Hungary; S, Sweden; AUS, Australia; USA, United States America; CH, Switzerland; RUS, Russia; P, Portugal.

Additional file 2 Primer sequences and PCR conditions for the candidate genes used in this study

Rres v4.0 annotation	Gene ID	Gene size (bp)	+/- Strand	Chromosome	Position in Fg genome (bp)	Primer name	Sequences of the primers	Ta (C°)	Expected product (bp)	Sequenced region relative to the ATG
FGRRES_12164	*FGP1*	1029	-	1	8,347,781-8,348,809	Fgp1F	CGGGACAGAACTTCAGGGTC	64	356	635-990
						Fgp1R	TTGGTGGACGTTTTGGTGGA			
FGRRES_06878	*CMK1*	1390	+	4	1,536,725-1,538,122	Cmk1F	ATGCTAAACAGGCTTCACGG	58	619	154-772
						Cmk1R	TCCGTTGATATCACGCACCT			
FGRRES_02342_M	*CUT*	857	-	1	7,565,910-7,566,766	Cut12F	ACACCATACAAACGCTTAGCC	58	513	359-872
						Cut12R	TGGAAAGGGTGGAGTTTGGG			
						Cut2F	TCCACCAGTAACAACGGGAG	56	613	(-89)-524
						Cut2R	CCTGTCTCGTTCCCAGTCAG			
FGRRES_08811	*EFTU*	1765	+	2	2,739,809-2,741,573	EfTu12F	GGAGAGCTTGCCAGACTTGA	65	552	499-1051
						EfTu12R	TACGCCTGGGTTCTTGACAA			
						EfTu2F	AAATTACGACAAAGCCGCAAA	59	558	-211 - 346
						EfTu2R	GCATACCCGGTTCAAGCATC			
FGRRES_05906	*FGL1*	1160	-	3	3,739,711-3,740,870	Fgl1F	ATCATGCTTGCACCAGAATG	58	630	603-1232
						Fgl1R	TCGTTCAAGGTCGTCTCCG			
FGRRES_15765	*LAEA*	1347	-	1	2,108,510-2,109,771	LaeA1F	TCACAACTACCCTGGCCAAG	58	605	-64 - 805
						LaeA1R	GGTGTGACGATGATGAGCG			
FGRRES_16620	*FLBA*	1697	+	3	4,724,323-4,726,775	Flb3F	CGGGATACTCACTGCGAAGA	59	596	1131-1726
						Flb3R	GCATGGCCGGATTTCCTTC			
FGRRES_09614	*GPA*	1354	-	4	5,903,655-5,905,008	Gpa21F	ATGTGTCTGTTGCTTGCGTC	59	617	661-1277
						Gpa21R	GCGAACGAAGACAACAGGTA			
FGRRES_00838	*HSP70*	2045	-	1	2,729,806-2,731,850	Hsp1F	AAAGTACATAGACCACCTTAGCTT	58	629	1584-2213
						Hsp1R	GTCATCACCAACGACAAGGG			

Additional file 2 continued

Rres v4.0 annotation	Gene ID	Gene size (bp)	+/- Strand	Chromosome	Position in Fg genome (bp)	Primer name	Sequences of the primers	Ta (C°)	Expected product (bp)	Sequenced region relative to the ATG
FGRRES_00472	*SCH9*	2747	-	1	1,500,657-1,503,402	Sch1F	CCTTCTTTCGCACTTGGTACA	58	638	949-1586
						Sch1R	AGTCGGGACCCTTATGTTGT			
FGRRES_06874	*TOP1*	3124	-	4	1,521,180-1,524,303	Top1F	CGCCAACCCTTCTTACGTTG	58	627	2520-3146
						Top1R	ACTCGACGTTGACTCAAGCT			
FGRRES_16251	*TRI6*	673	+	2	5,402,308-5,402,964	Tri6F	ATGGAGGCCGAATCTCAC	54	600	35-558
						Tri6R	CCACCCTGCTAAAGACCCT			
FGRRES_08531	*ERF2*	2040	+	2	1,732,866-1,734,960	Erf2F	AGGCATCTTTGTTGTTGTG	52	750	1193-1825
						Erf2R	TTGGTAATACGTGGGTTGT			
FGRRES_09612	*HOG1*	1074	+	4	5,916,303-5,917,943	Hog11F	AAATGTCGCCGTCAAGAAGATC	57	583	265-848
						Hog11R	GAATGTTGCTAGGCTTGAGGTC			
FGRRES_09435	*SHO1*	1008	+	4	6,563,943-6,564,950	Sho11F	TCGTACTCTCTCAGCTCTCCCT	65	537	1-536
						Sho11R	CGAGGAGTTGAAGATGGGTTGG			
FGRRES_16491	*STE11*	2777	+	3	2,434,319-2,437,095	Ste111F	CAGGGTCAAGAAGTTATCCGCG	65	598	813-1411
						Ste111R	TCAGATGCAATCAACTCGCTGG			
FGRRES_05633	*MSB2*	3321	+	3	2,920,242-2,923,562	Msb21F	AGCTTTCATCTCCGCTCCTGTA	65	573	3270-3843
						Msb21R	TATCCCTTGCCATCCCATTCCA			

Additional file 3 Best linear unbiased estimates (BLUEs) for mean aggressiveness and DON production calculated across four environments (location × year combinations) for 100 *F. culmorum* isolates

Name.	Mean FHB with arcsin transformation	DON with arcsin transformation	Mean FHB after detransformation	DON after detransformation
7D7	0.562964243	0.368782662	28.48307063	12.99459796
7D9	0.555484343	0.33596876	27.81031711	10.86914886
9D32	0.586934282	0.368715	30.67066041	12.99004812
FC50	0.464586615	0.069534411	20.07517335	0.482724686
S129	0.422229704	0.151845828	16.7932234	2.288048851
S290	0.560598592	0.369046801	28.26977258	13.01236617
S293	0.580975597	0.387069417	30.12250451	14.24883297
7D22	0.556490056	0.327257868	27.90048677	10.33285854
7D23	0.58129955	0.346982439	30.15223402	11.56419165
7D24	0.589864776	0.359455333	30.94125655	12.37382136
7D26	0.54107802	0.229037769	26.52871327	5.154740038
7D27	0.566523252	0.374702317	28.8048732	13.39527183
7D28	0.613881971	0.399893688	33.18278254	15.15703899
7D34	0.567777644	0.395341689	28.91855098	14.83201441
7D6	0.579557517	0.382679146	29.9924644	13.943291
8D13	0.561110112	0.360871783	28.31585257	12.46725468
8D14	0.529088891	0.310283458	25.47695548	9.322553584
8D17	0.576988207	0.323729947	29.75726532	10.11907667
8D2	0.59112634	0.353099313	31.05794913	11.95828727
8D20	0.579275768	0.360028287	29.96664669	12.41157879
8D28	0.568161089	0.351524499	28.95332681	11.85627919
8D3	0.572010899	0.369188266	29.30316018	13.02188651
8D33	0.559188259	0.375199021	28.14284154	13.42912552
8D4	0.570125204	0.381594097	29.13165188	13.86820425
8D5	0.584207599	0.385443669	30.41947997	14.13536602
8D6	0.575273849	0.318375099	29.60062699	9.798385678
9D1	0.567542384	0.366721751	28.89722074	12.85631888
9D11	0.561877547	0.402978592	28.38502892	15.37895252
9D18	0.568769503	0.417839519	29.00853102	16.466292
9D22	0.594183025	0.471328124	31.34118582	20.61795642
9D31	0.577681372	0.339296468	29.82066642	11.07716497
9D34	0.577245404	0.393726852	29.78078559	14.71740994
9D37	0.573478897	0.301619912	29.43688193	8.824902789
9D38	0.54668665	0.374727874	27.02540586	13.39701284

Additional file 3 continued

Name.	Mean FHB with arcsin transformation	DON with arcsin transformation	Mean FHB after detransformation	DON after detransformation
9D40	0.610570748	0.328471348	32.8713228	10.40684897
9D5	0.612869267	0.385890762	33.08744677	14.16653256
FC104	0.608091099	0.405916978	32.63857361	15.59155186
FC2	0.59612429	0.425852584	31.52142889	17.0649436
FC3	0.605770881	0.457638034	32.42117576	19.52141249
FC33	0.609131063	0.430810447	32.73613681	17.43958854
FC37	0.54174275	0.287710231	26.58742782	8.051821685
FC40	0.578011063	0.402258628	29.85083559	15.32704344
FC46	0.568670885	0.325670884	28.99958092	10.23644687
FC60	0.364655836	0.078597715	12.71833746	0.61648904
FC65	0.617425569	0.406280978	33.51691658	15.61797098
FC68	0.521179617	0.082871648	24.79078818	0.685200261
FC69	0.56202343	0.32986695	28.3981846	10.49223231
FC7	0.534742915	0.273662231	25.97124022	7.304003076
FC70	0.541839628	0.291284828	26.59598835	8.247417184
FC72	0.583796848	0.387151031	30.38169212	14.25453905
FC74	0.533980995	0.335444091	25.90445125	10.83650957
FC75	0.565383268	0.352336424	28.70167907	11.90882415
FC89	0.522021175	0.307925774	24.86350047	9.185907848
FC90	0.593958464	0.401739925	31.3203538	15.2896898
FC95	0.606263928	0.420631647	32.4673413	16.6739205
FC98	0.577990279	0.366528605	29.84893351	12.84339189
R1011	0.607788457	0.449075268	32.61019561	18.84711806
R1111	0.581632658	0.404474036	30.18281234	15.48700251
R1211	0.585712078	0.3686511	30.55800016	12.98575184
R1311	0.583770044	0.387396759	30.37922663	14.27172509
R1411	0.620486978	0.384716207	33.80625106	14.08471648
R1511	0.569533946	0.316514038	29.07793667	9.688008405
R1611	0.583511257	0.299492129	30.35542633	8.704563976
R1811	0.588186966	0.377610031	30.78625019	13.59396402
R1911	0.585043066	0.346473253	30.49638112	11.53164454
R2211	0.572576505	0.403542312	29.35466083	15.41964655
R2311	0.607711696	0.407620107	32.60299888	15.71532183
R311	0.539002313	0.354938854	26.34563696	12.07792078
R411	0.606570031	0.429538828	32.49601131	17.34319088
R711	0.594855769	0.344952729	31.40361718	11.43469048
R811	0.619575567	0.390637914	33.72004945	14.49921518
R911	0.617682393	0.385087454	33.54116548	14.11055508
S021	0.627938327	0.374481152	34.5129952	13.38020955

Additional file 3 continued

Name.	Mean FHB with arcsin transformation	DON with arcsin transformation	Mean FHB after detransformation	DON after detransformation
S022	0.612252421	0.418251972	33.02941099	16.4968972
S023	0.609660967	0.350585802	32.78587805	11.7956553
S043	0.58154838	0.428302166	30.17507497	17.24964586
S045	0.563274905	0.396301244	28.51111722	14.90028759
S060	0.55562885	0.399849971	27.82326779	15.15390367
S109	0.632591766	0.432351885	34.95611845	17.55672264
S220	0.599182318	0.437005477	31.80592538	17.91221596
S222	0.58382179	0.329934583	30.38398632	10.49637795
S229	0.589649496	0.338816031	30.92135562	11.04702598
S256	0.555152853	0.29152701	27.78061618	8.260746213
S259	0.567497646	0.32851654	28.89316499	10.40960896
S264	0.557396754	0.322792693	27.98185574	10.06261513
S265	0.568948359	0.29615145	29.02476543	8.51713834
S267	0.566294345	0.323172054	28.78414309	10.08545152
S274	0.557843172	0.337559651	28.02194482	10.96838032
S275	0.621730761	0.449401442	33.92397532	18.8726372
S276	0.606845294	0.375062092	32.52179845	13.41978928
S280	0.537762928	0.131587719	26.236518	1.7215618
S289	0.581212368	0.383133045	30.14423239	13.97475173
S296	0.569633755	0.401781678	29.08700217	15.29269515
S299	0.615656258	0.412331908	33.34997958	16.05980651

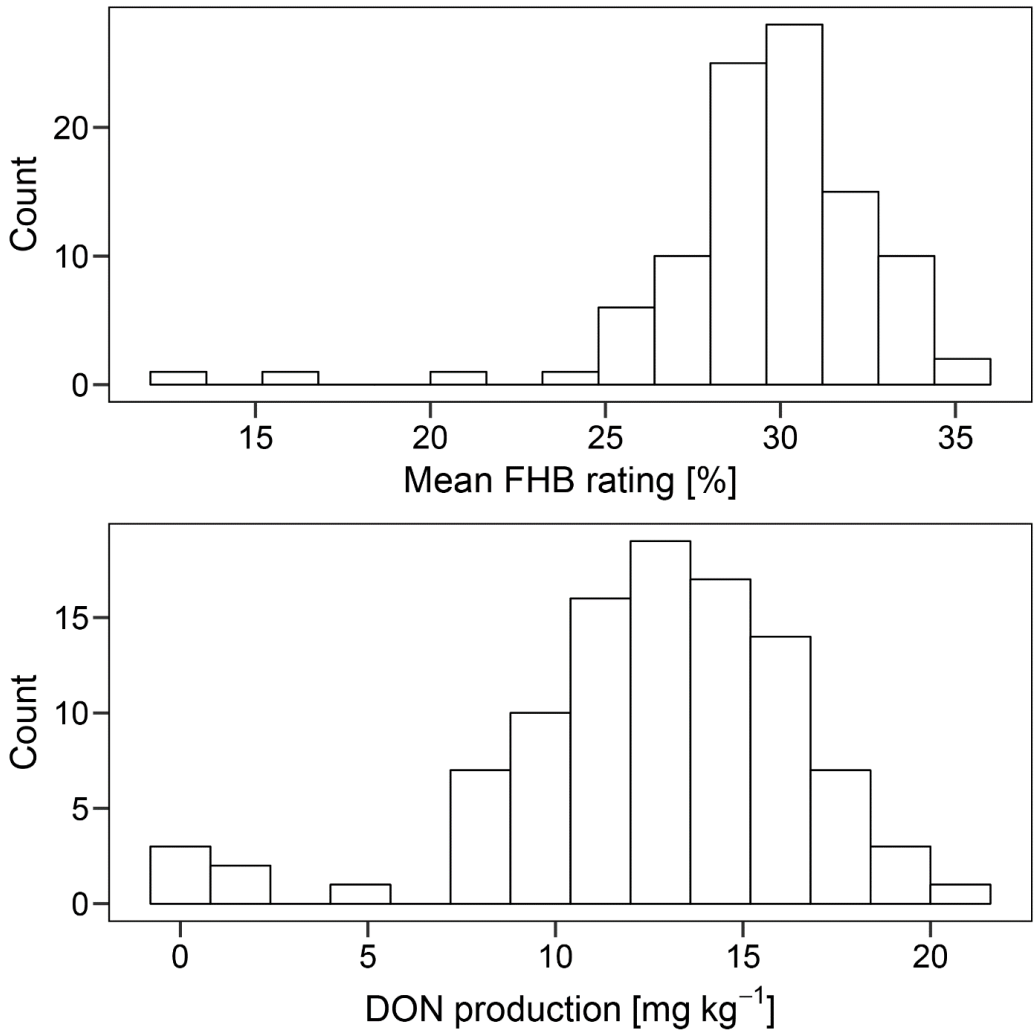
Additional file 4: Genotyping information of 100 isolates of *F. culmorum*.

Name	Chr	Pos	Strand	7D22	7D23	7D24	7D26	7D27	7D28	7D34	7D6	7D7	7D9	8D13	8D14	8D17	8D2	8D20	8D28	8D3	8D33	8D4	8D5	8D6	8D8	9D1	9D11	9D18	9D22	9D31	9D32	9D34	9D37	9D38	9D40	9D5	FC104	FC106	FC2	FC3	FC33	FC37	FC40	FC46	FC50	FC60	FC65	FC68	FC69	FC7	FC70	FC72	FC74
CMK407	4	1537132	+	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT-60	1	7565970	-	A	A	A	A	A	A	A	A	A	0	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CUT56	1	7565854	-	A	A	A	A	T	A	A	T	T	T	T	A	T	T	T	A	T	A	T	T	T	T	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CUT57	1	7565853	-	A	A	A	A	T	A	A	T	A	T	T	T	T	A	A	A	A	A	A	A	A	A	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CUT58	1	7565852	-	C	C	C	C	C	G	C	C	C	G	G	G	G	G	G	C	G	C	G	G	G	G	C	C	C	C	C	C	C	C	C	C	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
CUT59	1	7565851	-	C	C	C	C	C	G	C	C	C	G	G	G	G	G	G	C	G	C	G	G	G	G	C	C	C	C	C	C	C	C	C	C	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
CUT60	1	7565850	-	A	A	A	A	T	A	A	T	A	T	T	T	T	A	A	A	T	A	T	T	T	T	T	A	A	A	A	A	A	A	A	A	A	T	T	A	A	A	A	A	A	T	T	A	A	A	A	A	A	A	A
CUT61	1	7565849	-	T	T	T	T	A	T	T	T	A	T	T	T	T	A	A	A	T	A	T	T	T	T	T	A	A	A	A	A	A	A	A	A	A	T	T	A	A	A	A	A	A	T	T	A	A	A	A	A	A	T	T
CUT62	1	7565848	-	C	C	C	C	C	G	C	C	C	G	G	G	G	G	G	C	G	C	G	G	G	G	C	C	C	C	C	C	C	C	C	C	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
CUT63	1	7565847	-	G	G	G	G	G	C	G	G	G	C	C	C	C	G	G	G	C	G	C	C	C	C	C	G	G	G	G	G	G	G	G	G	G	C	C	G	G	G	G	G	G	C	C	G	G	G	G	G	G	C	G
CUT64	1	7565846	-	C	C	C	C	C	G	C	C	C	G	G	G	G	G	G	C	G	C	G	G	G	G	C	C	C	C	C	C	C	C	C	C	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
CUT65	1	7565845	-	C	C	C	C	C	G	C	C	C	G	G	G	G	G	G	C	G	C	G	G	G	G	C	C	C	C	C	C	C	C	C	C	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
CUT66	1	7565844	-	G	G	G	G	C	G	G	G	C	C	C	C	C	G	G	G	C	G	C	C	C	C	C	G	G	G	G	G	G	G	G	G	G	C	C	G	G	G	G	G	G	C	C	G	G	G	G	G	G	G	G
CUT67	1	7565843	-	G	G	G	G	C	G	G	G	C	C	C	C	C	G	G	G	C	G	C	C	C	C	C	G	G	G	G	G	G	G	G	G	G	C	C	G	G	G	G	G	G	C	C	G	G	G	G	G	G	G	G
CUT536+1	1	7565374	-	G	G	G	G	C	G	G	G	C	C	G	G	G	G	G	G	C	G	C	C	C	C	C	G	G	G	G	G	G	G	G	G	G	C	C	G	G	G	G	G	G	C	C	G	G	G	G	G	G	G	G
CUT536+2	1	7565374	-	A	A	A	A	T	A	A	T	T	T	A	A	A	A	A	A	A	A	A	A	A	A	T	T	A	A	A	A	A	A	A	A	A	T	T	A	A	A	A	A	A	T	T	A	A	A	A	A	A	A	A
CUT536+3	1	7565374	-	A	A	A	A	T	A	A	T	T	T	A	A	A	A	A	A	A	A	A	A	A	A	T	T	A	A	A	A	A	A	A	A	A	T	T	A	A	A	A	A	A	T	T	A	A	A	A	A	A	A	A
CUT785	1	7565125	-	T	T	T	T	C	T	T	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	T	T	T	T	T	T	C	C	T	T	T	T	T	T	T	T
EFTU148+1	2	2739809	+	G	G	G	G	C	C	G	G	C	C	C	C	C	G	G	G	C	G	C	C	C	C	C	G	G	G	G	G	G	G	G	G	G	C	C	G	G	G	G	G	G	C	C	G	G	G	G	G	G	G	G
FGL671	3	3739040	-	G	G	G	G	A	G	G	G	G	G	G	G	G	G	G	G	0	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FGL743	3	3738968	-	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C	0	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
FGL791	3	3738920	-	G	G	G	G	A	G	G	G	G	G	G	G	G	G	G	G	0	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FGL908	3	3738803	-	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	0	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
HOG380	4	5916683	+	T	T	T	T	T	T	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
HOG382	4	5916685	+	T	T	T	T	T	T	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	0	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
HOG724	4	5917027	+	A	A	A	A	A	A	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	0	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
HSP1997	1	2729806	-	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
MSB3585	3	2923827	+	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
MSB3699	3	2923941	+	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
MSB3743	3	2923985	+	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
STE1262	3	2434319	+	G	G	G	G	G	G	A	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
TRI147	2	5402455	+	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
TRI468	2	5402776	+	A	A	A	A	A	A	A	A	G	G	A	A	G	G	G	A	A	G	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

Additional file 4 continued

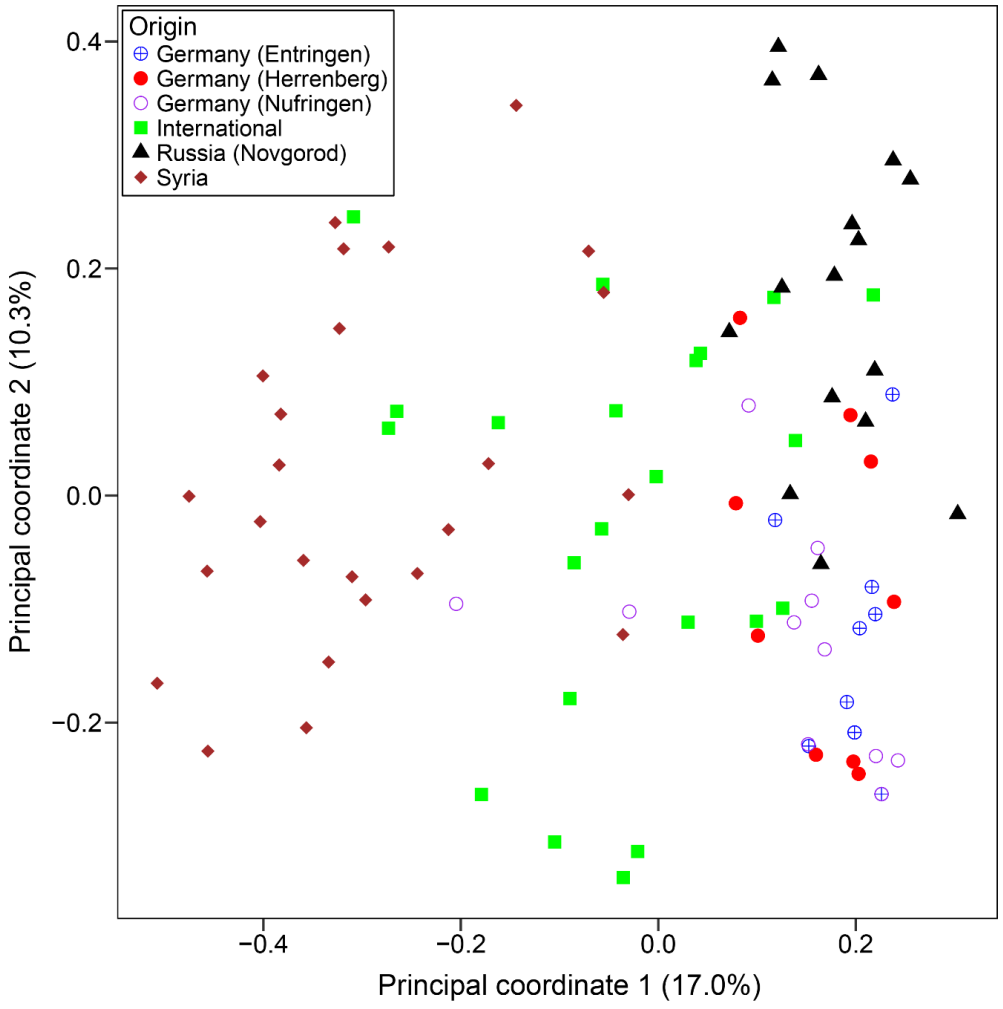
Name	Chr	Pos	Strand	FC75	FC89	FC90	FC95	FC98	R1011	R111	R1111	R1211	R1311	R1411	R1511	R1611	R1811	R1911	R2011	R2111	R2211	R2311	R311	R411	R711	R811	R911	S021	S022	S023	S043	S045	S060	S109	S129	S220	S222	S229	S256	S259	S264	S265	S267	S274	S275	S276	S280	S283	S289	S290	S293	S296	S299
CMK407	4	1537132	+	A	A	G	A	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	A	G	G	A	A	G	G	A	A	O	A	A	A	A	A	G	G	G	O	G	G	A	G	G	G	G	G
CUT-60	1	7565970	-	A	0	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CUT56	1	7565854	-	T	0	T	T	T	A	T	A	A	T	T	A	A	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
CUT57	1	7565853	-	T	0	T	T	T	A	T	A	A	T	T	A	A	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
CUT58	1	7565852	-	G	0	G	G	G	G	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT59	1	7565851	-	G	0	G	G	G	G	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT60	1	7565850	-	T	0	T	T	T	A	T	A	A	T	T	A	A	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
CUT61	1	7565849	-	A	0	A	A	A	A	T	A	A	T	T	A	A	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
CUT62	1	7565848	-	G	0	G	G	G	G	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT63	1	7565847	-	C	0	C	C	C	C	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT64	1	7565846	-	G	0	G	G	G	G	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT65	1	7565845	-	G	0	G	G	G	G	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT66	1	7565844	-	C	0	C	C	C	C	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT67	1	7565843	-	C	0	C	C	C	C	C	G	C	C	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT536+1	1	7565374	-	C	G	C	G	C	C	C	G	G	G	C	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
CUT536+2	1	7565374	-	T	A	T	A	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CUT536+3	1	7565374	-	T	A	T	A	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CUT785	1	7565125	-	C	T	C	T	C	T	A	T	T	T	C	T	T	T	C	T	T	T	T	T	C	C	T	T	C	C	T	T	C	C	T	T	C	C	T	T	T	T	C	C	T	T	T	T	C	C	T	C	T
EFTU148+1	2	2739809	+	C	0	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
FGL671	3	3739040	-	G	G	G	A	G	A	A	G	G	A	G	G	A	A	G	G	A	A	G	A	G	G	A	A	G	G	A	A	G	G	A	A	O	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FGL743	3	3738968	-	C	C	C	T	C	T	A	T	T	C	C	T	T	C	T	T	C	T	T	C	C	T	T	C	C	T	T	C	C	T	T	O	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
FGL791	3	3738920	-	G	G	G	G	A	G	G	A	A	A	A	G	G	A	G	G	A	G	A	A	G	G	A	G	G	A	A	O	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FGL908	3	3738803	-	A	A	A	C	A	C	C	A	A	C	A	C	C	A	C	C	C	C	A	C	A	A	C	C	A	A	C	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
HOG380	4	5916683	+	T	T	T	T	T	T	O	T	T	T	T	T	T	T	T	T	O	O	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
HOG382	4	5916685	+	T	T	T	T	T	T	O	T	T	T	T	T	T	T	T	T	O	O	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
HOG724	4	5917027	+	A	A	A	A	A	A	O	A	A	A	A	A	A	A	A	A	O	O	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
HSP1997	1	2729806	-	C	C	T	C	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
MSB3585	3	2923827	+	T	C	T	T	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
MSB3699	3	2923941	+	T	C	C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
MSB3743	3	2923985	+	T	T	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	G
STE1262	3	2434319	+	A	G	G	G	G	G	G	G	G	A	G	G	A	A	G	G	G	G	G	G	A	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
TRI147	2	5402455	+	G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
TRI468	2	5402776	+	A	G	G	G	G	G	G	G	G	G	A	G	G	A	G	G	G	G	G	G	A	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

Additional file 5 Histograms for mean aggressiveness and DON production



Histograms of best linear unbiased estimates (BLUEs) for mean aggressiveness (top) and DON production (bottom) calculated across four environments (location × year combinations) for 100 *F. culmorum* isolates

Additional file 6 Principal coordinate analysis for 100 *F. culmorum* isolates.



Population structure and familial relatedness based on 10 SSR markers. Principal coordinate analysis for 100 *F. culmorum* isolates, based on modified Rogers' distance. Number in parentheses refer to the proportion of variance explained by the principal coordinate

Additional file 7 Characteristics of identified single nucleotide polymorphisms (SNPs) in the candidate genes and *p* values for association tests against mean aggressiveness and DON production.

SNP Name	Chromosome	Position[a]	Strand	A1*	A2*	Number of measured isolates	Calling Rate	*p* value for DON production association	*p* value for aggressiveness association
HOG380	4	34468138	+	T	C	94	0.94	0.01321006	0.003991487
HOG382	4	34468140	+	T	C	94	0.94	0.01321006	0.003991487
HOG724	4	34468482	+	A	G	94	0.94	0.01321006	0.003991487
TRI147	2	17163405	+	G	A	93	0.93	0.05794438	0.280532593
CUT536+1+	1	7565374	-	G	C	97	0.97	0.22950222	0.073213761
STE1262	3	23192827	+	G	A	99	0.99	0.25614153	0.873537442
CUT536+2+	1	7565374	-	A	T	97	0.97	0.27484246	0.094920517
CUT536+3+	1	7565374	-	A	T	97	0.97	0.27484246	0.094920517
MSB3585	3	23682335	+	T	C	100	1.00	0.36511497	0.509699849
MSB3699	3	23682449	+	C	T	100	1.00	0.38002164	0.865411244
CUT-60	1	7565970	-	A	G	96	0.96	0.43501359	0.470405700
HSP1997	1	2729806	-	C	T	96	0.96	0.55146394	0.474098206
CUT785	1	7565125	-	T	C	96	0.96	0.57761370	0.199607867
FGL908	3	24497311	-	A	C	98	0.98	0.62580793	0.867228116
CUT67	1	7565843	-	C	G	97	0.97	0.66899609	0.871665015
CUT66	1	7565844	-	C	G	97	0.97	0.66899609	0.871665015
CUT65	1	7565845	-	G	C	97	0.97	0.66899609	0.871665015
CUT64	1	7565846	-	G	C	97	0.97	0.66899609	0.871665015
CUT63	1	7565847	-	C	G	97	0.97	0.66899609	0.871665015

Additional file 7 continued

SNP Name	Chromosome	Position[a]	Strand	A1*	A2*	Number of measured isolates	Calling Rate	*p* value for DON production association	*p* value for aggressiveness association
CUT62	1	7565848	-	G	C	97	0.97	0.66899609	0.871665015
CUT61	1	7565849	-	A	T	97	0.97	0.66899609	0.871665015
CUT60	1	7565850	-	T	A	97	0.97	0.66899609	0.871665015
CUT59	1	7565851	-	G	C	97	0.97	0.66899609	0.871665015
CUT58	1	7565852	-	G	C	97	0.97	0.66899609	0.871665015
CUT57	1	7565853	-	T	A	97	0.97	0.66899609	0.871665015
CUT56	1	7565854	-	T	A	97	0.97	0.66899609	0.871665015
CMK407	4	30088587	+	G	A	97	0.97	0.68693541	0.422480293
FGL791	3	24497428	-	G	A	98	0.98	0.71224831	0.934119245
FGL743	3	24497476	-	C	T	98	0.98	0.71224831	0.934119245
FGL671	3	24497548	-	G	A	98	0.98	0.71224831	0.934119245
TRI468	2	17163726	+	G	A	92	0.92	0.72213022	0.182486234
MSB3743	3	23682493	+	T	G	100	1.00	0.87876931	0.666161672
EFTU148+1+	2	14500759	+	C	G	97	0.97	0.98655669	0.777715708
ERF1338	2	14500759	+	T	A	58	0.58	0.99636143	0.978226238

[a] Position in the genome expressed in terms of base pairs (bp).

*Allele 1 and allele 2 for each of the identified polymorphisms

+ Insertion

4 Candidate genes for aggressiveness in a natural *Fusarium culmorum* population greatly differ between wheat and rye head blight

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Article

Candidate Genes for Aggressiveness in a Natural *Fusarium culmorum* Population Greatly Differ between Wheat and Rye Head Blight

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Abstract: *Fusarium culmorum* is one of the species causing Fusarium head blight (FHB) in cereals in Europe. We aimed to investigate the association between the nucleotide diversity of ten *F. culmorum* candidate genes and field ratings of aggressiveness in winter rye. A total of 100 *F. culmorum* isolates collected from natural infections were phenotyped for FHB at two locations and two years. Variance components for aggressiveness showed significant isolate and isolate-by-environment variance, as expected for quantitative host-pathogen interactions. Further analysis of the isolate-by-environment interaction revealed the dominant role of the isolate-by-year over isolate-by-location interaction. One single-nucleotide polymorphism (SNP) in the cutinase (*CUT*) gene was found to be significantly ($p < 0.001$) associated with aggressiveness and explained 16.05% of the genotypic variance of this trait in rye. The SNP was located 60 base pairs before the start codon, which suggests a role in transcriptional regulation. Compared to a previous study in winter wheat with the same nucleotide sequences, a larger variation of pathogen aggressiveness on rye was found and a different candidate gene was associated with pathogen aggressiveness. This is the first report on the association of field aggressiveness and a host-specific candidate gene codifying for a protein that belongs to the secretome in *F. culmorum*.

Keywords: association mapping; aggressiveness; candidate gene; cutinase; Fusarium head blight (FHB); quantitative trait loci (QTL); single-nucleotide polymorphism (SNP)

1. Introduction

Fungi are the most important pathogens that attack cereal crops in Central Europe. Among them, the genus *Fusarium* is a worldwide threat to many agricultural crops and commodities reducing not only the yield, but contaminating the grain with mycotoxins [1]. They induce seedling blight, foot and root rot, and head blight in the field. Fusarium head blight (FHB) is one of the most common and harmful diseases that affect all small-grain cereals and some forage grasses worldwide [1]. From infected ears, about 13 different species can be isolated, among them *Fusarium graminearum*, *F. culmorum*, and *F. avenaceum* are the most common in Europe [1]. Outbreaks of FHB result in yield losses and quality reduction, while mycotoxins produced by the pathogen lead to contamination of grain. There is substantial evidence of risks to human and animal health posed by FHB mycotoxins [2]. An estimated $7.67 billion loss was caused by FHB in wheat and barley production alone in the period between 1993 and 2001 in the USA [3].

F. culmorum (W.G. Smith) Sacc., firstly described in 1892, is a soil-borne pathogen and the principal origins of inoculum are crop residues containing fungal mycelium and long-living chlamydospores in the soil [4]. Main risk factors for FHB infection are maize as previous crop, reduced soil management,

especially no tillage, a susceptible wheat cultivar, and favorable weather conditions. Cereal plants are most vulnerable to FHB infection during flowering till the soft dough stage. Wet and warm weather in the periods of crop anthesis and maturation can increase the risk of development of FHB [5]. When the macroconidia reach the ear, they germinate and the fungus can grow into cereal florets either passively by natural openings, for example, the stomata [6], or actively by direct penetration of the cuticle and cell walls. This is facilitated by a great range of hydrolyzing enzymes such as cutinases, cellulases, pectin lyases and xylanases, which are released by the fungus during the penetration process [7].

F. culmorum and *F. graminearum* belong to the category of hemibiotrophic pathogens. Hemibiotrophs present a short biotrophic phase throughout the primary phase of infection and then switch to necrotrophy with secretion of mycotoxins and enzymes for degradation of host cell walls [8,9]. Trichothecenes are mostly produced by proteins and regulators encoded by the *TRI* genes located at the trichothecene gene cluster [2,10]. Among the trichothecenes, deoxynivalenol (DON) is the most common mycotoxin, but also nivalenol is produced by some isolates of both species. Additionally, all isolates produce zearalenone, a compound exhibiting oestrogenic properties in mammals.

From the host perspective, the genetic basis of FHB resistance in cereals has been explored in a large number of studies that observed a quantitative inheritance [5,7–9]. This type of resistance is controlled by many genes, each with a small phenotypic effect and affected by the environment (locations, years). Quantitative resistance is not race specific, i.e., the same plant genotypes display an equivalent ranking against all pathogen isolates [11] and the resistance should be less prone to pathogen adaptation and, hence, more durable.

A key factor that determines parasitic fitness of an isolate is aggressiveness that describes the quantitative pathogenicity and should, hence, be quantitatively measured [11]. Aggressiveness is frequently evaluated by directly assessing epidemic rates [12], and reflects several basic quantitative traits of the fungal life cycle, such as infection efficiency, sporulation, sizes of the lesion, and toxin production [13]. Mycotoxin production and their effects in aggressiveness have been studied in detail in Fusarium species. Cumagun & Miedaner [12] reported a positive correlation ($r = 0.7$, $p < 0.01$) between aggressiveness and DON production using 50 isolates of *F. graminearum*. A similar outcome was reported for 100 *F. culmorum* isolates in wheat ($r = 0.67$, $p < 0.001$ [14]).

In contrast to a large number of studies on host resistance, studies on the genetic basis of fungal aggressiveness are very limited. Therefore, it is necessary to close this knowledge gap about genetic and environmental determinants of aggressiveness to make assumptions on the possible adaptation of the pathogens to host resistance. In the case of *F. culmorum* and *F. graminearum*, there is a high probability that many genes are associated with aggressiveness but the precise number and interaction between them are still to be established.

In the related species *F. graminearum* with frequent sexual recombination the development of mapping populations is possible. In a study [15] using this approach, two quantitative trait loci (QTL) for aggressiveness linked to the *TRI5* locus were identified. Unfortunately, this approach is not an option for *F. culmorum* because no teleomorph has been identified yet [16].

With the advantage of having the complete genome sequence of *F. graminearum* with four chromosomes comprising 36.6 Mbp [17,18], it is now possible to use other approaches such as candidate gene association mapping, a powerful tool to identify functional polymorphisms related with aggressiveness [19]. This requires the use of a panel of unrelated isolates that show a wide range of variation. Candidate genes are one option for association mapping. This approach is relatively economical and quick to perform when the full genomic sequence of the pathogen is available. It begins with the selection of a putative candidate gene according to its importance in the mechanisms of the trait being examined. Hence, previous knowledge about gene function is required [19]. The second step is to detect polymorphisms within the gene, which can affect the gene regulation or its product [20]. Finally, the polymorphisms in nucleotide diversity are verified for their association with phenotypic changes. With candidate gene association mapping, SNPs in three genes (TRI1, *MetAP1*, *Erf2*) were significantly associated with aggressiveness in *F. graminearum* in wheat [21]. An alternative is the

classical association mapping where the whole genome is saturated by molecular markers and distinct peaks show associations to phenotypic values. This has also been adopted in *F. graminearum* [22] and resulted in the identification of seven and five genes for aggressiveness and DON production, respectively. However, the function of the associated genes in relation to pathogenicity is not known.

F. culmorum has a broad host spectrum including all small-grain cereals [23]. In Europe, wheat and rye are the most widely distributed bread-making cereals. Bread wheat (*Triticum aestivum* L.) was grown on about 62.5 million hectares in 2016, rye (*Secale cereale* L.) across 3.6 million hectares [24]. Both cereals are mainly used as winter crops and have a very similar growth pattern, although winter rye is flowering about three weeks earlier than bread wheat. While bread wheat is a self-pollinating crop with homozygous line cultivars, rye is an outcrossing crop with a heterogeneous type of cultivars.

The goals of this research were to (i) untangle the relative importance of the components explaining the variance of aggressiveness measured in field experiments across two replications, two locations and two years, with an experimental, genetically homogeneous winter rye genotype as a host; (ii) compare the phenotypic information from rye and wheat; (iii) evaluate the association of SNPs in the candidate genes with *F. culmorum* aggressiveness quantified with two different Data sets (Table S1) using (a) only rye as host across two locations and two years (2015, 2016, Data set 1) and (b) the phenotypic information from rye and wheat across two locations in 2015 (Data set 2).

2. Materials and Methods

One hundred isolates of *F. culmorum* from a collection described in a prior study were used [25] (Table 1). They belong to four different field populations, one from Russia and three from Germany, one Syrian transect population and an international collection of the State Plant Breeding Institute, University of Hohenheim. Isolates were acquired from ears displaying observable FHB symptoms in the field.

Table 1. Population name, number of isolates, origin, host and year of sampling of the *Fusarium culmorum* populations used for inoculation.

Name	No. of Isolates	Origin	Host	Year of Sampling
7D	10	Entringen, Germany	Winter Wheat	2008
8D	12	Herrenberg, Germany	Winter Wheat	2008
9D	11	Nufringen, Germany	Winter Wheat	2008
R	19	Novgorod, Russia	Winter Wheat	1994
S	26	Coastal mountains, Syria	Spring wheat	2007
INT	22	International	Different cereals	1952–1995

Mycelial disks of Fusarium isolates were grown on synthetic nutrient-poor agar (SNA) medium and transferred in 2.5 mL Eppenmeyer tubes in distilled water at 6 °C for storage. One agar plug out of the stored isolates was placed in Erlenmeyer flasks with 400 mL of the SNA medium and incubated under constant shaking at 110 rpm and UV light for stimulation of sporulation during 1 week at 22–25 °C [26]. With a hemacytometer, the spores were counted for each isolate, from which the concentration of spores was calculated and aliquots frozen at −80 °C were prepared. Before application, the samples were thawed in water at 20 or 40 °C [27], and brought to a final concentration of 2×10^5 spores.

The spores were inoculated on the rye heads at full flowering with a manual atomizer and 100 mL suspension per square meter. A tractor was used to generate a stable air pressure of 3 bars to guarantee the even application of the spores on rye heads across the plot.

A susceptible, cytoplasmic-male sterile single cross of winter rye was used as host across the whole experiment (*Secale cereale* L., "L2177-P×L2184-N", HYBRO Saatzucht GMBH & Co., KG, Schenkenberg, Germany). The trial was made in two locations: Oberer Lindenhof (OLI, altitude 700 m, longitude 9°18′12″ E, latitude 48°28′26″ N) and Hohenheim (HOH, altitude 400 m, longitude 9°12′58″ E,

latitude 48°42′50″ N) in two years (2015 and 2016). For comparison, previously reported phenotypic data from wheat were used [14], corresponding to the measurements of aggressiveness of the same 100 *F. culmorum* isolates tested on a moderately susceptible winter wheat cultivar ("Inspiration", KWS LOCHOW GMBH, Bergen, Germany) with the same experimental conditions at the same locations and experimental design in 2014 and 2015. Comparison between crops was restricted to 2015, because only in this year the experiments were placed on the same field as split-plot design with crops as main plots and isolates as subplots. Means of annual temperature at OLI and HOH in 2015 were 8.88 °C and 10.86 °C and in 2016 were 8.5 °C and 10.12 °C, respectively. The mean precipitation at OLI and HOH were 709.8 mm and 492.1 mm in 2015 and 779.3 mm and 595.4 mm in 2016.

Seeds were grown in two-row plots with 1 m length and 0.42 m width. To decrease the drifting or secondary spore dispersal and avoid possible interference among plots, a chessboard-like design was used to arrange the plots that were bordered by long-strawed rye. The latter was a mix of two population cultivars: "Dukato" (Hybro Saatzucht GmbH & Co., KG) and "Conduct" (KWS LOCHOW GMBH) to secure pollination. Plots were sown with 220 kernels m^{-2}.

The experiment was arranged according to an alpha-lattice design with two replications per environment and an incomplete block size of ten plots. The randomization of genotypes was done by PLABPLAN (Version 1E, University of Hohenheim (350a), 70599 Stuttgart, Germany) within the program package PLABSTAT [28].

The ratings started with the initiation of symptoms about two weeks after inoculation and continued in 2 to 5 days intervals until the start of yellow ripening. Typical symptoms are the prematurely bleaching of infected cereal spikelets while the non-infected part of the head is still green [1,16]. In inoculation experiments, several to many adjoining spikelets are often affected by aggressive isolates under favorable weather conditions. In extreme, the whole head could turn white. FHB aggressiveness was evaluated visually three to five times as the percentage of infected spikelets per plot. This result sums up the percentage of infected spikes per plot and the percentage of infected spikelets per spike in one rating. For further calculations, the arithmetic mean of the ratings (=mean FHB ratings) was used.

The phenotypic data from each environment were separately screened for outlier detection with the Bonferroni-Holm method with re-scaled MAD standardized residuals as suggested by Bernal-Vasquez [29]. Additionally, the results from the wheat dataset combining the information from a previous study [14], were implemented in the analysis. The field data (FHB ratings) from rye and wheat could be combined because both hosts were inoculated with the same populations of *F. culmorum* in the same locations in one year (2015). Therefore, in the analysis of this Data set, we added a crop effect to the model.

We estimated variance components using the linear mixed model:

- Data set 1: Rye 2015 + 2016 across 2 locations per year

$$\begin{aligned} y_{ijn} = \mu + \mathrm{Iso}_i + \mathrm{Year}_j + \mathrm{Loc}_k + (\mathrm{Year} \times \mathrm{Loc})_{jk} + (\mathrm{Year} \times \mathrm{Loc} \times \mathrm{Rep})_{jkn} + (\mathrm{Iso} \times \mathrm{Year})_{ij} \\ + (\mathrm{Iso} \times \mathrm{Loc})_{ik} + (\mathrm{Iso} \times \mathrm{Year} \times \mathrm{Loc})_{ijk} + (\mathrm{Year} \times \mathrm{Loc} \times \mathrm{Block})_{ikm} + e_{ijkmn}, \end{aligned} \quad (1)$$

- Data set 2: Rye & wheat 2015 across 2 locations

$$\begin{aligned} y_{ijn} = \mu + \mathrm{Iso}_i + \mathrm{Crop}_l + \mathrm{Loc}_k + (\mathrm{Crop} \times \mathrm{Loc})_{lk} + (\mathrm{Crop} \times \mathrm{Year})_{jk} + (\mathrm{Crop} \times \mathrm{Loc} \times \mathrm{Rep})_{lkn} + \\ (\mathrm{Iso} \times \mathrm{Crop})_{il} + (\mathrm{Iso} \times \mathrm{Loc})_{ik} + (\mathrm{Iso} \times \mathrm{Crop} \times \mathrm{Loc})_{ilk} + (\mathrm{Crop} \times \mathrm{Loc} \times \mathrm{Rep} \times \mathrm{Block})_{iklm} + e_{ilknm}, \end{aligned} \quad (2)$$

where y_{ijn} is the aggressiveness of the ith isolate in the jth year at the kth location, mth block and lth crop. Iso, Loc, Rep and e_{ilknm} denote isolate, location, replication or their interactions and the residual error, respectively.

The variance components were estimated by applying the restricted maximum likelihood (REML) approach and their significance was verified by model comparison with likelihood ratio tests [30].

Heritability (h^2) was estimated on an entry-mean basis as the ratio of genotypic to phenotypic variance according to Piepho and Möhring [31]. Furthermore, fixed genotypic effects were assumed to calculate the best linear unbiased estimates (BLUEs) of the genotypic values for the two Data sets (Table S1). All statistical analyses were performed with ASReml version 3.0 (VSN International Ltd., Hemel Hempstead, UK) [32].

Ten candidate genes previously found as polymorphic in our set of *F. culmorum* isolates [14] were used for this study (Table 2). For details on DNA extraction, PCR amplification, sequencing and SNP calling refer to Castiblanco et al. [14]. Finally, 97 isolates could be genotyped.

Table 2. Candidate genes under study and number of SNPs with minor allele frequencies >5% [14].

Rres v4.0 Annotation [a]	Gene	No. of SNPs [b]	Function and References
Genes encoding transcription factors			
FGRRES_08811	*EFTU*	1	Elongation factor 1α elicits an immune response in the host (Pathogen Associated Molecular Pattern, PAMP) and was identified as differentially secreted [33]
Genes encoding proteins involved in signal transduction			
FGRRES_06878	*CMK1*	1	Predicted virulence associated protein [34], probable CMK1/2 protein kinase type I [35]
FGRRES_16491	*STE11*	1	Belongs to MAPK module regulating fungal development and pathogenicity in *F. graminearum* [36]
FGRRES_08531	*Erf2*	1	Associated with aggressiveness [21]
FGRRES_09612	*HOG1*	3	Regulates hyphal growth, stress responses and plant infection in *F. graminearum* [37]
FGRRES_16251	*TRI6*	2	Global transcription regulator in *F. graminearum* associated with affected severity in *F. culmorum* [38]
Genes encoding membrane proteins			
FGRRES_05633	*MSB2*	3	Transmembrane sensor that regulates invasive growth and plant infection in fungi [36,39]
Genes encoding secreted proteins			
FGRRES_02342_M	*CUT*	17	Predicted cutinase, required to penetrate the host cuticle [33]
FGRRES_05906	*FGL1*	4	Secreted fungal effector lipase [40,41]
FGRRES_00838	*HSP70*	1	Involved in heat-shock response and found to be secreted differentially under pathogenicity conditions in *F. graminearum* [33]

[a] The given ID (FGSG) is the entry number of the Rres v4.0 annotation *F. graminearum* genome database [42]; [b] SNPs detected among the 100 isolates of *F. culmorum* analyzed in this study.

The association analysis was calculated using principal coordinate (PCo) and pairwise kinship coefficients [43] for correction of population structure. All subpopulations were grouping together in a common point cloud, only the Syrian subpopulation was partially shifted to the right [14]. A mixed linear model combining the two main principal coordinates as fixed effect and a kinship matrix for the random isolate effect was used to identify marker-trait associations in the Data sets (Table S1) [44]. The obtained p values were corrected for potential inflation [44]. The significance of marker–trait associations was based on a false discovery rate (FDR) and an adjusted p value of <0.05 as the cutoff. The proportion of genotypic variance (p_G) explained by each SNP was derived from the sums of squares of the SNP in a linear model divided by h^2. All calculations were done with statistical software R version 2.14.2 (The R Foundation for Statistical Computing, Vienna, Austria) [45] including packages GenABEL version 1.8 [44,46] and APE version 3.5 [47,48].

3. Results

FHB symptoms were successfully observed in rye after inoculation with *F. culmorum*, and large differences among the tested isolates were found as shown by the ranges (Table 3). The mean FHB rating (=aggressiveness) across the four environments (=location × year combinations) was 14.85%,

varying from a minimum of 0.5% to 45%. FHB symptoms in the non-inoculated plots across the environments were not observed.

Table 3. Means and isolate ranges of mean Fusarium head blight (FHB) rating of rye after inoculation with 100 *Fusarium culmorum* isolates at two locations in two years (four environments).

Environment	Mean FHB Rating	
	Mean (%)	Isolate Range (%)
2015—Hohenheim	11.01	0.50–32.25
2015—Oberer Lindenhof	19.36	1.00–35.20
2016—Hohenheim	11.16	0.66–41.66
2016—Oberer Lindenhof	17.88	2.20–45.00
Combined	14.85	0.50–45.00

We analyzed the aggressiveness of the same 100 isolates of *F. culmorum* on wheat as a host in 2015 and on rye in 2015 and 2016 at each of two locations (Figure 1). A comparison of phenotypic data between rye and wheat is possible in 2015 where both crops were planted simultaneously in the same field and under the same experimental design. Mean FHB rating was considerably higher for rye in this year and wider ranges of aggressiveness were obtained on this crop in both locations.

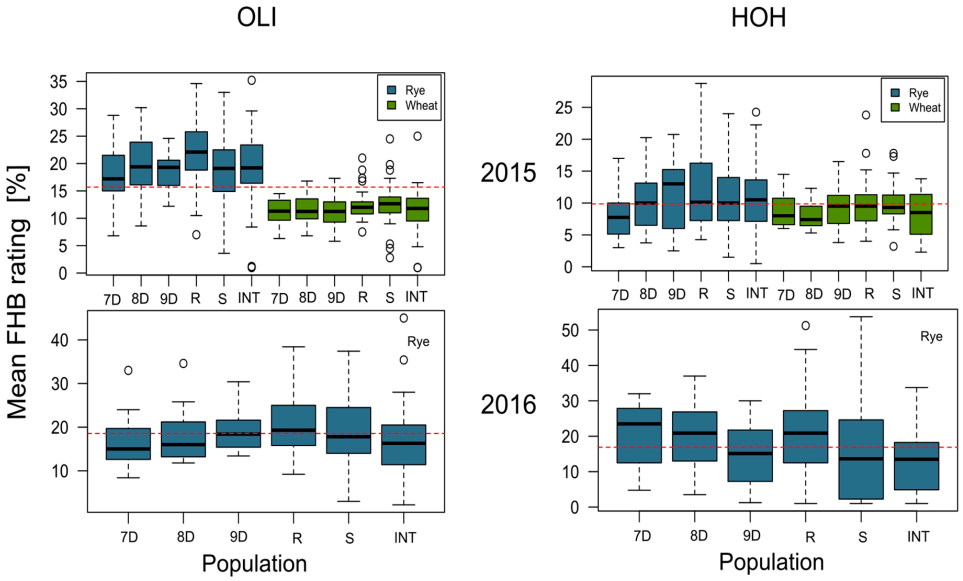

Figure 1. Boxplot of mean Fusarium head blight (FHB) rating (%) of five field populations (7D, Entringen, 8D, Herrenberg, 9D, Nufringen, R, Novgorod/Russia, S, Syrian transect) and the international collection (INT) of a total of 100 *Fusarium culmorum* isolates across two locations (OLI = Oberer Lindenhof, HOH = Hohenheim) in two years (2015 and 2016) and two crops (wheat, rye); the red dashed line is the grand mean across all populations, the open circles refer to outliers.

The frequency distribution of the best linear unbiased estimators (BLUES) calculated from the mean FHB rating followed a normal distribution (Figure 2) as expected for quantitative traits. The BLUES in the rye Data set ranged from −4.23% for isolate FC60 to 21.47% for isolate FC95 (Table S1). The mean across the isolates was 8.78%. In the wheat Data set the BLUES ranged from 18.92% for isolate FC60 to 34.80% for isolate S109.

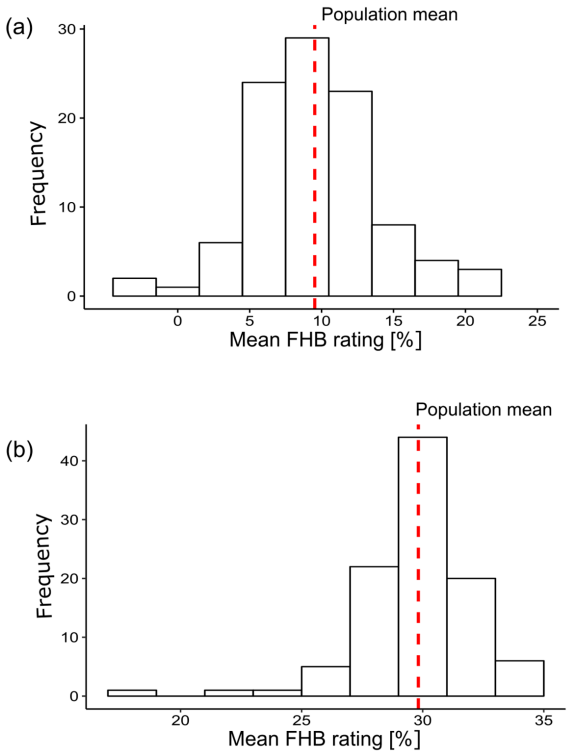

Figure 2. Histogram of the best linear unbiased estimators (BLUES) for mean Fusarium head blight (FHB) rating among 100 *Fusarium culmorum* isolates calculated across two years and two locations in (**a**) rye (2015 + 2016) and (**b**) wheat (2014 + 2015).

The correlation of mean FHB aggressiveness on rye and wheat was significant (Figure 3).

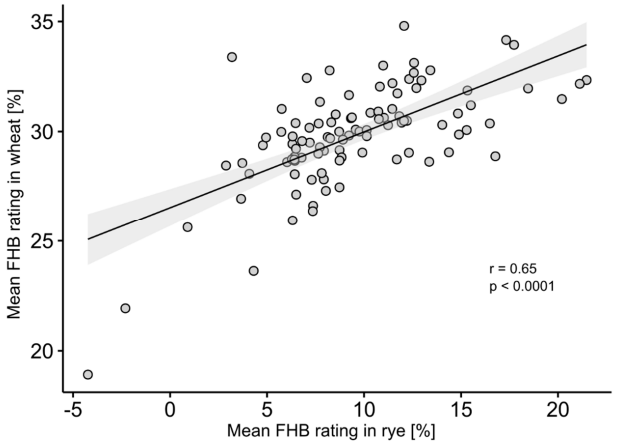

Figure 3. Relationship between wheat and rye calculated as BLUES with mean Fusarium head blight (FHB) rating of 100 *Fusarium culmorum* isolates across four environments; indicated is the regression line and the standard deviation (in grey); *r* = coefficient of correlation, *p* = probability of error.

The SNP located at position −60 in the gene *CUT* (FGRRES_02342_M) was associated with field aggressiveness in both analyzed Data sets. Figure 4a shows the significance of the 17 SNP polymorphisms located in that gene, each bar represents one SNP. At position +56 to +77, 12 SNPs were closely linked resulting in a thick bar in the graph. The SNP at position −60 explained 16.05% of the proportion of the genotypic variance and was significant at $p < 0.001$.

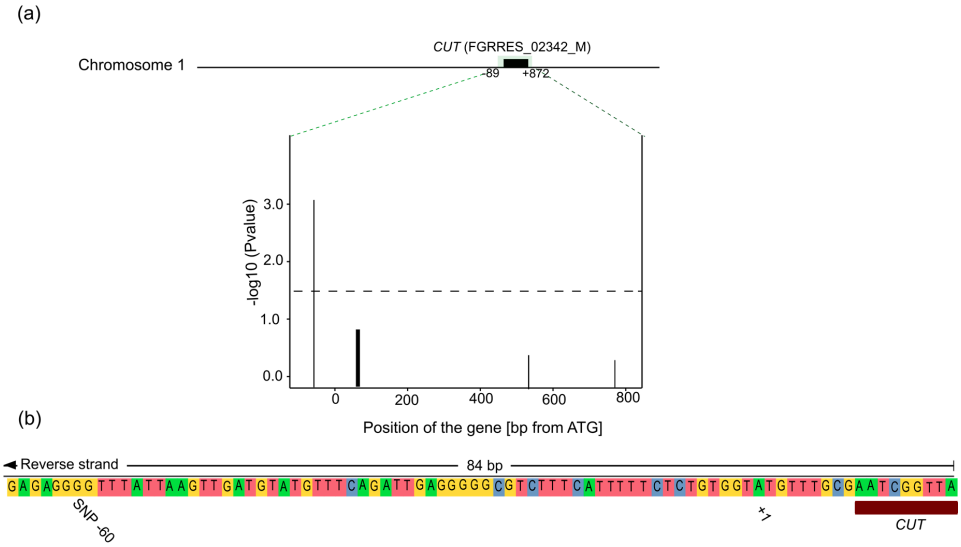

Figure 4. Significant association of SNPs with aggressiveness of 100 *Fusarium culmorum* isolates in the candidate gene cutinase (*CUT*). (**a**) Amplified region of the *CUT* gene on chromosome 1. The significance ($-\log_{10}$ of p value) of 17 SNPs was tested for this gene, each bar corresponds to one SNP. The significance by the cut-off at p <0.05 is shown by the dashed horizontal black line. (**b**) Location of the significantly associated SNP according to the ATG codon.

The variance components were estimated for Data set 1, which corresponds to two years and two locations in rye (Figure 5a). The isolate variance was significant ($p < 0.01$) for mean FHB aggressiveness. The isolate-by-year and the three-way interaction variances were also significant ($p < 0.001$), isolate-by-location interaction variance was not important. The entry-mean heritability for Data set 1 was 0.80.

When aggressiveness measured during 2015 on rye and wheat was combined (Data set 2, Figure 5b), there was a smaller, albeit significant, isolate variation than in Data set 1. The isolate-by-crop and the three-way interaction variances were small and significant only at p <0.05. The genotype-by-location interaction variance was not significant in this analysis and the entry-mean heritability was 0.83.

The two haplotypes found for the associated SNP had a significantly different aggressiveness with the isolates having the SNP with the minor allele frequency being more aggressive in both Data sets (Figure 5c,d). The percent of explained genotypic variance was considerably larger for Data set 1 than for Data set 2 (16.05% vs. 5.96%).

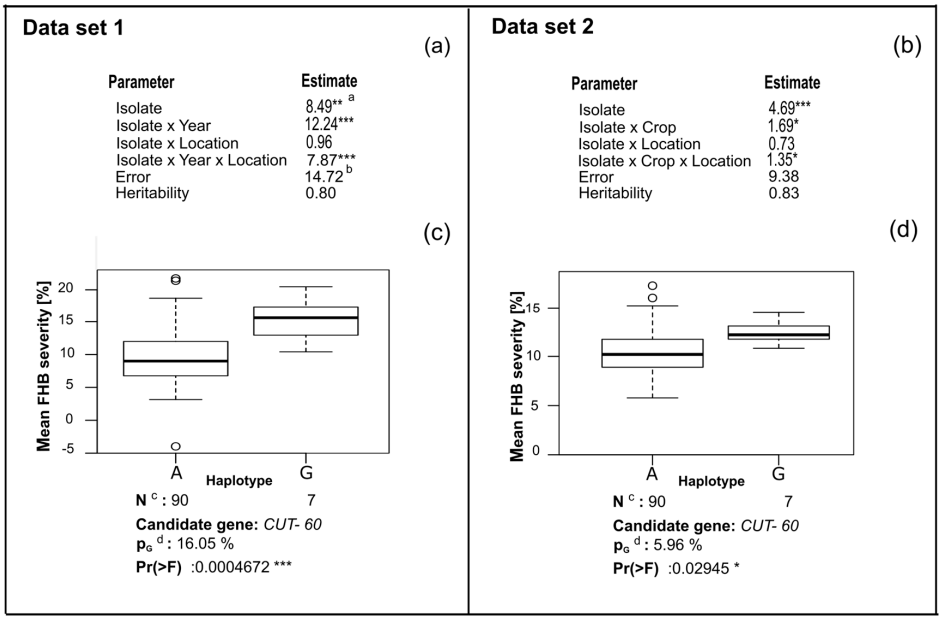

Figure 5. Estimates of variance components (**a**,**b**) and boxplots for mean FHB severity (%) among the two haplotypes of the associated SNP (**c**,**d**), significances and percentages of genotypic variance explained by *CUT-60* (p_G) in Data set 1 (rye in 2015 + 2016) and Data set 2 (rye + wheat in 2015) after inoculation with 100 *Fusarium culmorum* isolates (Table S1). [a] *** Significance at p <0.001, ** significance at p <0.01, * significance at p <0.05; [b] Since heterogeneous variance for error was assumed, the reported value is the mean value of the individuals errors, [c] Number of isolates representing the haplotypes, [d] Percentage of the genotypic variance explained.

4. Discussion

Fusarium head blight is a disease with global relevance since it causes large economic losses and harmful mycotoxin contamination of the grain. In contrast with the numerous investigations on the genetics of quantitative resistance to FHB in cereals, studies on the genetic basis of aggressiveness components in *Fusarium* and other fungi are limited. Increasing our knowledge of the genetic mechanisms by which pathogens damage their hosts is of particular importance for the efficient protection of cultivated host plants and may allow us to monitor pathogenicity pathways necessary for fitness or adaptation.

Even though the importance of genome-wide association studies (GWAS) has increased in recent years, candidate gene association studies allow a direct identification of genes, which play a role in the performance of the pathogen population, even when the genome information is still scarce [49]. This methodology has helped in the detection of genes for important traits in different organisms, such as maize [50,51], rice [52], wheat [53], Arabidopsis [54], and humans [20]. Moreover, this approach was successfully used to study aggressiveness and mycotoxin production in *Fusarium* species in wheat [14,21]. In this study, candidate gene association mapping was performed for *F. culmorum* aggressiveness in rye and compared with the outcome of a previous similar study in bread wheat [14]. From an international collection, 100 *F. culmorum* isolates were used to estimate the association of ten candidate genes, previously reported to be involved in pathogenicity (Table 2) with field aggressiveness.

4.1. Analysis of Phenotypic Data

F. culmorum populations displayed a high genotypic variance of field aggressiveness within individual field populations, similar to the variance displayed by the international collection (Figure 1). This pattern has been reported in other studies with winter rye seedlings inoculated with *F. culmorum* field populations in the greenhouse [55] and with wheat adult plants inoculated with *F. graminearum* in the field [56]. This high genetic variation allows phytopathogens to adapt quickly to new conditions such as a resistant crop or changing environments [57]. The high variability of the *F. culmorum* populations increases their evolutionary potential, which is important to consider when developing successful control strategies [57,58].

In the analysis of the two datasets analyzed for FHB aggressiveness of 100 isolates, high heritabilities and significant ($p < 0.01$) isolate effects were obtained. Heritability is used in plant breeding as an indicator of the precision of the trials or a series of trials and for partitioning the total variance into the genetic and non-genetic components [31]. The mean heritability of the two datasets was 0.82, which is similar to a previous study with 42 *F. culmorum* isolates in winter rye, where the heritability value was 0.85 [59]. Significant quantitative isolate variation has previously been reported for aggressiveness studies of *F. graminearum* [26] and *F. culmorum* populations [14]. These results taken together allow the conclusion that the isolates used in this study displayed wide and consistent genetic differences in aggressiveness, which were systematically observed across a series of multi-environmental field trials.

When only the rye data were analyzed, corresponding to the years 2015 and 2016 (Data set 1), all interactions with isolate and year were significantly ($p < 0.001$) different from zero (Figure 5). This result is consistent with the contrasting weather conditions during both years. In 2015, the relative humidity was lower compared with other years, the total rainfall was 20% less than in 2016 and these differences were even larger if the rainfall patterns are compared during the experimental period. Accordingly, lower means and ranges of aggressiveness of the *F. culmorum* isolates under study were observed for both crops in 2015 (Figure 1). In contrast, 2016 was particularly favorable to fungal infection and disease development. In quantitative pathosystems, significant interactions with the environment are commonly reported [60–62]. The fact that the isolate-by-year interaction played a crucial role on the expression of field aggressiveness, but not the isolate-by-location interaction suggests that trials with different years must be used in order to get reliable results when testing for pathogen aggressiveness.

Previous studies have addressed whether an isolate-by-host genotype interaction exists by using a few pathogen isolates on different host genotypes of one particular crop. Some of those studies have reported very low or lack of isolate-by-host interaction [63,64] and therefore no race specificity [65] in *F. culmorum* and *F. graminearum*. In contrast, other researchers have detected a significant interaction [66,67], but the authors argue in the discussion that the aggressiveness of isolates largely varied and the significance was rather produced by scaling effects [67]. Taking all studies together, we find contradictory and inconclusive results. The analysis of the Data set 2, which involved the comparison of the aggressiveness for the *F. culmorum* population in rye and wheat, revealed only a small, although significant ($p < 0.05$), isolate-by-crop interaction. Accordingly, the correlation between the aggressiveness of isolates for wheat and rye was significant ($r = 0.65$, $p < 0.0001$) i.e., the isolates ranked similarly on both crops (Figure 3). Despite the horizontal nature of Fusarium resistance, the significance in isolate-by-crop interaction should be examined in more detail in future because it might reflect changes in the dynamics of pathogen evolution in different cereal crops. Whether those changes are a hint for the beginning of a pathogenic specialization process as a product of the selection pressure imposed by agricultural ecosystems should be properly analyzed [68].

4.2. Candidate Gene Association Mapping

The sequence of the *F. culmorum* genome is still under development. Currently, two groups are working on it. Firstly, there is a fragmented assembly of an Australian strain CS7071 isolated from wheat crown rot (unpublished, Genebank accession CBMH010000000). The second group recently

presented a draft assembly for a British strain (UK99) from an infected wheat ear [69]. For the present study, the annotated *F. graminearum* genome sequence and the high homology between these two *Fusarium* species were exploited [70].

The SNP-60 in the *CUT* gene displayed significant association to FHB aggressiveness and was still significant after correction for population structure with a kinship matrix coupled by a principal coordinate analysis (PCoA). Using Data set 1, which involves the aggressiveness measured on rye alone, the SNP *CUT-60* explained 16.05% of the genotypic variance with a *p*-value of 0.001. In Data set 2 which analyzed data from wheat and rye, the genotypic variance explained by the SNP was 5.96% only with $p < 0.01$. Clearly, rye alone had a larger effect on this SNP than rye and wheat together.

Usually, susceptible plant genotypes allow the expression of larger aggressiveness differences when exposed to different pathogen isolates. In this study, the variability of aggressiveness expressed by the *F. culmorum* population was larger in rye than in wheat in 2015 (Figure 1), although rye is usually less susceptible to FHB than wheat [71–73]. This result is attributed to the characteristics of the selected experimental rye genotype combined with favorable weather conditions in 2015. Rye used for commercial production represents mainly complex hybrid cultivars that are phenotypically heterogeneous and genetically highly heterozygous. In order to measure reliable differences of isolate aggressiveness, a genetically homogeneous plant genotype was required. For the purpose of the presented research, a rye F_1 single cross between two inbred lines (A × B) was designed, which was genetically homogeneous and more susceptible than the commercial rye cultivars. The wheat genotype used for comparison was the moderately susceptible line cultivar "Inspiration". Consequently, the aggressiveness variation in rye was larger than in wheat in 2015.

Among all the candidate genes tested, *CUT* was the gene having most SNPs with a minor allele frequency (MAF) >5% (Table 2). The isolates that present the less common allele of the associated SNP displayed on average higher aggressiveness values (Figure 5c,d). The allele frequencies of the associated SNP can give a hint of the type of selective forces influencing the trait. Since the SNP with a minor allele frequency of 0.07 at *CUT-60* represents an advantage for the pathogenic development of the fungus, it could be under positive selection and a recent selective sweep at this locus might explain the existence of rare alleles [74]. However, it cannot be ruled out that the significant polymorphism associated with aggressiveness could be in linkage disequilibrium (LD) with the causative SNP [75] present in the upstream region of the *CUT* gene that was not sequenced.

4.3. CUT Gene Is Associated with Aggressiveness in Rye

CUT was significantly associated with FHB aggressiveness and showed high nucleotide diversity. Comparative genomic studies have shown that genes involved in niche adaptation, such as the colonization of living plant tissue, appear to have a high diversity among isolates of the same *Fusarium* species [70]. Cutinase is an enzyme produced by several fungi and bacteria. It is a serine esterase that catalyzes the hydrolysis of cutin into fatty acid monomers. Basically, cutin and waxes are the major structural components of the plant cuticle [76], but the arrangement and composition of the cuticle varies largely among plant species, development stages and plant organs [77]. The cuticle is a shielding membrane of the aerial segments of plants such as non-woody stems, leaves and fruits, creating the first physical barrier that phytopathogens have to overcome and it is a source of nutrients for saprophytes.

In the field of host-pathogen interactions, different functions have been attributed to the cuticle: spore attachment [78] and host signaling [79]. The penetration process assisted by cutinase has been debated for many years [80]. This role of cutinase was proved in some studies [81,82] and rejected by others [83]. In *F. culmorum* and *F. graminearum*, an active route for colonization is the invasion of the cuticle and cell wall with short hyphae [84,85]. Disruption of the cuticle was detected in a cytology study performed after *F. culmorum* inoculation on wheat [84]. The direct role of cutinase in this process, however, has not been proved yet. The *F. graminearum* genome preserves diverse cutinase genes [17] and 32 up-regulated genes, predicted as plant cell-wall degrading enzymes, among them cutinases,

were identified in a gene expression study of *F. graminearum* during infection on barley heads [17]. Accordingly, it was shown most recently, that a *Verticillium dahliae* extracellular cutinase (*VdCUT11*) is an important secreted enzyme affecting aggressiveness in *Nicotiana benthamiana* [86].

Based on this information and the results in this study, we hypothesize that variations in *CUT* regulation may influence the capacity of *F. culmorum* to penetrate the host in its initial biotrophic phase or may help in its saprophytic phase. Some authors suggested an essential role of the protein during saprophytic development [87].

A model was proposed in which cutin monomers that result from the action of *Fusarium* sp. cutinases stimulate host defense responses by creating a complex with plant nonspecific lipid transfer proteins (nsLTPs), and thus facilitating cutin repair [88]. It is not known whether the *CUT-60* polymorphism associated with aggressiveness produce a decrease or increase in the cutinase expression. Under this defense model of the host and an evolutionary scenario, e.g., host evasion from *Fusarium* sp., low expression of the enzyme could delay the recognition by the host defense system and thus increase aggressiveness.

The cuticle could have a role in the plant defense by influencing the deposition of inoculum in the initial stages of infection. Waxes on the plant surface can repel water and therefore prevent the formation of a water film that the pathogen needs to germinate. The role of the cuticle as a mechanical barrier is still not clear. In pathogens that enter the host plant only by direct penetration, a thick cuticle could increase resistance to infection. *F. graminearum* and *F. culmorum*, however, can enter passively by innate openings, such as stomata, or actively by direct penetration, where the plant cuticle still may play a role in resistance. Yoshida et al. [89] evaluated the relationship between FHB resistance in barley and different traits, among them wax coating. According to their results, the wax coating might have a small effect to reduce FHB infection. The authors hypothesize that this could be due to water repellency of the spike.

Interestingly, the gene *HOG1* previously reported as associated with *F. culmorum* aggressiveness in wheat as host [14] was not significantly associated with aggressiveness when rye was used as a host plant. One reason why no SNP within *HOG1* was significantly associated with aggressiveness, although there was nucleotide diversity also in rye, might be that the effect of the SNP is not stable across environments. QTL-by-environment interactions are typical for quantitative traits [90]. Some QTL vary in the magnitude of their allelic effects or they are active in certain environments but not in others. These interactions are possible mechanisms that preserve the genetic variation of quantitative traits in the population [91]. On the other hand, the association study of aggressiveness for *F. culmorum* in wheat [14], although it revealed one SNP in the *CUT* gene (*CUT* position 536 + 1) significant at $p < 0.1$, did not display any significance with *CUT-60*. Given the nature of the cutinase protein, codified by the *CUT* gene of *F. culmorum*, a possible explanation for the differences could be due to differences in the cuticles of wheat and rye. Cuticles vary significantly in their architecture, for example changes in thickness according to the species and ontogeny [92]. There are differences between wheat and rye in the epicuticular wax layer: Rye ears, stems and leaves look gray and those of wheat green. The thick, gray waxy layer of rye can be easily rubbed off illustrating that it is really wax [93,94]. Therefore, it might be no surprise that the fungal cutinase has a larger impact in rye than in wheat. Accordingly, Harris et al. [95] observed in a transcriptomics study four days after infection host-specific gene expression among wheat, barley, and maize as hosts of *F. graminearum*.

4.4. Location of the SNP

The associated polymorphism was located −60 bp upstream from the start codon in the *CUT* gene (Figure 4b) and is caused by a change in the base pair G/A. One possible explanation for the identification of an SNP associated with aggressiveness and located in the upstream non-transcribed region of the gene could be that the SNP is in LD with the "real" polymorphism responsible for the trait variance, in closely located genes or in another region of the *CUT* promoter that was not sequenced. Another explanation might be that the polymorphism is in fact located within the promoter region,

given that those regions are normally directly adjacent to the gene. Therefore, a change in this region could influence the gene transcription levels. Regulation elements of the cutinase gene promoter have been identified in the upstream non-transcribed region of the cutinase gene in *F. solani* f.sp. *pisi* [96]. According to this study, the effects are manifold: Firstly, a silencer between −287 and −249 bp from the ATG codon keeps basal gene expression low and affects the inducibility of the gene. Secondly, an antagonist of the silencer at −360 and −310 bp was detected. Thirdly, mediated basal transcription is located within first 141 base pairs of the cutinase promoter. Finally, there is a GC-rich palindrome at −171 bp, which forms the binding site of cutinase transcription factor CFT1.

5. Conclusions

This study demonstrates the potential of candidate gene association mapping to reveal genes that affect fitness traits for populations of plant-pathogenic fungi under field conditions. This approach is an alternative to traditional QTL studies, especially when recombinant mapping populations are not available. Natural field populations of *F. culmorum* possessed a high genetic diversity in aggressiveness that enables the infection process and increases FHB damage. The identified cutinase gene should be further analyzed by gene expression studies to validate its importance in *F. culmorum* aggressiveness using different cereal hosts including rye. Whole-genome sequencing of the fungus in future will enable a verification of our association mapping study, allow detection of more genes relating to aggressiveness and improve our understanding of the genetics that contributes to this important, quantitatively inherited trait.

Supplementary Materials: The following are available online at www.mdpi.com/2309-608X/4/1/14/s1. Table S1: Best linear unbiased estimates (BLUEs) for 100 *F. culmorum* isolates in rye (Data set 1) and wheat+rye (Data set 2).

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Author Contributions: Thomas Miedaner was responsible for the project, the research plan, helped with analyses and interpretation of data, revised the manuscript and finally approved it. Valheria Castiblanco was responsible for all field experiments and the selection of the candidate genes, analyzed all data from wheat and rye in both years, and wrote the paper. Hilda Elena Castillo helped with the field experiments in 2016, conducted all lab experiments of this study, analyzed the data from rye, and helped with writing the paper. All authors read and approved the final manuscript.

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Supplementary material

Table S1. Best linear unbiased estimates (BLUEs) for 100 *F. culmorum* isolates in rye (Data set 1) and wheat/rye (Data set 2).

Name	Mean FHB (Data set 1)	Mean FHB (Data set 2)
7D22	6.02996208	7.026035841
7D23	4.783959899	9.32667303
7D24	9.567929844	11.40888751
7D26	4.085511852	6.272004522
7D27	6.305742364	7.792814215
7D28	7.0472762	12.02333653
7D34	8.717224372	10.7144464
7D6	11.02548582	9.541806156
7D7	9.762118551	12.11685281
7D9	13.35986824	10.24860952
8D13	7.932579076	8.196516495
8D14	7.371765296	6.246690897
8D17	7.202349919	8.622956021
8D2	11.45239205	12.67311073
8D20	10.1355119	9.96455015
8D28	6.80660376	9.273322832
8D3	14.8928292	11.8164075
8D33	6.432052481	9.928018495
8D4	8.123812245	10.88568785
8D5	15.29040787	13.46829237
8D6	8.736143272	10.59995312
8D8	11.81896909	11.93197962
9D1	7.718832191	9.132302991
9D11	6.778704587	10.24857189
9D18	8.841593314	8.821060701
9D22	10.32627843	13.15177003
9D31	6.46895175	7.176876878
9D32	12.69862297	13.27772719
9D34	12.21168898	10.79381433
9D37	9.220184249	9.408678217
9D38	7.925836708	9.336535945
9D40	9.22230271	12.29534403
9D5	12.95725778	13.35976826
FC104	11.71981413	15.55422342
FC106	7.301953639	9.763324609
FC2	15.50671146	13.69555682
FC3	12.32412826	13.16894999

Name	Mean FHB (Data set 1)	Mean FHB (Data set 2)
FC33	11.46376967	14.03612338
FC37	8.733090228	9.814494185
FC40	8.252606321	11.1535056
FC46	12.31325909	13.35240756
FC50	4.309048539	6.20885782
FC60	-4.237592853	0.094455534
FC65	3.190563794	11.26880413
FC68	6.314777244	7.933894896
FC69	8.736990951	9.455522949
FC7	3.659595197	7.673468829
FC70	7.817520279	8.732535454
FC72	6.318132125	10.81876022
FC74	6.500164436	7.191543381
FC75	9.913760069	10.07955108
FC89	7.357148394	7.333544172
FC90	9.325131167	13.42238563
FC95	21.47313526	13.61512624
FC98	7.659554608	10.27887188
R1011	20.19923061	12.62275988
R111	9.374168944	11.95334925
R1111	5.733770882	9.902388597
R1211	7.181354638	10.53442347
R1311	11.93657778	12.57485893
R1411	12.57678783	15.65902347
R1511	4.945504711	6.928369845
R1611	8.309681833	8.601720821
R1811	14.03178312	12.16658991
R1911	16.48452245	11.78492468
R2011	12.05810596	11.25816568
R2111	10.13972981	11.25429398
R2211	6.414982181	8.339285341
R2311	15.3294195	14.9005141
R311	8.03183091	8.424443199
R411	18.45707113	16.47092361
R711	10.73321286	12.95647064
R811	17.73151451	13.97961324
R911	13.41871457	13.05674637
S021	17.32632893	13.9958185
S022	12.56616577	13.94740566
S023	8.908226892	11.15610424

Name	Mean FHB (Data set 1)	Mean FHB (Data set 2)
S043	21.12043297	13.8439849
S045	16.77046243	11.3643689
S060	2.883787399	8.50984134
S109	12.06492759	17.7207364
S129	-2.304532717	2.380355492
S220	7.725976677	10.37252396
S222	14.84108378	12.65112739
S229	5.754289743	11.49430783
S256	6.280923616	8.848624254
S259	6.489782502	9.939180571
S264	14.37590642	9.816297313
S265	7.63808642	9.689855439
S267	3.723573885	10.11695873
S274	6.426130163	11.54901996
S275	10.98428653	11.33545261
S276	10.81476726	11.35209654
S280	0.901540817	5.893721665
S283	11.68779352	12.11102087
S289	10.76439234	11.56144517
S290	11.23775162	11.7568284
S293	8.537010603	12.69128669
S296	6.4077697	10.22009255
S299	8.225054891	10.6302285

5 General discussion

The understanding of the molecular mechanisms controlling quantitative plant-pathogen interactions is key to formulate effective and durable disease management strategies. Research on plant pathology has mainly focused on the understanding of qualitative (vertical) resistances (gene-by-gene interactions) and quantitative (horizontal) pathogenicity remains highly unknown. In agriculture, the vast majority of plant-microbe interactions of economic importance are governed by quantitative interactions. While Fusarium head blight is a model pathosystem for the study of quantitative resistances in small-grain cereals, other economically important examples of this polygenic interaction include *Botrytis cinerea* in multiple hosts (Corwin et al., 2016) and *Rhynchosporium commune* in barley (Stefansson, Mcdonald, & Willi, 2014).

Advances in genomics, phenomics, and biostatistics have provided major innovations to disentangle the complexity of quantitative plant-microbe interactions. This thesis focused on the understanding of phenotypic and molecular aspects of the *Fusarium culmorum* - cereal pathosystem. Previous studies demonstrated that the Fusarium-cereal pathosystem follows the classic quantitative interaction proposed by Vanderplank (Zadoks & Schein, 1988) where the most resistant host genotypes remain resistant against all isolates regardless of their aggressiveness, and vice-versa, the most aggressive isolates remain as aggressive, independently of the host genotype (Tóth et al., 2008; van Eeuwijk et al., 1995). According to quantitative-genetic theory, such

interaction is governed by many individual genes that have only small to moderate effects, i.e. quantitative-trait loci (QTLs), that are prone to environmental changes (Van Der Plank, 1966). This quantitative inheritance applies also for the pathogens, *F. culmorum* and *F. graminearum* (Cumagun et al., 2004; Voss et al., 2010). The environment in case of the pathogen includes not only location and years, but also the host (host species, host genotype) and the habitat (living host, host residues, soil).

In the first paper, we explored the relative conbution of genotypic variation and environmental plasticity to the total phenotypic variation among isolates of *Fusarium culmorum* from natural field populations (Castiblanco et al., 2020). We designed a field experiment to deepen our understanding of the environmental effects on the pathogen, especially the host species-isolate and isolate-location-year interaction. We wanted to test whether an isolate-host interaction is evident when we use different species of cereals as hosts, and not only different cultivars of the same species as previously reported (Van Eeuwijk et al., 1995). As a result, of the comparison of a set of *F. culmorum* isolates into four cereal hosts, we identified high isolate ranking and genotypic correlation among hosts suggesting no isolate-host interaction. According to these results, in the next papers we studied the molecular aspects associated with the aggressive reaction of the pathogen across different host species. The second and third paper apply the methodology of candidate gene association mapping to corroborate the role of previously reported putative genes on the quantitative variation in aggressiveness of *F. culmorum* (Castiblanco et al., 2018, 2017). The comparison of the association of putative aggressiveness genes under two different hosts (wheat and rye), reveled host-specific QTLs, suggesting that the quantitative interaction also may include specific aspects.

Phenotypic diversity: Specific methods for quantitative assessment

Phenotyping has been a constraint when studying quantitative traits in plant pathology (Corwin et al., 2016). Contrastingly, qualitative traits as the hypersensitive response (HR), which is characteristic of gene-for-gene interactions, are measured as presence-absence characteristics in young-plant stage under standardized environmental conditions (seedling or leaf-segment tests). On the contrary, quantitative traits like aggressiveness are highly affected by the environment, therefore laboratory or greenhouse methods often are not representative of the reality in the field leading to low correlations only.

The expression of aggressiveness is influenced by factors acting in different stages of the disease process. Before the interaction with host, the key influential factors are pathogen and the environment. The assessment of the influence of those factors is often approached by studying several genotypes of the pathogen under several environmental conditions (Lannou, 2012). Most common response variables for the effect of these factors are the viability/longevity of the spores, germination rate, and the growth capacity of the mycelium. The next stage takes place when the pathogen reaches the host. Therefore, a conclusive understanding of aggressiveness should involve not only several levels of the pathogen and the environment but also several genotypes of the host. Typical response variables to assess this triple interaction are efficiency of penetration, growth, colonization of host tissue and sporulation. Given the above-described complexity in the study of aggressiveness and its expression, two main bottlenecks should be solved to establish a reliable and efficient phenotyping strategy. Firstly, an efficient experimental design allowing to capture the variability caused by environmental factors, by the genotype of the fungus and by the genotype of the host. This design derives in large size experiments conducted at multiple locations and years. Secondly, an integrative, reliable and affordable response variable that reflects the effect of the three factors should be identified. The

measurement strategy implemented to quantify the response variable should be cost-effective and high-throughput, considering the large size of the experiment and the complexity of the triple interaction.

Studies focused on the reproductive biology of the pathogen allowed the development of methods to grow *Fusarium* in the laboratory and massively produce spores of the fungus in liquid media. These inoculum production methods have already been implemented for large-scaled field experiments (Mather et al., 1992; Talas et al., 2012; Voss et al., 2010). It must be assured, however, that the observed and measured symptoms are truly caused by the inoculated isolate. We, therefore, used a chess-board like design with each plot surrounded by four plots of a tall triticale cultivar and rated visually visible symptoms of the plot several times during pathogenesis as already described by Gang et al. (1998). Simultaneously, the progress of multivariate data analysis methods, such as mixed-model analysis, has generated the necessary tools to disentangle the magnitude of the effect of each factor (environment, pathogen genotype or host genotype) on the variability of the aggressiveness. While the effect of the factor pathogen genotype can be assessed by the magnitude of the variance explained by the genetic make-up of the isolates, all factors associated with environment reflect the importance of plasticity in the development of the disease (Scheiner & Lyman, 1989).

Phenotypic diversity: The role of the environment in the expression of the aggressiveness

Plasticity is defined as the ability of a pathogen to detect changes in its environment and the use of signal transduction pathways to alter its phenotype in response to environmental changes (Irwin et al., 2003). The mixed model approach allows us to estimate the variance components associated to plasticity and its heritable fraction (Scheiner & Lyman, 1989). Firstly, the plastic variance is considered as the sum of the environmental variance and the

isolate-by-environment interaction variance. Secondly, plasticity is computed as the ratio between plastic variance and total phenotypic variance. Finally, the heritable fraction of plasticity is the proportion of the total phenotypic variance attributed to the genotype by environment interaction effect. From these definitions, another way to define plasticity would be all changes in the phenotype attributed to changes induced by the environment.

We had three different data sets, each replicated across two years and two locations obtained under field conditions over the period of 2014 to 2016. The first dataset consisted in a population of 110 *F. culmorum* isolates inoculated on bread wheat (Castiblanco et al., 2017), the second consisted in the same 110 *F. culmorum* isolates inoculated on rye (Castiblanco et al., 2018) and the third, a sub-sample of 28 *F. culmorum* isolates inoculated on four host species (winter forms of bread wheat, durum wheat, rye, triticale) (Castiblanco et al., 2020). For all experiments, the plastic variance exceeded the isolate (genotypic) variance, for instance in Castiblanco et al. (2020) the environmental variance exceeded eight times the isolate (genotypic) variance. However, focusing on the heritable fraction of the plasticity presents a different view as less than 17% of the plastic variance corresponds to the isolate-by-environment interactions. Further, we partitioned the isolate by environment interaction into isolate by year, isolate by location and isolate by host and their interactions.

The isolate-year interaction as well as triple interactions involving year, accounted for a high amount of variation sometimes even exceeding the isolate variance. Strong changes in the weather were observed among the years under study, for instance, a contrastingly dry spring in 2015 and a humid spring in 2016. High and significant interactions with year reflect that although the dry conditions experienced in 2015 allowed certain isolates to perform well, the performance of the same isolates did not translate when conditions were humid in 2016, regardless of whether the host was the same or not (Castiblanco et al., 2020). Differentiation on the isolates that perform well under dry conditions compared to humid ones could be an evidence of

an incipient process of pathogen adaptation. The small fraction of heritable plasticity and the lack of a constant selection pressure given the erratic pattern of seasonal changes could hinder a speciation process as suggested by Voss et al., (2010). However, previous studies reported regional differences in pathogen composition and temporal dynamics, e.g. for the chemotype (Kelly et al., 2015), which might be also supported by the phylogeny of the *F. graminearum* species complex being shown to have biogeographically structured lineages (O'Donnell et al., 2004).

From the perspective of the pathogen, the definition of the environment must involve not only the climatic conditions but also the host it inhabits. Across the experiments, we found that when using different cereals species as hosts, the isolate- host species interaction was either very low (Castiblanco et al., 2018) or not significant at all (Castiblanco et al., 2020). Accordingly, isolate ranking did not significantly change and high genetic correlations for aggressiveness among host species were detected. This pattern has also been revealed by previous studies, using different genotypes of wheat (Tóth et al., 2008; Van Eeuwijk et al., 1995). To the best of our knowledge, this is the first study that has investigated the *Fusarium culmorum* isolate by host species interaction using different cereal species as hosts. Considering that each cereal species presents differences in plant morphology, which may play a role in the plant defense reaction (such as the structure of epidermis and cell walls in general), the ability of the isolates to cope with those differences in a similar way reflects a high plasticity with low heritable component. In contrast to our results, Akinsanmi et al., (2007) found changes in the fitness of the pathogen due to passage through alternative hosts, suggesting an incipient process of speciation rather than a plastic behavior for the pathogen. Some specific aspects should be considered when comparing both studies. First, in the study of Akinsanmi et al., (2007) the changes in aggressiveness are studied only after one unique generation of passage through an alternative host. It should be considered carefully if one generation is enough to evidence genetic changes leading to adaptation, or whether it might be instead attributed to epigenetic changes (Gijzen et al., 2014; Gómez-Díaz et al., 2012). Secondly,

although pathogen species in both studies belong to Fusarium clade, the study of Akinsanmi et al., (2007) involves *F. graminearum* and *F. pseudograminearum*, which differ from *F. culmorum* in the sexuality behavior, as in *F. culmorum* no teleomorph has been found so far. Further studies are required to elucidate the significance of both studies at the light of evolution.

Phenotypic diversity: Comparison between aggressiveness and deoxynivalenol (DON) data

We found a high correlation between DON accumulation and aggressiveness in the cereal species used as a host (Castiblanco et al., 2018, 2017). The close association between aggressiveness in wheat and DON production has been demonstrated in several field studies (Gang et al., 1998; Mesterházy et al., 2002; Miedaner et al., 2000; Talas et al., 2012; Voss et al., 2010). Multiple reviews tackle the role of DON in aggressiveness during the pathogenic phase and in antagonistic efficiency during the saprophytic phase (Audenaert et al., 2013; Tunali et al., 2012). Temporal differences rather than presence/absence changes in the biosynthetic modulation of DON determine the role of this mycotoxin in aggressiveness. The isolates triggering the production of DON at the appropriate time to neutralize the host defense response, are more efficient to colonize the host tissue (Audenaert et al., 2013; Diamond et al., 2013). Moreover, Bai et al., (2002) demonstrated that isolates with the DON biosynthetic pathway disrupted were able to infect the host but not to colonize and complete the disease process. Then, although DON is not needed for the initial infection, it is required for the colonization and spread across the host tissues, explaining the close relationship between aggressiveness and DON accumulation observed in our study (Bai et al., 2002; Diamond et al., 2013).

The mixed model analysis applied to DON accumulation in grain (Castiblanco et al., 2020) revealed a significant contribution of the factor

host species but not an isolate-host interaction. Concerning the host effect, most of the isolates induced a higher DON accumulation in the mature grain of durum wheat than in the grain of other hosts. Consistently, the study of Gaikpa et al., (2020) showed that DON concentration in mature grain is higher in most of the tested genotypes of durum wheat, in comparison with other small grain cereals (bread wheat, triticale and rye). Those results however, should not be interpreted as a sign of specificity of isolate by host, as the ranking of the isolates for DON accumulation did not change significantly according to host species (Castiblanco et al., 2020). As mentioned earlier, the heritable fraction of plasticity is the proportion of the total phenotypic variance attributed to the genotype by environment interaction effect. The low heritable fraction of plasticity in DON accumulation data let us conclude that there is no evidence of a specialization process. These results are aligned with our conclusion regarding aggressiveness.

Molecular diversity: Criteria to choose candidate genes

Literature reports on the molecular mechanisms that underlie quantitative resistance and aggressiveness are rather limited and inconclusive (Poland et al., 2009; Zhang et al., 2019). However, there is an overall agreement on the role of gene co-expression networks (sets of simultaneously activated genes) in plant pathogen interactions (Zhang et al., 2019). The activation of the network and regulation of the signals in space and time determine the effectiveness and therefore fitness of the individual (Zhang et al., 2019). Several theories have been proposed to explain the quantitative nature of aggressiveness. An initial attempt suggested that quantitative interactions correspond to the shadow effect of multiple gene-by-gene interactions established during the coevolution process between the pathogen and the plant, i.e., "the zigzag model of plant immune system" (Jones & Dangl, 2006). Then, secreted effectors would play a major role on the establishment of the plant-pathogen

recognition and the subsequent trigger of the infection. More recently, it has been suggested that quantitative interactions depend little on the factors involved in the direct recognition between the plant and the pathogen, but on master regulators, such as transcription factors (Talas et al., 2012). Miedaner (personal communication, 2014, 27 August) suggested that plant passive defense mechanisms (e.g. papilla or thickening of cell walls by callose depositions) are of major importance and therefore enzymes, toxins and other secondary metabolites in the pathogen would be the highest determinants for aggressiveness.

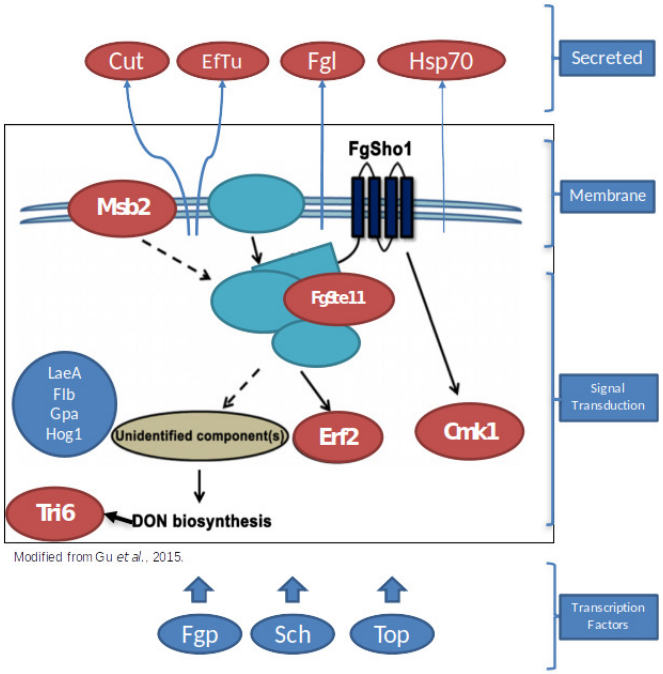

Figure 1: Modification of the proposed model for the role of FgSho1 in Fusarium graminearum (Gu et al., 2015) On the DON biosynthesis pathway.

Aiming to shed light on the regulation of aggressiveness, we chose for our association study seventeen candidate genes encoding for proteins with a confirmed or predicted role in pathogenesis in different categories of the signaling pathway for aggressiveness (Castiblanco et al., 2017). In detail, four genes encoding transcription factors, eight genes encoding proteins involved in signal transduction or transport activities, two genes codifying for proteins

located in membrane and three secreted proteins corresponding to a putative secreted enzyme and two putative secreted effectors (Fig. 1). Specific primers for amplification of the most variable regions within selected genes were designed and used to characterize 100 isolates of *F. culmorum*. Amplicons for each isolate and gene combination were sequenced and aligned against the reference genome of *F. graminearum* (http://fungi.ensembl.org) to identify single nucleotide polymorphisms (SNPs). We did not include genes with lethal or sub-lethal effects or housekeeping genes involved in basic metabolism because we expect few to any polymorphisms within those genes, which are the fuel for association studies. Our approach offers a wider coverage of aggressiveness factors signaling pathway in comparison with similar studies, focused mainly on the DON biosynthesis pathway (Talas et al., 2012).

Molecular diversity: Nucleotide variation within candidate genes

The genes encoding proteins secreted and located in the membrane (*SHO1, MSB2, CUT, FGL1, HSP70*) displayed the highest genetic diversity in our study. The first report on the genome sequence of *Fusarium graminearum* concluded that genes involved in the plant-pathogen interaction are located at highly polymorphic regions (Cuomo et al., 2007). Current genomic analyses found that genes related to plant-pathogen interaction, pathogenicity and host adaptation are not only preferentially located within polymorphic islands but also up-regulated *in planta* (Wang et al., 2017). Moreover, these genes display greater diversification rate than genes fulfilling basal functions (Laurent et al., 2017). Thus, we suggest that as in *Fusarium graminearum*, the recognition of the host through secreted proteins, plays an important role in the aggressiveness of *Fusarium culmorum*. The high level of genetic diversity observed in secreted proteins could arise from an intense selection pressure caused by the interaction of *Fusarium* and its plant hosts. This

pressure in the long term favors the accumulation of new and sometimes extreme variants, the process known as positive or disruptive selection (Hill, 2017). Decoding the role of molecular recognition in the aggressiveness of *Fusarium*-cereal pathosystems should be an intense and fruitful research field in coming years.

In three out of the four transcription factors studied (*FGP1, SCH, TOP1*), no polymorphisms were found, which suggests a highly conserved function and pleiotropic control of basic physiology (Castiblanco et al., 2017). In our study, the genes involved in control and transport showed levels of intermediate diversity compared to the transcription factors and secreted proteins. When analyzing the linkage disequilibrium (LD) of the studied regions, both Talas et al. (2012) and our study (Castiblanco et al., 2017) observed pairs of genes with high and significant LD, even when located at different chromosomes. This result can be explained in two ways: firstly, the allelic variants of pairs of genes in high LD are involved in the same physiological network and therefore evolve simultaneously. Secondly, additional unknown genes/factors at a very small physical distance to our regions of study are responsible for the LD between genes located at different chromosomes.

Combining phenotypic and molecular diversity: Candidate gene association study outcome

Candidate gene association mapping revealed allelic variants of *HOG1* and *CUT* as significantly associated with aggressiveness and DON production in bread wheat and rye, respectively. In bread wheat, *HOG1*, a kinase involved in signal transduction, explained 10.29% of variance for aggressiveness and 6.05% of variance for DON accumulation in mature grain (Castiblanco et al., 2017). In rye, a cutinase (*CUT*) explained 16.05% of variance for aggressiveness (Castiblanco et al., 2018). Although an insertion in *CUT* (CUT536 + 3)

could be associated with aggressiveness in bread wheat, the corresponding p-value ($p = 0.07$) did not indicate statistical significance. While these results might rise an hypothesis about specificity of pathogen alleles to trigger the disease in certain crops, the study of Castiblanco et al. (2020) revealed a high genotypic correlation for the level of aggressiveness of different isolates among host species. Taken together, our results suggest the existence of a specific molecular machinery underlying the isolate-host interaction, which despite the specificity is resulting in similar phenotypic reactions across different crops. Similar results have been reported for the differential expression of genes associated with transport, secondary metabolism, integral membrane proteins and chitinases of *Fusarium graminearum* when colonizing wheat, barley and maize (Harris et al., 2016).

In our second and third paper (Castiblanco et al., 2018, 2017), we found only two out of 17 genes being significantly associated with aggressiveness and DON production in bread wheat or rye. However, the 15 non-significantly associated genes should not be regarded as not involved in aggressiveness or DON production. Instead, the nature of the association approach implemented only allows to conclude that *SNPs* in *CUT* and *HOG1* explain a large proportion of the variance among the different levels of aggressivess in the isolates under study. As mentioned by Zhu et al., (2008), association approaches might fail when rare allelic variants are present in the population. The same holds true when important alleles are fixed, what might be the case in our population, because all isolates displayed at least a moderate level of aggressiveness. Future studies might improve the detection power by including isolates with a predominant saprophytic rather than pathogenic behavior or by enhancing the population size. From the pathogen perspective, not only the climatic conditions but also the host could be regarded as the environment. Then, the differential association of SNPs in *CUT* and *HOG1* against the studied hosts can be interpreted as pathogen-QTL by environment interaction. For instance, in our third paper, we reported the polymorphism-60 in *CUT* as significantly associated with aggressiveness of *F. culmorum* in rye, while the same SNP plays a neutral role in the aggressiveness in bread

wheat. This interaction is frequently studied in the literature of complex traits, and it is also associated with environment-specific allelic effects and antagonistic pleiotropy. Antagonistic pleiotropy occurs when a certain polymorphism is especially beneficial under a certain environment, although it is neutral or even detrimental in a different environment (Anderson et al., 2014; Fournier-Level et al., 2013). Previous studies argued that changes in environment may induce changes in both the QTL effect and the direction of selection, which may explain the persistence of allelic polymorphism at pathogen-QTL (Fournier-Level et al., 2013). Thus, continuous changes in environmental conditions maintain allelic diversity within the genome, and in turn lead to plasticity. The results of our first paper suggest that QTLs associated with aggressiveness in *F. culmorum* might be under antagonistic pleiotropy when exposed to different environments. This hypothesis, as well as the evolution of QTLs associated with aggressiveness in *F. culmorum* is a wide research field to be explored.

In bread wheat and rye, the SNPs associated with aggressiveness and DON production were located in non-codifying regions. This can be explained by i) an indirect association, where the SNP is in high linkage disequilibrium with the causal variant of the association; or ii) because of a direct association as the detected SNP might be involved in post-transcriptional regulation, such as alternative splicing. Strain-specific expression of virulence factors has been reported in *Z. tritici* (Palma-Guerrero et al., 2016). Then, further studies about the expression patterns of factors involved in aggressiveness would be required to understand the role of molecular variation at non-coding regions in the aggressiveness of *F. culmorum*.

Studied genes grouped in different categories of the signaling pathway for aggressiveness. Only the categories secreted and signal transduction were involved in differences of aggressiveness among isolates. While *HOG* is involved in the signal transduction category, as well as the *MetAP1* and *Erf2* genes reported by Talas et al. (2012), *CUT* is a protein that belongs to the secretome, and therefore is involved in the first line of communication with the

host. The results of our second and third paper are novel as they suggest that changes in the expression of secreted proteins can also explain differences in aggressiveness among isolates in natural infections. Several approaches have been used to study secreted molecules mainly in *F. graminearum*. For instance, Yang et al., (2012) used a gelbased proteomics approach to identify the proteins secreted by *F. graminearum* in the interaction with barley and wheat; Rampitsch et al., (2013) compared the secretomes of the wildtype with two nonpathogenic deletion mutants of *F. graminearum*; Brown et al., (2012) predicted a refined secretome by combining several bioinformatic approaches applied on the genome of *F. graminearum*, and Lu & Edwards, (2016) selected putative candidate effector proteins through proteomics and sequence/transcriptional analyses in the genome of *F. graminearum*. While all these studies predict proteins that could be related with aggressiveness, our third paper is the first report to associate a secreted protein (CUT) with the aggressiveness of natural populations of *F. culmorum*.

Many genes involved in fungal secondary metabolism are arranged in clusters, which are easily impacted by epigenetic modifications (Chen et al., 2019). In *F. graminearum*, the enzymes required for trichothecene production are encoded by 15 *TRI* genes, located at three clusters on different chromosomes. The first cluster is composed by 12 *TRI* genes, the second clusters two genes at the *TRI1-TRI16* loci, and the third correspond to the gene *TRI101* (Chen et al., 2019). A similar cluster pattern for the *TRI* genes is presumed for *F. culmorum* (Schmidt et al., 2018). The study of Talas et al., (2012) tested the role of five *TRI* genes (*TRI1*, *TRI5*, *TRI6*, *TRI10* and *TRI14*) in aggressiveness using candidate gene association mapping. However, none of the genes tested displayed significant association with aggressiveness, neither with DON production. In our second paper, we found significant association between three SNPs (positions 380, 382 and 724 relative to the start codon) within HOG1 and DON accumulation in mature grains of bread-wheat. As the three associated SNPs in the study are located at the non-coding regions, the authors hypothesized a role for these SNPs in post-transcriptional regulation. Studies using rice grains, wheat kernel cultures and in planta, found

a reduced DON and NIV production in mutants of *HOG MAPK* belonging to different *F. graminearum* strains (Zheng et al., 2012). These evidences suggest that the level of DON biosynthesis and DON accumulation might be determined by genes outside rather than inside the DON biosynthetic pathway.

Consequences for disease management and resistance breeding

Integrated plant disease management use crop rotations to reduce the amount of inoculum as well as growth and survival of plant pathogens. However, the success of rotational strategy depends on the crops selected and their ability to reduce disease development (Larkin, 2015). The high genetic correlation of aggressiveness of *F. culmorum* isolates observed among bread wheat, durum wheat, rye and triticale by Castiblanco et al., (2020) suggest that these crop species display a similar defense profile. Therefore, crop rotation strategies alternating only these four cereals would not serve the purpose of breaking nor delaying the disease cycle. Furthermore, it is well known that rotation involving other *Poaceae* species, especially using maize as pre-crop to bread wheat, is one of the main causes for FHB problems worldwide (Dill-Macky & Jones, 2000). Contrastingly, some crop species are known as disease suppressive by its ability to play an active role in disease reduction. For instance, crops in the *Brassicaceae* family, produce compounds that can suppress some pathogens and diseases. In the specific case of Fusarium head blight mulch layers of white mustard, Indian mustard or clover consistently suppressed *F. graminearum* infection and decreased mycotoxin contents in wheat grain (Drakopoulos et al., 2020). Thus, efficient crop rotational strategies for the management of Fusarium head blight in small cereals should involve the regular use of crop species outside the *Poaceae* family.

In addition to crop rotation, varieties improved for disease resistance should be grown. Plant breeding for durable resistance must combine QTL with

complementary modes of action. Major QTL's have proven to be a valuable, and feasible to introgress, source for resistance breeding. However, finding major QTL's in quantitative plant-pathogen interactions is rare and the durability on their resistance under field conditions is questionable. Combining major QTLs with small effect QTLs is a promising approach for extending the effectiveness of resistance breeding (Pilet-Nayel et al., 2017). This approach seems to be appropriate in the pathosystem *F. culmorum*/small grain cereals. As the results of Castiblanco et al., (2020) suggest, the high plasticity of *F. culmorum* populations requires a high diversity in resistance mechanisms to be selected in breeding programs. For instance, in wheat breeding major QTL of Chinese origin should be simultaneously selected with small effect QTLs of native origin (Miedaner et al., 2019). In the plant genomics era, the simultaneous selection process of major and minor QTLs might be facilitated by tools as genomic selection. Prediction models incorporating major QTLs as fixed effects have shown promising results when applied within and among winter wheat breeding populations (Herter et al., 2019). Further research about the impact of genomic selection in the long-term maintenance of high genetic diversity in resistance mechanisms would promote the adoption of the strategy by practitioners. In parallel, the aggressiveness of *Fusarium* populations should be monitored, because a shift to higher aggressiveness might be possible, when resistant cultivars are grown on a large acreage. *Fusarium* species can only be managed durably, when both parts of the pathosystem are acknowledged. New molecular tools, like whole-genome sequencing or RNA seq, will greatly contribute to our knowledge and may open new avenues for *Fusarium* control.

6 Summary

Fusarium head blight is one of the most devastating diseases of cereals globally and responsible for large harvest losses, not only due to the reduction in productivity but also due to the contamination of the grain with mycotoxins. The major causal agent worldwide is *Fusarium graminearum*, in Europe also other *Fusarium* species, among them *Fusarium culmorum* (FC) play an important role. The interaction between *Fusarium* species and cereals has been categorized as quantitative according to previous phenotypic and genetic observations.

We studied the molecular and phenotypic diversity of natural populations of FC and how they interact with four cereals (bread wheat, durum wheat, triticale, rye) as host. Specifically, we sought (i) to understand the interaction between host and isolate, and between isolate and environment using the variance partition approach offered by mixed models applied to analyze multi-environmental studies; (ii) to identify or validate the association of *Fusarium* genes previously assigned as candidates using field aggressiveness and deoxynivalenol (DON) production; and (iii) to compare the application and results of the candidate gene association mapping approach applied to the same population of FC isolates but with different phenotypic data obtained from inoculation in different hosts-bread wheat and rye.

Phenotyping was based on multi-environmental field experiments where each plot of the host plant was artificially inoculated with spores of the respective

isolate in accordance with the experimental design. Aggressiveness was visually quantified as the percentage of spikelets with symptoms per plot and was repeatedly evaluated over time. The content of the mycotoxin deoxynivalenol (DON) in the harvested grain was evaluated by double enzyme linked immunosorbent assays (ELISA). Genes previously reported in the literature as related to aggressiveness were selected for sequencing. Using the available *F. graminearum* genome sequence, specific primers were constructed to amplify and sequence the most variable regions of the respective genes.

The partitioning of the phenotypic variance using mixed models, for a subpopulation of 38 FC isolates in four cereal hosts, allowed to disaggregate the magnitude of the genotypic and environmental variance, and the environmental variance in turn into its different components. The genotypic variance was significant, but was exceeded by the magnitude of the environmental variance and its interactions with genotype, showing that the role of plasticity in the pathosystem of *Fusarium* culmorum and its cereal hosts is highly important. In contrast, the variance associated with the host factor and the interactions with host were not significant, confirmed by high values of genetic correlation amogn host. This result supports the categorization of the cereal/*Fusarium culmorum* interaction as unspecific and quantitatively inherited also from the view of the pathogen. For the present study, plasticity was understood as the changes in the phenotype of the pathogen that could be attributed to changes induced by the environment. Our data revealed the year as factor with the highest influence on plasticity, meaning that the isolates with high performance values under humid conditions did not exhibit the same high values under dry conditions. Because the environmental conditions are erratic between the years, the lack of a constant selection pressure in the same direction reduces the probability of achieving a speciation event per environment. The phenotypic data of the DON content in harvested grain showed a high correlation with the aggressiveness data.

An association mapping study with 17 candidate genes for aggressiveness using a population of 100 isolates of FC inoculated on bread wheat revealed the

significant association of the HOG1 gene, explaining 10.29% of the genetic variance of aggressiveness and 6.05% of the genetic variance corresponding to the accumulation of DON in mature grain. HOG1 is a kinase-like protein involved in the communication within the oxidative metabolism of the fungus. In a similar study using the same population of FC isolates and the same candidate genes but rye as host, the gene CUT showed a significant association with aggressiveness, explaining 16.05% of the genetic variance. The CUT gene encodes a cutinase protein, belonging to the secretome and involved in the process of unleashing the membranes and cuticles of the host plant.

Taken together, our results suggest that i) field trials of breeding for resistance to FC in cereals should be carried out in several years to properly account for the genotype-by-year interaction; ii) despite the fact that molecular communication may present some type of host specificity the high plasticity guarantees that the effects on the phenotype are very similar among the cereal hosts; and iii) the high genetic correlation of aggressiveness for different cereals invites to involve non-cereal crops in the rotation plans focused on *Fusarium* disease management.

7 Zusammenfassung

Fusarium ist weltweit eine der verheerendsten Krankheiten von Getreide und verantwortlich für große Ernteverluste, nicht nur aufgrund der verminderten Produktivität, sondern auch wegen der Kontamination des Getreides mit Mykotoxinen. Der weltweit wichtigste Erreger ist *Fusarium graminearum*; in Europa spielen aber auch andere Fusarienarten, darunter *Fusarium culmorum* (FC), eine wichtige Rolle. Die Interaktion zwischen *Fusarium* spp. und Getreide wurde aufgrund früherer phänotypischer und genetischer Beobachtungen als quantitativ eingeordnet.

Wir untersuchten die molekulare und phänotypische Vielfalt der natürlichen FC-Populationen und ihre Interaktion mit verschiedenen Getreidearten (Brotweizen, Durumweizen, Triticale, Roggen) als Wirt. Insbesondere untersuchten wir (i) die Interaktion zwischen Wirtsart und Isolat sowie zwischen Isolat und Umweltfaktoren mit Hilfe von gemischten Modellen; (ii) den Einfluss von Fusarium-Genen, die zuvor als Kandidatengene bekannt waren, auf die Aggressivität und die Produktion von Deoxynivalenol (DON) bei Weizen im Feldversuch an zwei Orten und zwei Jahren und (iii) die Anwendung und Ergebnisse der Kandidatengen-Assoziationskartierung mit Hilfe derselben FC-Population im Vergleich von Brotweizen und Roggen.

Die Phänotypisierung basierte auf Feldexperimenten in mehreren Umwelten mit künstlicher Infektion der Wirtsarten durch Sporen des jeweiligen Isolates. Die Aggressivität wurde visuell als Prozentsatz der symptomtragenden Ährchen je Parzelle mehrfach im Infektionsverlauf quantifiziert. Der Gehalt

des Mykotoxins Deoxynivelanol (DON) im geernteten Getreide wurde mit Hilfe eines, *enzyme-linked immunosorbent assays*' (ELISA) bewertet. Für die Sequenzierung wurden Gene ausgewählt, die zuvor in der Literatur in Zusammenhang mit Aggressivität beschrieben wurden. Unter Verwendung der verfügbaren Genomsequenz von *F. graminearum* wurden spezifische Primer entworfen, um die Regionen der jeweiligen Gene mit der höchsten Variabilität zu amplifizieren und zu sequenzieren.

Die Schätzung der phänotypischen Varianz unter Verwendung gemischter Modelle für eine Subpopulation von 38 FC-Isolaten in vier Getreidearten erlaubte es, die genotypische und umweltbedingte Varianz - und die umweltbedingte Varianz wiederum in ihre verschiedenen Komponenten - aufzuteilen. Die genotypische Varianz war signifikant, wurde jedoch von der Größe der Umweltvarianz und ihren Wechselwirkungen mit dem Genotyp übertroffen, was zeigt, dass die Rolle der Plastizität im Pathosystem von *Fusarium culmorum* und seinen Getreidewirten von großer Bedeutung ist. Im Gegensatz dazu waren die mit der Wirtsart assoziierte Varianz und die Interaktionen mit der Wirtsart nicht signifikant, was durch hohe Werte der genetischen Korrelation zwischen den Wirtsarten bestätigt wurde. Dieses Ergebnis unterstützt die Einordnung der Interaktion zwischen Getreide und *Fusarium culmorum* als unspezifisch und quantitativ vererbt auch von Seiten des Pathogens. Für die vorliegende Studie wurde Plastizität als die Veränderung des Pathogen-Phänotyps verstanden, welche auf umweltbedingte Veränderungen zurückgeführt werden konnte. Unsere Daten zeigten, dass der Faktor Jahr den höchsten Einfluss auf die Plastizität hatte, was bedeutet, dass die Isolate mit hoher Leistung unter feuchten Bedingungen nicht die gleiche hohe Leistung unter trockenen Bedingungen aufwiesen. Da die Umweltbedingungen in den einzelnen Jahren schwanken, macht das Fehlen eines konstanten Selektionsdrucks in eine gleichbleibende Richtung eine spezifische Anpassung an die jeweilige Umwelt unwahrscheinlich. Die phänotypischen Daten des DON-Gehalts im Erntegut wiesen eine hohe Korrelation mit den Aggressivitätsdaten auf.

Eine Assoziationskartierung mit 17 Kandidatengenen für Aggressivität unter

Verwendung einer Population von 100 FC-Isolaten, mit denen Brotweizen inokuliert wurde, zeigte einen signifikanten Einfluss des HOG1-Gens, welches 10,29% der genetischen Varianz für Aggressivität und 6,05% der genetischen Varianz für die Akkumulation von DON in reifem Getreide ausmachte. HOG1 ist ein Kinase-ähnliches Protein, das an der Kommunikation innerhalb des oxidativen Metabolismus des Pilzes beteiligt ist. In einer ähnlichen Studie mit der gleichen Population von FC-Isolaten und den gleichen Kandidatengenen, aber mit Roggen als Wirt, zeigte das Gen CUT eine signifikante Assoziation mit Aggressivität, was 16,05% der genetischen Varianz erklärte. Das CUT-Gen kodiert ein Cutinase-Protein, welches zum Sekretom des Pilzes gehört und am Prozess der Durchdringung der Membranen und der Kutikula der Wirtspflanze beteiligt ist.

Zusammengefasst zeigen unsere Ergebnisse, dass i) Feldversuche zur Züchtung von Resistenz gegen FC bei Getreide in mehreren Jahren durchgeführt werden sollten, um die Genotyp-Jahr-Interaktion angemessen zu berücksichtigen; ii) obwohl die molekulare Kommunikation auch eine gewisse Wirtsspezifität aufweisen kann, stellt die hohe Plastizität von *F. culmorum* jedoch sicher, dass alle geprüften Getreidearten ähnlich betroffen sind; iii) die hohe genetische Korrelation der Aggressivität für verschiedene Getreidearten dazu führen sollte, Nicht-Getreidepflanzen in die Fruchtfolgepläne einzubeziehen, um Fusarien-Erkrankungen besser zu kontrollieren.

8 Resumen

La fusariosis, espiga blanca o golpe blanco es una de las enfermedades más devastadoras de los cereales a nivel mundial y responsable de grandes pérdidas de cosecha, no solo por la reducción de la productividad sino también por la contaminación del grano con micotoxinas. El principal agente causal a nivel mundial es *Fusarium graminearum*, en Europa también juegan un papel importante otras especies de Fusarium, entre ellas *Fusarium culmorum* (FC). La interacción entre las especies de Fusarium y los cereales se ha categorizado como cuantitativa de acuerdo con observaciones fenotípicas y genéticas previas.

Estudiamos la diversidad molecular y fenotípica de poblaciones naturales de FC y cómo interactúan con cuatro cereales (trigo harinero, trigo duro, triticale y centeno) como hospedero. Específicamente, buscamos (i) comprender la interacción entre el hospedero y el aislamiento, y entre el aislamiento y el medio ambiente, utilizando el enfoque de partición de la varianza que ofrecen los modelos mixtos aplicados al análisis de estudios multiambientales; (ii) identificar o validar la asociación de genes de Fusarium previamente reportados en la literatura como candidatos, utilizando como variable respuesta la agresividad de campo y producción de desoxinivalenol (DON); y (iii) comparar la aplicación y resultados de la aproximación de mapeo por asociación en gen candidato, aplicada a la misma población de aislamientos de FC, pero con datos fenotípicos obtenidos a partir de la inoculación en dos diferentes hospederos, trigo harinero y centeno.

La fenotipificación se basó en experimentos de campo multiambientales donde cada parcela de la planta hospedera fue inoculada artificialmente con esporas del respectivo aislamiento de acuerdo con el diseño experimental. La agresividad se cuantificó visualmente como el porcentaje de espiguillas con síntomas por parcela y se evaluó repetidamente a lo largo del tiempo. El contenido de micotoxina deoxinivalenol (DON) en el grano cosechado se evaluó mediante ensayos inmunoabsorbentes ligados a enzimas dobles (ELISA). Se seleccionaron para la secuenciación genes previamente reportados en la literatura como relacionados con la agresividad. Usando la secuencia del genoma de *F. graminearum* disponible, se construyeron cebadores específicos para amplificar y secuenciar las regiones más variables de los genes respectivos.

La partición de la varianza fenotípica mediante modelos mixtos, para una subpoblación de 38 aislamientos de FC en cuatro cereales hospederos, permitió desagregar la magnitud de la varianza genotípica y ambiental, y la varianza ambiental a su vez en sus diferentes componentes. La varianza genotípica fue significativa, pero fue excedida por la magnitud de la varianza ambiental y sus interacciones con el genotipo, lo que demuestra que el papel de la plasticidad en el sistema patogenico de FC y sus cereales hospederos, es muy importante. Por el contrario, la varianza asociada con el factor hospedero y las interacciones con el hospedero no fueron significativas, lo que se confirmó por los altos valores de correlación genética entre los hospederos. Este resultado apoya la categorización de la interacción cereal/FC como "no-específica" y de herencia cuantitativa, aun desde el punto de vista del patógeno. Para el presente estudio, se entendió por plasticidad a los cambios en el fenotipo del patógeno que podrían atribuirse a cambios inducidos por el ambiente. Nuestros datos revelaron el año como factor de mayor influencia en la plasticidad, lo que significa que los aislamientos con altos valores de desempeño en condiciones húmedas no exhibieron los mismos valores altos en condiciones secas. Debido a que las condiciones ambientales son erráticas entre los años, la falta de una presión de selección constante en una única dirección, reduce la probabilidad de lograr un evento de especiación por ambiente. Los datos fenotípicos del contenido de DON en el grano cosechado

mostraron una alta correlación con los datos de agresividad.

Se realizó un estudio de mapeo por asociación con 17 genes candidatos para agresividad utilizando una población de 100 aislamientos de FC inoculados en trigo harinero, el cual reveló la asociación significativa del gen HOG1, explicando el 10,29 % de la varianza genética de agresividad y el 6,05 % de la varianza genética correspondiente a la acumulación de DON en grano maduro. HOG1 es una proteína, tipo quinasa involucrada en la comunicación dentro del metabolismo oxidativo del hongo. En un estudio similar que utilizó la misma población de aislamientos de FC y los mismos genes candidatos, pero centeno como hospedero, el gen *CUT* mostró una asociación significativa con la agresividad, lo que explica el 16,05 % de la varianza genética. El gen *CUT* codifica una proteína cutinasa, perteneciente al secretoma e involucrada en el proceso de destrucción de las membranas y cutículas de la planta huésped. Tomados en conjunto, nuestros resultados sugieren que i) los ensayos de campo de mejoramiento para la resistencia a FC en cereales deben llevarse a cabo en varios años para manejar adecuadamente la interacción genotipo por año; ii) a pesar de que la comunicación molecular puede presentar algún tipo de especificidad por el hospedero, la alta plasticidad garantiza que los efectos sobre el fenotipo sean muy similares entre los hospederos de cereales; y iii) la alta correlación genética de agresividad para diferentes cereales invita a involucrar cultivos no cereales en los planes de rotación enfocados al manejo integrado de Fusarium.

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Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Molecular and phenotypic diversity in populations of Fusarium culmorum on cereal hosts

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Cali - Colombia, 27.02.2021	Valheria Castiblanco Vargas
Place, Date	Signature

Annex 4

Instructions on the importance and criminal legal consequences of the declaration in lieu of an oath

according to Sec. 18(3) sentence 6 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

The University of Hohenheim requires a declaration in lieu of oath on the independence of the scientific work done in order to ensure that the doctoral candidates have done the scientific work independently.

Because the legislators place a particular importance on declarations in lieu of oath and these declarations can have serious consequences, the legislators have placed criminal penalties on false declarations in lieu of oath. If a person willfully (that means knowingly) submits a false declaration, the punishment can be imprisonment for up to three years or a fine.

If a person negligently submits a false declaration (that is, it is submitted even though the person should have realized that the declaration was not correct), then the punishment can be imprisonment for up to one year or a fine.

The criminal provisions can be found in Sec. 156 of the Criminal Code (StGB, false declaration in lieu of oath) and in Sec. 161 StGB (negligent false oath, negligent false declaration in lieu of oath).

Sec. 156 StGB: False Declaration in Lieu of Oath

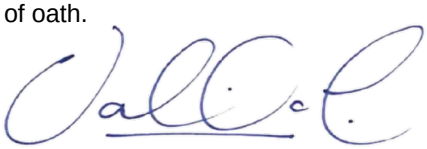
Persons who make a false declaration in lieu of oath to an institution responsible for accepting such declarations or persons who make false statements on such a declaration are subject to imprisonment of up to three years or a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Declaration in Lieu of Oath

161(1): If an action described in Secs. 154 and 156 are done negligently, the punishment is imprisonment of up to one year or a fine.

161(2): There is impunity if the perpetrator corrects the false declaration in a timely manner. The provisions in Sec. 158(2 and 3) apply mutatis mutandis.

I acknowledge the instructions on declarations in lieu of oath.



Cali - Colombia, 27.02.2021	Valheria Castiblanco Vargas
Place, Date	Signature