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Distribution of *Fusarium* species in maize and aspects of their chemical control in wheat

Dissertation

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List of abbreviations

ABC	ATP-binding cassette
AcDON	monoacetyl-deoxynivalenol
ANOVA	analysis of Variance
ATP	adenosine triphosphate
BEA	beauvericin
bp	base pair
cv	cultivar
ca	circa
Ct	cycle threshold
CYP51	cytochrome P450 14 alpha-demethylase
DAcDON	diacetyl-deoxynivalenol
DAS	deacetoxyscirpenol
DMI	sterol 14 α -demethylation inhibitors
DNA	deoxyribonucleic acid
d NTP	2`-deoxynucleoside 5`-triphosphate
DON	deoxynivalenol
dpi	days post inoculation
DSMZ	deutsche Sammlung von Mikroorganismen und Zellkulturen
et al	et alii (multiple authors)
EC50	effective concentration inhibiting growth by 50%
F.	<i>Fusarium</i>
FAO	food and agriculture organization
FB1	fumonisin B1
FB2	fumonisin B2
FB3	fumonisin B3
FHB	<i>Fusarium</i> head blight
FDK	<i>Fusarium</i> -damaged kernels
FUS	fusarenone-X
FUP	Fusarproliferin
G.	<i>Gibberella</i>
GTP	guanosin triphosphate
HT2	HT2-toxin

LC/MS	liquid chromatography mass spectrometry
MIC	minimum inhibitory concentration
MgCYP51	CPY51 of <i>Mycosphaerella graminicola</i>
MON	moniliformin
NEO	neosolaniol
NIV	nivalenol
PCR	polymerase chain reaction
q-PCR	quantitative PCR
PDA	potato dextrose agar
PDB	potato dextrose broth
QoIs	quinone outer binding site inhibitors
RNase	ribonuclease
SBI	sterol–Biosynthesis Inhibitors
SDS	sodium dodecylsulfate
SNA	synthetic nutrient agar
sp.	species
T2	t2-toxin
TAE	tris-Acetate-EDTA
Taq	<i>Thermus aquaticus</i>
Tris	tris (hydroxymethyl)- aminomethane
W	weight
V	volume
ZEA	zearalenol
ZOH	zearalenol

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1. Introduction

Cereals occupy about 50 % of arable land globally. Domestication of crops occurred around 10.500 years before present (bp) for wheat, 9000 years (bp) for maize, and 8.000 years (bp) for rice. However, wheat is the most important cereal crop for mankind in multiple ways, it is globally grown in more than 70 countries and takes first place in terms of tillage area among cereals cultivated in Europe (Dixon, 2007; Kotowicz et al., 2014).

To this day, this plant does not lose its importance and with maize and rice, feed much of the world, providing 44% of total dry matter and 40% of food crop energy (Dixon, 2007). Also Wheat plays an important role in Germany. In 2013, the production of European wheat was 143. 3 metric tons (MT), while, this production of wheat in Germany during the same year reached 25 MT, on the other hand, the world production was 713 MT, making it the third most-produced cereal followed by maize (1.016 MT) and rice for the same year (745 MT) (FAOSTAT, 2014).

Several diseases cause yield loss and reduce grain quality in wheat. They include leaf spots, rusts, bunts, powdery mildew, and *Fusarium* head blight (FHB). Also called ear blight or scab, it is the major fungal disease affecting several gramineous hosts including wheat and barley (Parry et al., 1995; McMullen et al., 2012). The increasing occurrence of FHB in the last decades along with its effect on yield (Wilcoxson et al., 1988; Alvarez et al., 2010), grain quality and grain contamination with mycotoxins (Gilbert et al., 2013; Pereira et al., 2014) make this pathogen one of the most intensively studied fungal plant pathogens (Goswami and Kistler, 2004). The disease occurs throughout much of the world and is associated with several *Fusarium* species.

Fusarium graminearum is the prevalent species in many of the affected regions, and other species, which can also be highly pathogenic, are frequently found in association with the disease (Desjardins, 2006; Leslie and Summerell, 2006). Different *Fusarium* species have been described as producers of toxic secondary metabolites that affect human and animal health. Among these mycotoxins, trichothecenes are the most important (Schollenberger et al., 2007). *F. graminearum* produces several mycotoxins, including nivalenol, deoxynivalenol (DON) and its derivatives, zearalenone (ZEN), fusarin C and aurofusarin (Bennett and Klich, 2003; McCormick, 2003; Trail, 2009). The primary economic and health consequence of FHB

is due to DON contamination despite its relatively low acute toxicity (Paul et al., 2005). DON is a potent protein biosynthesis inhibitor affecting the digestive system and major organ function in humans and animals. When ingested in sufficient doses, it causes nausea, vomiting, and diarrhea. Farm animals fed with contaminated grain show weight loss and food refusal (Bennett and Klich, 2003); for this reason DON is also called vomitoxin or food refusal factor. DON is a virulence factor in wheat, causing tissue necrosis (Proctor et al., 1995; Desjardins et al., 1996). Common management strategies to control FHB include host resistance, fungicides, biological control and cultural practices. However, application of fungicides is still the major control method.

The present work consists of four chapters: chapter I provides a general literature review for FHB in wheat and maize, chapter II presents materials and methods for the following trials:

- 1- Sensitivity of *Fusarium* species isolates to metconazole and epoxiconazole and comparison of CYP51 sequence of *Fusarium* species with CYP51 in *Septoria tritici*.
- 2- *In vitro* sensitivity of some fungal isolates of *Fusarium* species to carbendazim and investigation of the contribution of β -tubulin to its sensitivity.
- 3- Application timing and dose rate of triazoles alone or in mixture with pyraclostrobin for the control of *Fusarium graminearum* in wheat and maize.
- 4- Aggressiveness of *F. graminearum* isolates using several assays.
- 5- Distribution of *Fusarium* species in maize plant.
- 6- Systemic growth of *Fusarium* species in maize plant.

Chapter III presents the results and the last chapter provides the discussion.

The first experiment was aimed to characterize the sensitivity of *F. graminearum* isolates towards metconazole and epoxiconazole. Additionally, to investigate possible mechanisms of reduced sensitivity to demethylation inhibitors fungicides (DMIs) and its correlation with alterations in the target-encoding CYP51 gene. And also to investigate if the mutations of *Fusarium* CYP51 confer resistance by comparing with *S. tritici* CYP51 which resistance towards DMI fungicides was previously reported.

The second experiment was conducted to determine sensitivity to carbendazim in *Fusarium* isolates collected from wheat and maize in Germany as well as to investigate the contribution of *tub2* to the sensitivity of *Fusarium* spp. to carbendazim.

The third experiment was aimed to evaluate the effect of protective and curative application and dose rate on the efficacy of metconazole, single applications of epoxiconazole and pyraclostrobin fungicides or in mixture (pyraclostrobin and epoxiconazole) in reducing *Fusarium* occurrences in maize and wheat. Additionally, to compare the effect of the mixture on *Fusarium* development and DON accumulation in wheat and maize grain.

The fourth experiment was conducted to investigate the relationship between the results obtained by Petri-dish test and two other assays to determine whether the performance of the most aggressive isolate is stable across the tested assays (Petri-dish test, single floret inoculation, DNA quantification) and, in addition, to investigate the pathogenicity of *F. graminearum* isolates. And to evaluate the aggressiveness of *F. graminearum* isolates sampled from different geographic origins. The fifth one was conducted to determine the possibility of seed transmission of *F. graminearum* to the whole plant through certain organs and evaluate the DNA content in these organs. Finally, the last experiment was aimed to assess and compare the appearance and distribution of *Fusarium* species in different organs of forage maize.

2. Chapter I

General literature review

2.1 Wheat

It was found through historical inscriptions that wheat is the earliest field crop used for human food processing. It has been the main food of the major civilizations of Europe, West Asia and North Africa. However, its appearance was in Southwestern Asia, which was first cultivated about 10000 years ago, as a part of “Neolithic Revolution”. And some of the earliest remains of wheat have been discovered in Syria, Jordan and Turkey. Around 600 million tonnes of wheat are global produced each year. Wheat had been helped for the emergence of city-based societies at the beginning of civilization because of being the first crops was easily cultivated on a large scale, as well as, it had the additional advantage of yielding a harvest that had long-term storage of food (Curtis, 2002; Shewry, 2009).

This crop provides plenty of uses, for instance, as a forage crop for livestock as well as the straw can be used as construction material for roofing thatch, the milling of the whole grain give the white flour besides, the by- products such as the bran and germ

Wheat is classified under the genus *Triticum*, family Poaceae, order Poales, class Liliopsida, division Magnoliophyte and Kingdom Plantae (Curtis 2002; Gustafson et al., 2009).

Durum wheat (*Triticum durum* L.) and common wheat (*Triticum aestivum*) are the most important commercial wheat types. According to its growth habits, wheat can be split into spring wheat and winter wheat, these classification depends on many factors such as the seeding and harvesting time, additional, yield and protein content (Curtis, 2002; Bockus et al., 2009; Gustafson et al., 2009).

Wheat is mainly divided into three types based on ploidy level; diploid (14 chromosomes), tetraploid (28 chromosomes) and hexaploid (42 chromosomes) (Gooding and Davies, 1997; Gustafson et al., 2009).

The environmental conditions for wheat growing are wider than other crops such as rice and corn. The most suitable growing temperature is 25 °C, with a range from 3-4 °C to 30-32 °C.

For that reason, wheat has a large range of locations having various environmental conditions (Curtis, 2002). In other words, wheat is mostly grown under dryland environments, however, irrigated wheat is also grown (Baenziger et al., 2006).

Wheat is considered a major source of energy, protein as well dietary fiber in human nutrition. It is also rich in vitamins and minerals for instance, potassium, zinc, manganese, and thiamin. For that reason, wheat has been one of the main food sources for human and livestock.

Several variety of foods are made from wheat flour such as bread, biscuits, cookies, cakes, pasta, noodles, and couscous moreover, using fermentation to produce beer, alcoholic beverages and biofuels (McMullen et al., 1997; Pena, 2002)

2.1.1 *Fusarium* head blight of wheat

FHB, also called scab, can significantly reduce grain yield and quality in many crops including wheat, barley and oats (Parry et al., 1995). The first description of FHB was in England in 1884. It was characterized as wheat scab and considered to be a primary threat to the production of small grains during the early years of last century. In 1890, an FHB outbreak was reported in Indiana. Then, in 1920 Antanasoff used the “*Fusarium* blight”. Currently, both of terms “scab and head blight” are used to describe this disease (Bai and Shaner, 1994; Stack, 2003).

It is known that FHB is a pre-harvest disease, but *Fusarium* species may grow post-harvest if drying for the wet grain is inefficiently and quickly. Yield reduction results from shriveled, small, chalky grains which may be removed from the combine because of their light weight. Infected kernels which are not eliminated from the combine reduce grain weight. In terms of indirect losses, FHB results in reducing seed germination and seedling blight, as well, poor stands (Kotowicz et al., 2014).

Several factors have caused the increasing in FHB epidemics, they include frequent precipitation, the use of susceptible cultivars to FHB, and increasing in the area under corn cultivation which had reduced or no tillage practices, as well as, pathogen factors including adaptation and virulence (Lehoczki et al., 2010). FHB-infected grains may contain a high content of mycotoxins such as DON and zearalenone which pose a serious threat to both of animal and human health (McMullen et al., 1997; Pereira et al., 2014). These mycotoxins have been associated with livestock toxicoses, feed refusal, diarrhea, emesis, and contact dermatitis, in contrast, they cause in human several effects such as, toxicosis, nausea, vomiting, anorexia, and convulsions (Bennett and Klich, 2003). Due to the hazardous effects on eukaryotic cells, the DON and ZEN contents tolerated in marketable cereals were defined by European and national laws, in order to protect consumers from the health hazardous connected with the intake of these mycotoxins (EC, 2006; Pereira et al., 2014). Due to that,

the pathogen population in FHB system has been received a high scientific attention and has been the subject of various recent research papers (Bottalico and Perrone, 2002; Doohan et al., 2003; Gale, 2003; Goswami and Kistler, 2004; Xu et al., 2005)

FHB occurs throughout much of the world and is associated with several *Fusarium* species. At least 17 different *Fusarium* species have been accompanied with the disease in wheat and other small grains (Parry et al., 1995). The environmental requirements and influence their geographical distribution (Table 1). In warmer regions, through parts of the world, *F. graminearum* is the most important species causing FHB. In contrast, in cooler regions, *F. culmorum* is the predominant species such as the U.K., Northern Europe, and Canada and is also associated with DON, ZEN, and/or NIV, meanwhile, *F. avenaceum* has been found over range of climates, but typically exists solely in small proportion of *Fusarium* isolates (Parry et al., 1995; Desjardins, 2006).

In this regard, this species (*F. avenaceum*) was reported to be dominant in the colder areas of northern Europe (Yli-Mattila et al., 2004) and Canada (Turkington et al., 2002), as well as, it can be also present in central Europe (Wiśniewska et al., 2014).

Moreover, *F. poae* has been found in various countries, along with the most frequently isolated FHB pathogens, such as Argentina (González et al., 2008), Canada (Bourdages et al., 2006) and also in some northern European areas (Birzele et al., 2002). In Italy, these two species have also been frequently detected in the last years both in wheat (Covarelli et al., 2012, Infantino et al., 2012) and in barley (Giannini et al., 2013).

In Northwestern Europe the major casual agents of FHB described are *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch.), *F. culmorum* (W. G Smith) Sacc, *F. avenaceum* (Fr.) Sacc. (Teleomorph: *Gibberella avenacea* R. J. Cook), and *F. poae* (Peck) Wollenw. (Parry et al., 1995; Bottalico and Perrone, 2002; Waalwijk et al., 2003; Büttner, 2006).

On the whole, Many reports has been suggested that, *F. graminearum* and *F. culmorum* are thought to be highly pathogenic and the most predominant species and widespread pathogen causing FHB in many countries (Brennan et al., 2003; Stack and McMullen, 1985.). While, the other species mentioned are less aggressive on wheat and barley, which means, their proliferating is small but they still can infect (Imathiu et al., 2014).

Table 1. Environmental requirements affecting some species of *Fusarium*.

Fungal development processes	<i>F. graminearum</i>	<i>F. culmorum</i>	<i>F. poae</i>	<i>F. avenaceum</i>	Source
Reported optimum temperature or moisture (tested range)					
Mycelial growth	25 °C (10–30 °C)	20–25 °C (10–30°C)	20–25 °C (10–30°C)	20 °C (10–30°C)	Brennan et al., 2003
Sporulation (conidia)	32 °C;–0.14 MPa	32 °C;–1.5 MPa		28 °C;–1.5 MPa	Xu, 2003
Conidia	100% 0 to–6.0 MPa	100% 0 to–6.0 MPa		100% 0 to–6.0 MPa	Sung and cook, 1981
Germination	delay when drier	delay when drier		delay when drier	Sung and cook, 1981
Infection	29 °C;> 48 h	26.5 °C; 72 h		29 °C; 72 h 25 °C;	Rossi et al., 2001
Frequency	with time	10–35 °C, 8–72 h		24–48 h	Rossi et al., 2001
Perithecial	20–24 °C;–0.45				Dufault et al., 2006
Ascospores	4h;at–2.0 MPa				Sung and cook, 1981
Germination	24 h at–6.0 MPa (0 to–8.0 MPa)				Sung and cook, 1981
Infection by ascospores	Increases in rate to 35 °C	-	-	-	Lu et al., 2001
Wheat seedling inhibition	25 °C (10–30 °C)	25 °C (10–30 °C)	20 °C (10–30 °C)	25 °C (10–30 °C)	Brennan et al., 2003

MPa: osmotic pressure

2.1.2 Taxonomy

Over 70 fungal species were reported to belong to the genus *Fusarium* occurring in natural conditions in different regions of the world. They typically live saprophytes in the soil, as well as, they can find on plant residues and other organic substrates (Lesile and Summel, 2006;

Nucci and Anaissie, 2007). Species of the genus *Gibberella* belong to the kingdom Fungi, division Ascomycota, class Sordariomycetes, order Hypocreales, family Nectriaceae. The teleomorphic stage was not identified for some *Fusarium* species, for that reason, the anamorphic stage was relocated to the subclade Deuteromycotina, class Hypomycetes, order Tuberculariales as independent species (CABI, 2001).

2.1.3 Symptoms of FHB

FHB symptoms are defined by the appearance of slightly water-soaked, brownish spots present at the base or the middle of the glume, or on the rachis. Other symptoms appear as tan to brown discoloration (blight) of the rachis particular at the base of the spike (Parry et al., 1995; Pirgozliev et al., 2003). Subsequently, this water-soaked appearance and discoloration spread and increase in size until the whole spikelet is covered. Depending on weather conditions, the spots spread further from the point of invasion to the neighbouring spikelets.

When all conditions are very favourable for FHB development, a growth of salmon pink to red coloured owing to the presence of fungal growth and its sporulation . This red coloured can be seen on the base of the spikelet and develops through the entire head (Parry et al., 1995; Pirgozliev et al., 2003)

Eventually, the infected spikelets become necrotic, discoloured, shrunken and chalky in appearance (Fig 1), these kernels are often referred to as *Fusarium* damaged kernels (FDK), scabby kernels or tombstones. Later, black raised spots formed by perithecia may appear, such perithecia are usually associated with *Gibberella zeae*, the sexual stage of *F. graminearum*.

Apparently healthy kernel also may be infected, particular if the infection occurred late in kernel development.



A



B



C

Figure 1. Symptoms of *Fusarium* head blight on wheat. A. Water soaked brown-coloured spots at the base of the glume or on the rachis. B. Partial bleaching of wheat head. C. Sound (right) and *Fusarium*-damaged winter wheat (cultivar Ritmo) kernels.

2.1.4 FHB disease cycle

The *Fusarium* inoculum originates either from the soil or from *Fusarium*-infected crop residues of several plants, such as wheat, corn, barley, soybean and rice on the soil surface which act as the primary sources of inoculum (Shaner, 2003). Macroconidia, chlamydospores and *Fusarium* mycelium serve as a source of inoculum. The shape and size of *Fusarium* spores are spore type and species dependent. Ascospores are described as the major source of inoculum initiating the infection. They are formed in special fruiting structures called perithecia. The name of the sexual stage of the *Fusarium* is *Gibberella*.

The fruiting structures can discharge ascospores coercive into the air, in contrast, macroconidia only depend on inactive transport by the kinetic energy of rain or wind (Shaner,

2003; Bai and Shaner, 2004). Moreover, sowing cereal seed into *Fusarium*-infected soil may cause the infection of plants and subsequently result in the development of both seedling blight and foot root.

Later in the growing season, air-borne inoculum may infect the spikes of plants, causing FHB. However, their discharge and survival firmly depends on environmental conditions (Fig 2, Table 2). Under high relative humidity or rain, infected spikes may produce pinkish mycelia and sporodochia, causing in production of macroconidia, which subsequently infect secondary tillers. While, under warm and windy environmental conditions, the ascospores are picked up by air currents and may travel big distances in the air (Shaner, 2003).

The infection occurs when the ascospores (and also macroconidia) land on a susceptible wheat heads. It is thought that the extruded anthers during flowering stage is the site of primary infection. Subsequently, the fungus germinate and enter the tissues using natural openings in the lemma, glume and palea through the anther and colonize the developing caryopsis, floral bracts and rachis. Consequently, the florets are killed and the kernels will not develop (Trail, 2009; Xu and Nicholson, 2009).

Florets which are infected later will produce diseased kernels that are shrivelled, wilted and tombstone in appearance and DON production starts almost immediately after infection of *F. graminearum*. In contrast, the kernels that are colonized during the late kernel development, may not appear to be affected, but may still contaminated with the mycotoxins (Jansen et al., 2005; Trail, 2009)

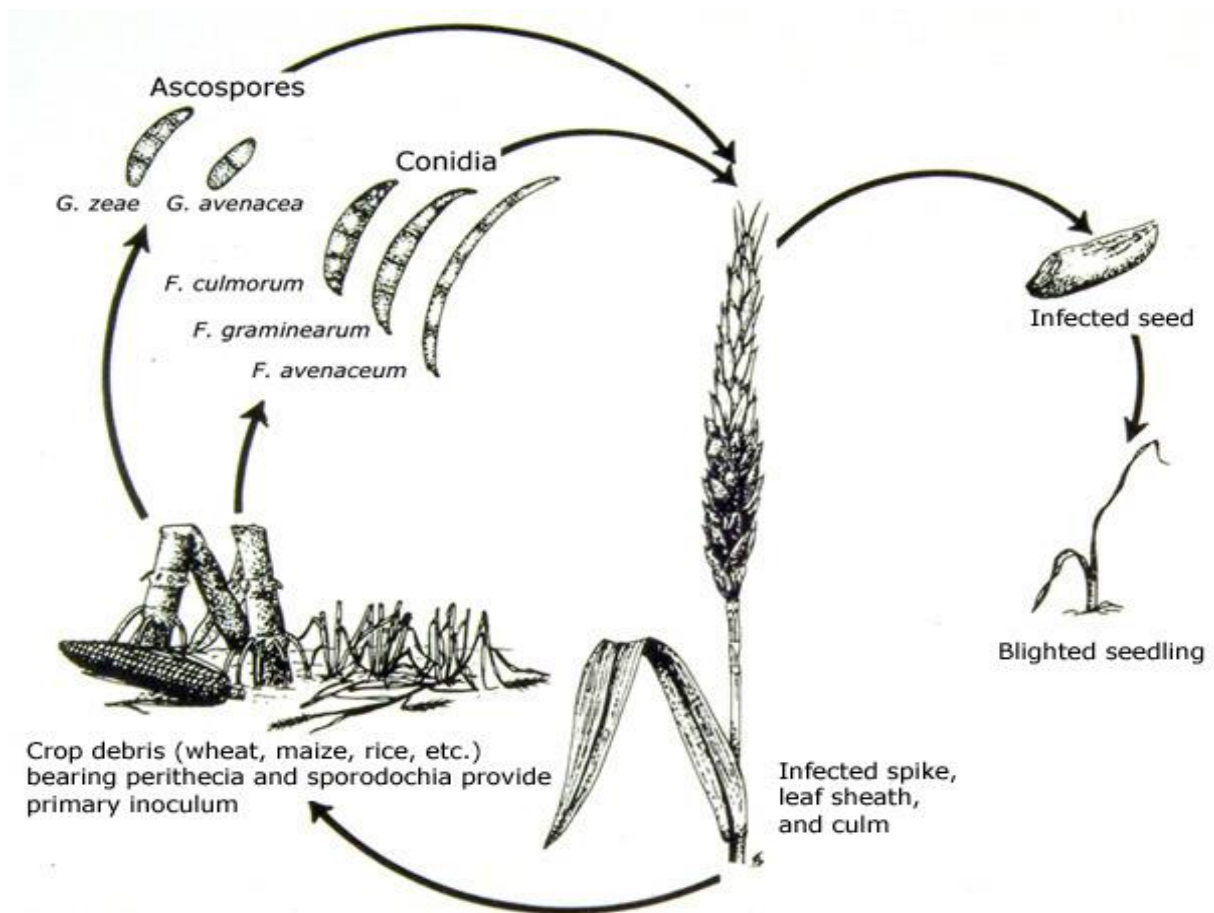


Figure 2. Disease cycle of *Fusarium* head blight pathogens (Schilder and Bergstrom, 2009).

In case of using infected kernels as seed for a subsequent wheat crop, these infected seeds may give rise to blighted seedlings (Guenther and Trail, 2005).

In wheat, the window for FHB infection is brief and occurs from anthesis stage through early grain development. Due to that, FHB infection is restricted to one infection cycle with no secondary infection (Shaner, 2003). Therefore, the level of primary inoculum that is available to infect the host plant will have a significant effect on epidemic development (Sutton, 1982).

Table 2. *Fusarium* species and spore types (Booth, 1971; Joffe, 1986).

Asco: ascospores. Chlamydo: chlamydospores. Macro: macroconidia.

Anamorph	Teleomorph	Macro	Chlamydo	Asco
<i>F. graminearum</i>	<i>G. zeae</i>	spindle shaped, curved 28-78 x 3.2-5.0µm	rare	1-4 septate 13- 28µm
<i>F. culmorum</i>	-	compact, dorsiventral, mostly 5 septate 25-68 x 4.8-8.2 µm	yes	absent
<i>F. avenaceum</i>	<i>G. avenacea</i>	small, thin 3-7 septate 31-92 x 2.5-4.1 µm	no	elliptical 1-3 septate 12-24 µm
<i>F. poae</i>	-	sparse, small, curved 3 septate 17-36 x 4.0-6.6 µm	yes	absent

2.2 *Fusarium* mycotoxins

Mycotoxins contamination in the kernel is considered one of the most important elements of health risk. It is described as the primary problem in food safety which have harmful effects to both animals and humans (Gilbert and Tekauz, 2000; Ngoko et al., 2008).

Some of mycotoxins are produced before the harvest (zearalenone, trichothecenes, fumonisins), but others are produced post the harvest (aflatoxins, ochratoxin A). *Fusarium* species are described as mycotoxin producer before harvest (Champeil et al., 2004b).

Placinta et al. (1999) found that zearalenone, deoxynivalenol and derivatives,

nivalenol, T2-toxin and HT2-toxin are the primary toxins produced by *Fusarium* species

Since toxigenic *Fusarium* species are common phytopathogens, *mycotoxin contamination* of cereal grain is unavoidable. Table 3 demonstrates the key of *Fusarium* spp. which are responsible for head blight in cereals, and the major toxins produced by them (D'Mello et al., 1999).

Table 3. Important species of *Fusarium* infecting cereals, their frequency of occurrence in Europe, and mycotoxins produced (D’Mello et al., 1999).

Species	Frequency	Aggressiveness to head	Major mycotoxins produced
<i>F. culmorum</i>	+++++	high	deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), nivalenol (NIV), fusarenone (FUS), zearalenone (ZEN)
<i>F. graminearum</i>	+++++	high	DON, 3 ADON, 15ADON, NIV, FUS, ZEN
<i>F. sporotrichioides</i>	++	low	T2-toxin, HT2-toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS), FUS, ZEN
<i>F. poae</i>	+++	average	T2-toxin, HT2-toxin, NIV, DAS, FUS
<i>F. verticillioides</i>	++++	average	fumonisin, moniliformin, fusarin C
<i>F. proliferatum</i>	++	average	fumonisin, moniliformin, fusarin C
<i>F. avenaceum</i>	+++++	average	moniliformin, beauvericin
<i>F. cerealis</i>	++	high	NIV, FUS, ZEN
<i>F. tricinctum</i>	++	low	moniliformin
<i>F. oxysporum</i>	++	saprophyte	moniliformin, wortmannin, fusaric acid
<i>F. equiseti</i>	++	saprophyte	fusarochromanone, ZEN, DAS

2.2.1 Trichothecenes

Several *Fusarium* species were reported as producers of toxic secondary metabolites, among these, trichothecenes have been considered the primary common mycotoxins produced by *Fusarium* (Schollenberger et al., 2007). Trichothecenes are tricyclic sesquiterpenoid mycotoxins with considerable differences in their oxygenation levels, however, they have molecular weights ranging from 200-500 Daltons. Their tricyclic ring structure characterized by a double bond at C9 and C10, and an C12, C13-epoxid ring (Fig 3) (Goswami and Kistler; Desjardins, 2006; Foroud and Eudes, 2009; Shank et al., 2011)

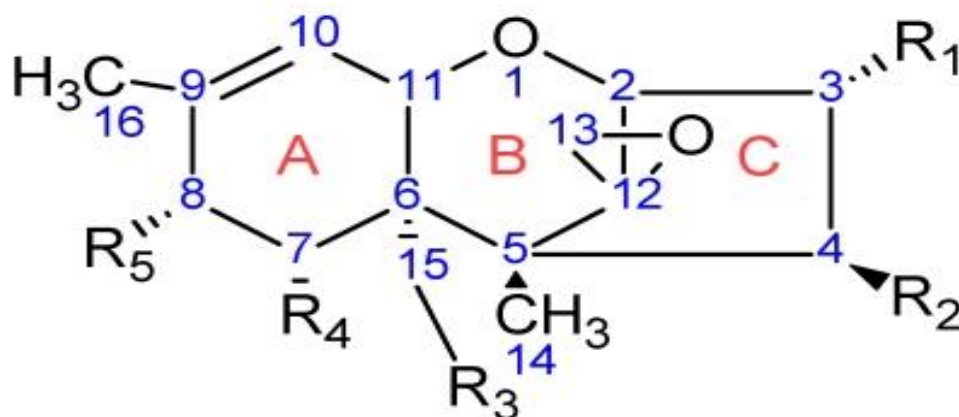


Figure 3. The chemical structure of the trichothecenes (Shank et al., 2011).

Four types of trichothecenes (type A to D) can be distinguished based on the type of substitution (a keto group) to the C8 position (Fig 4). However, Type A and B are more common in cereals than others. Type A trichothecenes which have hydrogen, hydroxyl or an ester group at C8, such as T2-toxin, HT-toxins, diacetoxyscirpenol (DAS) and scirpenol. In contrast, Type B trichothecenes which have a keto group at C8, include deoxynivalenol (DON, vomitoxin) and its derivatives: 3-acetyldeoxynivalenol (3ADON) and nivalenol (NIV) (Bottalico, 1998; Desjardins, 2006).

Irrespective of the structural composition, trichothecenes are generally associated with chronic hazardous toxicosis of mankind and animals. Its mode of action depending on the inhibit of eukaryotic protein synthesis associating with specific activity on ribosomal protein L3 through the 60S subunit causing an inhibition of peptidyl transferase activity and thereby protein biosynthesis (Rocha et al., 2005; Desjardins, 2006).

F. culmorum, *F. graminearum* and *F. sporotrichioides* are determined as the major producers of trichothecenes (Kotowicz et al., 2014). Trichothecenes biosynthesis proceeds from farnesyl phosphate, then followed by a sequence of cyclization, eight oxygenations and four esterifications (Desjardins, 2006).

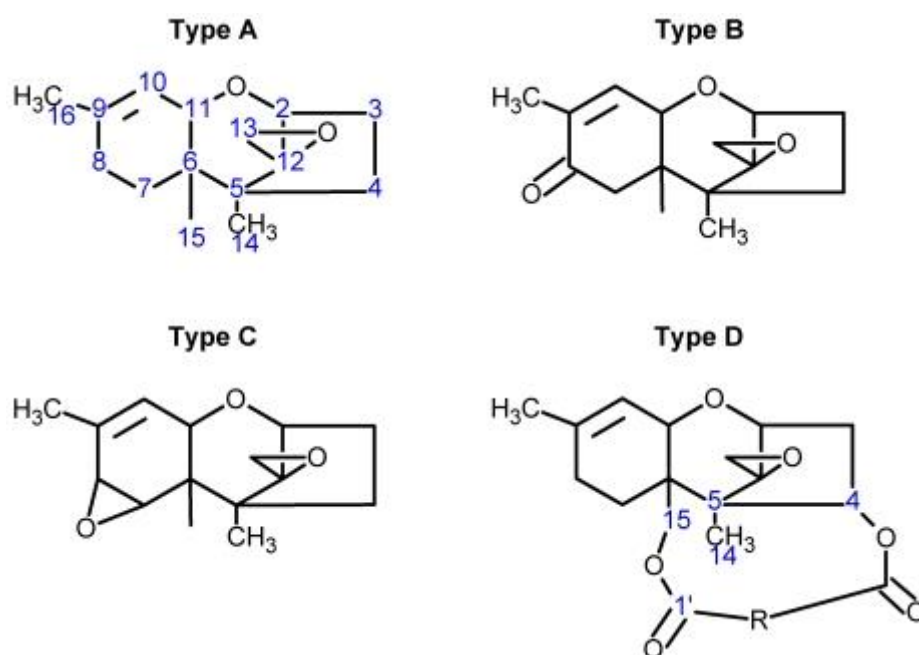


Figure 4. The general structures for Type A, B, C, and D trichothecenes (Shank et al., 2011).

Overall, DON is the most common mycotoxin of trichothecenes in the world. It has a low molecular weight reaches 296 Daltons. Also, its structure has one primary and two secondary hydroxyl groups. In the B-trichothecenes of *Fusarium*, Three strains chemotypes have been identified: 1- nivalenol and acetylated derivatives (NIV), 2- deoxynivalenol and 3-acetyl deoxynivalenol (3-ADON chemotype), and 3- deoxynivalenol and 15-acetyl deoxynivalenol (15-ADON chemotype). The differences between DON and NIV are due to the single change in hydroxyl group at C4. While, the 3-ADON and 15-ADON chemotypes are nature occurrences acetylated derivatives of DON (Mirocha et al., 2003; Desjardins, 2006) (Fig 5).

The main economic and health consequence of FHB is due to DON contamination despite its relatively low acute toxicity (Paul et al., 2005). The biological activity of DON exhibits its by binding to the 60S subunit of eukaryotic ribosomes with subsequent inhibition of the protein biosynthesis (Arunachalam and Doohan, 2013), in view of that, DON is described as a potent protein biosynthesis inhibitor occurring hazardous effects to the digestive system and major organ function in humans and animals. When ingested in enough doses, it causes nausea, vomiting, and diarrhea. Animals fed with DON-contaminated grain show weight loss and food refusal (Bennett and Klich, 2003); owing to that, DON is also called vomitoxin or food refusal factor. DON is a virulence factor in wheat, causing tissue necrosis (Proctor et al., 1995; Desjardins et al., 1996).

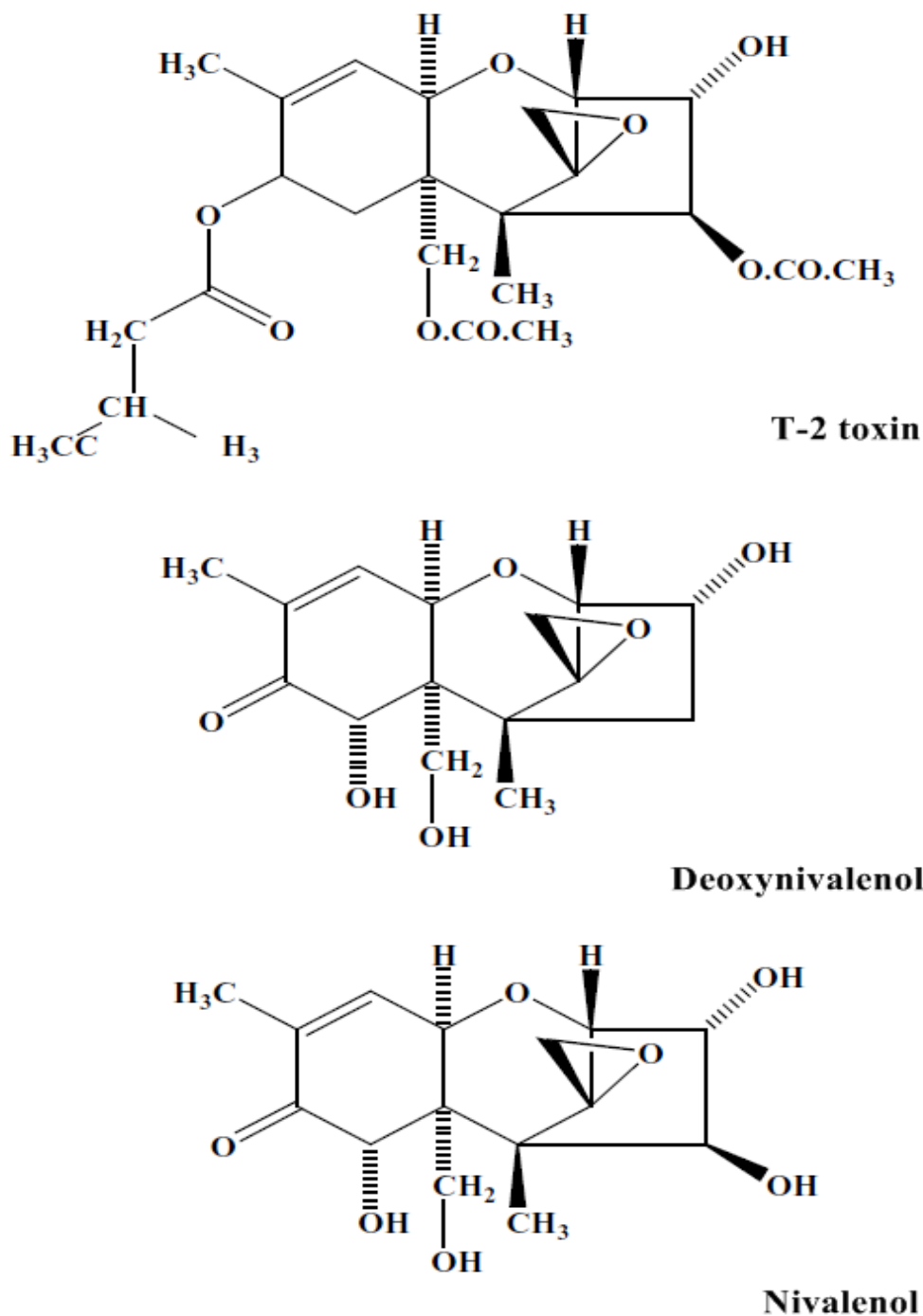


Figure 5. The general structures for T-2 toxin, deoxynivalenol and nivalenol (Shank et al., 2011).

In wheat grains, DON causes slowing down the germination and growth of wheat, as well as, retards the growth of the grain and the coleoptiles tissues (Snijders and Perkowski, 1990). On the other hand, in mature plants, DON seems to revolve in the phloem, with the concentration in plant following a descending gradient from the rachis, to the lemmas and grains to the

peduncle. Furthermore, from the fourth day after infestation, some parts of wheat head such the flower parts, rachis and peduncle include high amounts of DON below the point of infection than above it (Sinha et al., 1997; Savard et al., 2000).

Trichothecenes are considered as the major element of *F. graminearum* pathogenicity in most wheat cultures. In case of insufficient trichothecenes synthesis by *F. graminearum* resulted in susceptible plants being capable of retarding and even stopping *Fusarium* spread (Eudes et al., 2001).

Trichothecenes contamination result in worsening of milling and malting quality, in addition to, harmful effects on all organisms, hence, the consumption of contaminated food or forage with these substances will lead to hazardous diseases for both animal and human. It was reported that the absorption of trichothecenes result in blood and digestive disorders in mammals. As well, emesis is observed after the ingestion of at least 10 mg DON/ kg (Edwards, 2002; Champeil et al., 2004a).

On the whole, the toxicity level of trichothecenes are found to be various in different animals or even in plant cells, which indicated that, there are several consequences for chemotypes of FHB pathogen depending on their level of aggressiveness and fitness (Kimura et al., 1998; Goswami and Kistler, 2004). The effect of several trichothecenes in livestock is shown in Table 4.

On other hand, the absorption of trichothecenes in humans cause harmful effects, for instance burning sensations in the mouth and stomach, headaches, a decreasing in red blood cell count, necrosis of throat and stomach. It could also lead to death in some cases of trichothecenes poisoning (Champeil et al., 2004a).

Table 4. The effect of several trichothecenes in livestock.

Animal species/type	Trichothecene	Effects
Pigs	DON	emesis; reduced feed intake; decreased growth; stomach lesions; reduced blood levels of total protein, albumin, Ca and P; temporary decrease in serum protein levels; renal lesions
	NIV	profound reduction in feed intake; increased time to consume feed
	T2-toxin	dermatitis of nose, snout and buccal commissures; reduced growth; decreased blood glucose levels; increased blood levels of inorganic P and Mg; decreased red blood cell amount; reduced corpuscular volume and haemoglobin concentration; reduced leukocyte count; decreased antibody formation
Broiler chickens	DON	increased weight of heart, gizzard and bursa of Fabricius
	NIV	gizzard erosions; reduced weight of liver
	T2-toxin	decreased bodyweight gain; oral lesions
	DAS	decreased bodyweight gain; mouth lesions
Turkey poults	T2-toxin	reduced weight gain; oral lesions
	DAS	reduced feed intake and weight gain; oral lesions
Geese	T2-toxin	decreased egg yield and hatchability; inhibition of follicle maturation in ovaries; follicle degeneration; lesions in adrenal and thyroid glands
Cattle	DAS	anorexia; gastro-intestinal lesions; diarrhoea; reduced milk production

2.2.2 Zearalenone

Zearalenone (ZEN) is considered the most prevalent in temperate regions of America, Europe and Asia (Yazar and Omurtag, 2008; Mankeviciene et al., 2011). This toxin is commonly

isolated from maize; but it can also contaminate other cereals, such as, wheat, barley, oat and sorghum. It is resulted in a head rot and stalks rot in maize and associated with scab in wheat, furthermore it is a natural contaminant of hay (Coulombe, 1993; Krska et al., 2003).

These toxins are non-steroidal estrogenic mycotoxins that belongs to a very large family of fungal metabolites derived by cyclisations and modifications of non-ketide precursors (Fig. 6). ZEN has a chemical structure similar to antibiotic metabolites produced by many species of fungi (Desjardins and Proctor 2007).

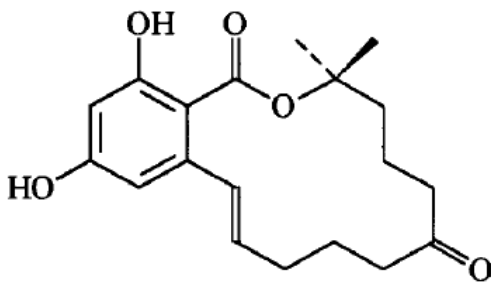


Figure 6. The chemical structure of zearalenone:

Zearalenone has a relatively low toxicity, which their LD50 value reach 2-10 g/kg body weight as determined with mice (Flannigan, 1991).

Fusarium fungi can produce both of trans (X-zearalenol) and cis (Y-zearalenol) forms of ZEN on grain. These metabolites can bind to estrogen receptors to varying extents, generating hyperestrogenism resulting in several problems in animals, particular in pigs. Owing to their susceptibility to this compound. Thus their estrogen receptors have a large affinity for X-zearalenol, which has from three to four times more estrogenic than ZEN, however, their toxic effects are not associated with any fatal mycotoxicoses in humans or even in animals (D'Mello et al., 1999; Moss, 2002). However, ZEN is also a major concern, despite its relatively low acute toxicity and carcinogenicity levels, it can lead to hyperestrogenism and reproductive problems in animals. For that reason, even small levels of these toxins in raw grain can cause them harmful effects to human or animal health (Desjardins, 2006).

It was reported that many *Fusarium* spp. Such as *F. graminearum*, *F. culmorum*, *F. equiseti*, *F. pseudograminearum*, *F. semitectum* and *F. crookwellense* are able to form zearalenones. In the contrast, the other species *F. oxysporum* and *F. solani* are not able to synthesize these mycotoxins (Desjardins, 2006).

On other hand, it was reported that zearalenone could be have a positive effect on plant growth which act as regulator of the flowering process.

It was concluded that application of ZEN in the culture of winter wheat. Addition to exhibits ZEN similar activity to plant hormones during some physiological processes. In the light of its hormonal role, the use of such compound in animal production may lead to negative effects because of being carcinogenic or genotoxic substances (Biesaga-Koscielniak and Filek, 2010; Kotowicz et al., 2014). In the European community, this implementation is forbidden (Moss, 2002). And the maximum accepted content of ZEN is 0.25 ppm in diets (D`Mello et al., 1999; Desjardins, 2006).

2.2.3 Fumonisin

The structure of the fumonisin is relatively simply compared to the other mycotoxins (Desjardins, 2006). Its chemical structure is long-chain amino polyalcohols consisting of a 20-carbon aliphatic chain with two ester linked hydrophilic flank chains. There is similarity in the structure of both fumonisins and sphingosine, a substantial phospholipid within cell. The toxicity of fumonisin is due to the competition with sphingosine in sphingolipids metabolism (Kotowicz et al., 2014). Up to date, around twenty- eight fumonisins have been found and classified into four groups known as A, B, C and P. the common natural contamination of cereals are FB1, FB2 and FB3. (Desjardins and Proctor 2007; Yazar and Omurtag, 2008).

Maize is considered to be the most frequently food source contaminated by these mycotoxins, but it was also reported the presence of fumonisins in wheat grain (De Girolamo et al., 2011; Scott 2012). Fumonisin are produced by various species of *Fusarium* including *F. verticillioides*, *F. proliferatum*, *F. anthropilum*, as well as *F. nygamai*. However, fumonisin B1 (Fig. 7) is the major toxic representative of these mycotoxins which is primarily produced by *F. proliferatum* and *F. verticillioides*. Additional, B1 is able to induce toxicity on contact in plant tissues. (Edwards et al., 2002; Yazar and Omurtag, 2008).

Fumonisin cause several diseases in animals, such as, equine leukoencephalomalacia, porcine pulmonary edema, hydrothorax in pigs, immunosuppression in poultry, and, hepatocarcinoma in experimental rodents. In this regard, Absorption of fumonisins leads to

form cancerous tumors in laboratory rodents and there is an epidemiological relation between the consumption of contaminated grain and human oesophageal cancer in some areas of the world (Kellerman et al., 1990; Rheeder et al, 1992, Bucci and Howard, 1996; Proctor et al., 2002; Desjardins, 2006)

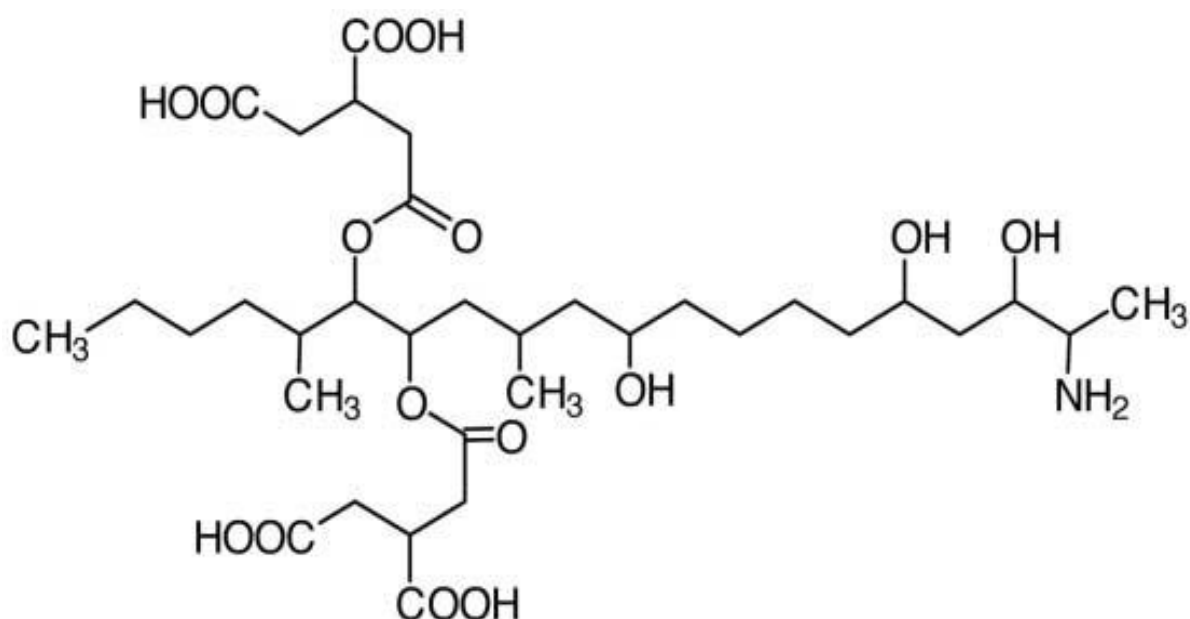


Figure 7. The chemical structure of fumonisin B1 (Kotowicz et al., 2014).

In respect of B1, it was reported that cereals infected with B1 can lead to human esophageal cancer and many cases were occurred in Africa, Northern Italy and Iran (Yazar and Omurtag, 2008).

2.3 Mycotoxin content limits

Due to the hazardous effects of mycotoxins on eukaryotic, the national and European laws have been limited the permissible contamination by selected mycotoxins in marketable cereals, in order to protect from health risks associated with the consumption of contaminated grain. These levels (Table 5) were regulated by Commission Regulation (EC) No. 466/2001 of 8th March 2001 as amended (Commission Regulation (EC) No. 466/2001 of 8th March 2001; Commission Regulation (EC) No. 856/2005 of 6th June 2005; Commission Regulation (EC) No. 1126/2007 of 28th September 2007 (EC, 2001; EC, 2005; EC, 2007).

2.4 Masked mycotoxins

This definition (masked mycotoxins) was firstly used by Gareis et al. (1990) to characterize a zearalenone glucoside: Since zearalenone glycoside is not detectable during conventional analysis, but hydrolysed during digestion, it appears probably that such masked mycotoxins are responsible in cases of mycotoxicoses (Kotowicz et al., 2014).

This term was recently used to refer to glycosylated derivatives of deoxynivalenol and zearalenone, which typically are undetectable and escape routine analytical detection designed to DON and ZEN, because of their diverse chemical behaviours and changed polarities and masses in comparison to the parental compounds (Kotowicz et al., 2014). Berthiller et al. (2013) suggested using the term “masked mycotoxins” only for plant metabolites of mycotoxins in order to avoid confusion.

Masked mycotoxins form by conjugation with glucose (glycosylation process) in wheat tissues in the second phase of plant metabolism, which occur in conjugated forms, typically formed by reaction of the parent compounds with sugars, amino acids or by covalent binding or non-covalent associations with macromolecules, for instance starch and proteins, in the food matrix (Cirlini et al., 2012). When the conjugation with glucose occur, DON can convert into DON-3 glucoside (DON-3G) and ZEN into ZEN-14 glucoside (ZEN-14G).

However, for other mycotoxins such as T-2 toxin many derivatives remain unknown, in addition, the analytical references standards are not available, thus, it causes difficulty to include masked conjugated mycotoxins in conventional analytical methods (De Boevre et al., 2012).

DON-3G was found in different cereal crops (Lancova et al., 2008), also in animal feed and foodstuff (Malachova et al., 2011; De Beoevre et al., 2012).

Table 5. The maximum allowed level of mycotoxin content. (EC, 2007).

Mycotoxin	Product	Maximum allowed level ($\mu\text{g}/\text{kg}$)
Deoxynivalenol	unprocessed cereals.	1250
	unprocessed durum wheat, oat.	1750
	unprocessed corn	1750
	intended for direct human consumption, cereal flour, bran.	750
	pasta	750
	bread (include bakery products), confectionery products, biscuits, cereals	500
	processed cereal-based food, food for infants and young children, baby products.	200
Zearalenone	unprocessed cereals	100
	unprocessed corn	350
	cereals intended for direct human consumption, cereal flour, bran	75
	refined corn oil	400
	bread, confectionery, biscuits, cereals	50
	processed cereal-based food, food for infants and young children, baby products.	20
	corn intended for direct human consumption, corn snacks, corn flakes.	100
	processed corn-based food for infants and young children.	20
Fumonisin	unprocessed corn.	4000
	corn intended for direct human consumption	1000
	corn snacks, corn flakes	800
	processed corn-based food for infants and young children.	200

2.5 Chemical control of FHB

2.5.1 DMI fungicides

Common management strategies to control FHB include host resistance, fungicides, biological control and cultural practices. However, application of fungicides is still the major control method; nevertheless it does not guarantee getting rid of the pathogen (Horsley et al.,

2006; Baturó-Ciesniewska et al., 2011). Fungicides containing triazoles as active ingredient (s) are currently the most effective plant protection agents against FHB pathogens (Edwards et al., 2001; Mesterházy, 2003; Paul et al., 2010). They may be applied preventively or as early-infection treatments. In contrast to other single site fungicides, resistance levels are usually low and the cross-resistance between their members are incomplete, despite of their long-term usage (Cools et al., 2013).

Results on the efficacy of fungicides and the effects on FHB and mycotoxin contamination are often conflicting. Mesterházy et al. (2003) reported inadequate or inconsistent control of FHB, rendering fungicides inefficient as a standalone control strategy. In some studies, triazole fungicides such as metconazole, prothioconazole and tebuconazole were found to be effective, resulting in reductions of FHB severity and mycotoxin contamination by 50- 80% and 5-90%, respectively (Matthies and Buchenauer, 2000). On the contrary, in another study, application of fungicides resulted in an increased trichothecene accumulation (Simpson et al., 2001).

In other studies, tebuconazole and metconazole showed high efficiency against *F. culmorum* and other fungal pathogens in wheat plants (Simpson et al., 2001; Ioos et al., 2005). In the same regard, the application of metconazole effectively reduced FHB and DON levels in grain, which indicates that this compound is very effective in controlling *F. culmorum* and *F. graminearum*. (Pirgozliev et al., 2002). Another triazole fungicide, prothioconazole, was effectively able to reduce the mycelial growth of some *Fusarium* species (*F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichioides*, *F. tricinctum*) (Kotowicz et al., 2014).

The variability in fungicide efficacy was also noted by Ransom and McMullen. (2008), who found that tebuconazole was not able to reduce FHB values to acceptable levels when the FHB severity was high. On the other hand, this fungicide more effectively reduced FHB than DON levels (Paul et al., 2007). In another experiment, this fungicide significantly reduced FHB, FDK, and DON in hard red wheat cultivars in USA (Jones, 2000).

The small effect of epoxiconazole was noted by Menniti et al. (2003), who reported that a single application of epoxiconazole reduced the incidence and severity of FHB when infection pressure was low to medium while it was ineffective in reducing DON content. An increase in

mycotoxin concentration after epoxiconazole treatment was also noted by other authors (Milus and Parsons, 1994; Klix et al., 2007). On the other hand, the application of epoxiconazole alone was effective and provided an excellent activity in reducing FHB (Chen et al., 2012).

The best time of DMI application is the period after the emergence of the head, consequently, the active ingredient moves in leaf tissues after being taken up. Owing to its systemic feature, it is not absolutely moving from leaves to head from the point of contact. The residual period of most DMI fungicides is approximately 14 days. The timing and rate of application are the two major critical points in controlling FHB. Mesterházy. (2003) suggested that the early application may protect only the leaves but not the heads. While this effect could be more hazardous for non-toxicogenic microorganisms when fungicides were sprayed several weeks before the flowering stage, it can boost subsequent spread of *Fusarium* species (Gilbert and Habert, 2013). On the other hand, some studies suggested that comparable control of FHB levels can occur when fungicides are sprayed just before or at the beginning of the anthesis stage and in some cases, 5 day into anthesis (McMullen et al., 2012).

It was concluded that the application of fungicides from both sides of the plots has the best effects in reducing FHB levels than the partial coverage of plants. However, the highest concentration of fungicides was found in the glumes and stepwise declines during the movement to lemma and embryo (Mesterházy, 2003).

On the whole, the variable effects of fungicides are due to, for instance, cultivar-resistance, climate, fungicide coverage, diverse epidemiology, the level of inoculum, besides the aggressiveness of the individual pathogens (Homdorket et al., 2000). Fungicides targeting FHB are typically applied at the mid-flowering stage because extruded anthers are the major infection factors, even though the window of vulnerability for infection can continue from flowering up to grain filling stages, depending on the variety (Del Ponte et al., 2007; Horeváj et al., 2011). Studies where fungicides were sprayed between the growth stages GS 32 and 50 did not provide any significant reduction of FHB; conversely, fungicides revealed significant reduction in both the severity of FHB and mycotoxin concentration when applied between the growth stages GS 59 and 70 (Pirgozliev et al., 2003). The effect of fungicides on FHB and DON levels may be due to either change in the proportion of trichothecene –producing *Fusarium* spp. or to change in the quantity of DON synthesis (Edwards et al., 2001).

2.5.1.1 The function and structure of CYP51

The sterol biosynthesis inhibitors (SBIs), and, among them, DMI (sterol 14 α -demethylation inhibitors), were first used agriculturally in 1970s. Their mode of action is the inhibition of CYP51 (sterol 14 α - demethylase), an enzyme that is located on the outer membrane of the endoplasmic reticulum. This enzyme is fundamental for ergosterol biosynthesis, consequently for cell survival (Klix et al., 2007). DMI fungicides interfere with this enzyme causing interruption in the conversion of lanosterol to ergosterol, an element of the fungal cell membrane. Thus, this effect results primarily from membrane dysfunction (Becher and Wirsal, 2012).

CYP51 belongs to the cytochrome P450 (CYP) monooxygenase superfamily. CYP proteins typically catalyse monooxygenations and are presently adjusted to 10 classes and 267 families. However, CYP51 is the oldest family; up to date, more than 100 CYP51 genes encoding sterol 14 α -demethylase have been sequenced from different organisms (Liu et al., 2011; Becher and Wirsal, 2012).

CYP51 is found in all kingdoms of life, it can be found in 46 out of 47 fungal species examined, including species from the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota, and multiple *CYP51* genes have also been identified in plants such as rice, oats and *Arabidopsis thaliana* (Becher and Wirsal, 2012).

Generally, most fungi have one CYP51 gene, such as *Candida albicans* and *Cryptococcus neoformans* but filamentous fungi, especially ascomycetes, have two or more CYP51 paralogues, for example *Penicillium digitatum* (two), *Aspergillus nidulans* (two), *Aspergillus flavus* (three), *Magnaporthe oryzae* (two), *Aspergillus fumigatus* possesses two distinct but related CYP51 proteins encoded by CYP 51A and CYP51B; CYP 51A correlated with the sensitivity to DMI fungicides, whereas CYP51B was responsible for the growth rate and shape maintenance in *A. fumigatus*.

Some species of *Fusarium* possess three CYP51 paralogues, including, *F. verticillioides*, *F. oxysporum* f. sp. *lycopersici* and *F. graminearum* (Fan et al., 2013). The reason for the existence of paralogous CYP51 genes in a single species remain unknown; however, it was interpreted that such paralogous CYP51 might have occurred during evolution in the process

of duplication of an ancestral gene followed by functional and structural specialization (Liu et al., 2011).

In the case of *F. graminearum*, the existence of three paralogous CYP 51 genes (CYP51A, CYP51B, and CYP51C) was revealed. Up to date their functions have not been investigated (Liu et al., 2011; Becher and Wirsfel, 2012). CYP51 sequences retained 22–30 % identical residues but in fungi, identities are about 42%; on the other hand, phylogenetic studies have shown that the amino acid sequence of CYP51A for *F. graminearum* and *Magnaporthe grisea* are 64.8% identical, while FgCYP51B is 77.7% identical to NcCYP51 from *Neurospora crassa* and FgCYP51C reveals 80.9% identity to FsCYP51 of *Fusarium solani* (Lepesheva and Waterman, 2011; Lui et al., 2011).

In comparison to most other CYP families, the function of CYP51 proteins have preserved a high specificity such as, the removal of the methyl group of sterol precursors, and they have maintained this high specificity throughout evolution. There are only five compounds structurally identical to 14 α -methylsterol substrates: lanosterol, 24, 25-dihydrolanosterol, 24-methylenedihydrolanosterol, obtusifoliol, and 4 β -desmethyl lanosterol (Lepesheva and Waterman, 2007).

Fungal CYP51 plays role in the postsqualene part of the fungal pathway and catalyzes the removal of the methyl group at the 14 α -position of sterol precursors, a key step in the biosynthesis of ergosterol, a sterol specifically located in fungal membranes that mediates the process of the permeability and fluidity in the fungal cell membrane (Daum et al., 1998). This reaction (Fig 8) contains three steps starting by successive conversion of a methyl group to hydroxymethyl, subsequently, into carboxyaldehyde intermediates, finally followed by the elimination of formic acid and concomitant formation of the 14,15 double bond in the resulting sterol product, 4,4-dimethyl-5 α -cholesta-8,14,24-triene-3 β -ol (Morrison et al., 2013).

Thus, deleterious 14 α -methylated sterols may accumulate as a result of an inhibition of CYP51 (Kelly et al., 1995), owing to that the rigidity of fungal membranes decreases (Abe et al., 2009). However, it has been reported that mutants in FgCYP51A, FgCYP51B, and FgCYP51C in *F. graminearum* did not impact vegetative growth in vitro (Liu et al., 2011).

All available structures of the cytochrome P450 show that the overall P450 structural fold is conserved during evolution from bacteria through mammals.

Before the availability of the three-dimensional structure of CYP51, the determined crystal structures of bacterial cytochrome P450 enzymes were utilized to deduce homology models of fungal CYP51 proteins (Boscott and Grant, 1994; Becher and Wirsal, 2012).

Thereafter, the identification of a bacterial CYP51 ortholog in *Mycobacterium tuberculosis* (MtCYP51), which reveals less than 30 % identity to fungal enzymes but is nevertheless able to oxidize the fungal CYP51 substrate lanosterol, lead to an improvement of these models (Aoyama et al., 1998; Bellamine et al., 1999). Also the structure of MtCYP51 lacks some information for consistent homology modelling of eukaryotic CYP51s, whereas its length reaches 451 amino acids (aa), while in fungi it ranges between 515 and 550 aa (Fraczek et al., 2011).

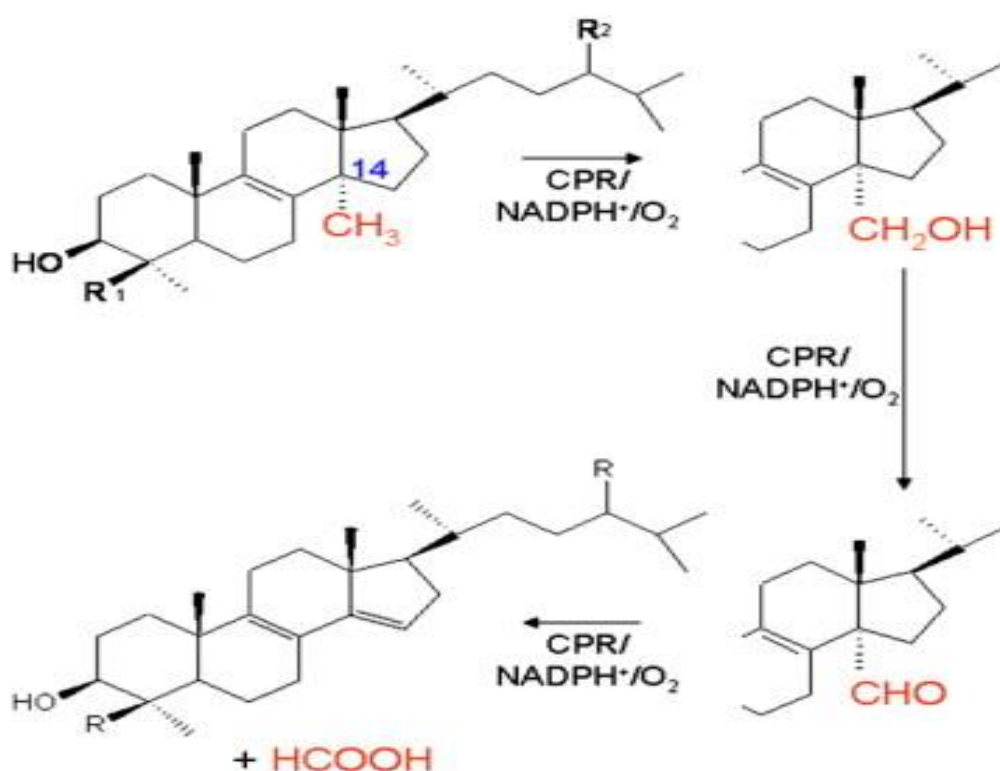


Figure 8. The ubiquitous sterol 14 α -demethylase (CYP51) biochemical reaction (Morrison et al., 2013).

The ligand access to MtCYP51 apparently occurs within an opening defined by the bent I helix and the open B/C loop (Fig. 9); in contrast, CYP51s eukaryotic is occurred by α -helices A', F'' and the β 4-1/ β 4-2 loop. The longest helix (I helix) in the molecule, is lacerated, whereas the N-terminal part moves away from the structural core shaping a 145° angle with the C-

terminal part. The Bowing appears at positions 253–255 in the 4-PI structure, then moves to positions 256–258 after FLU binding.

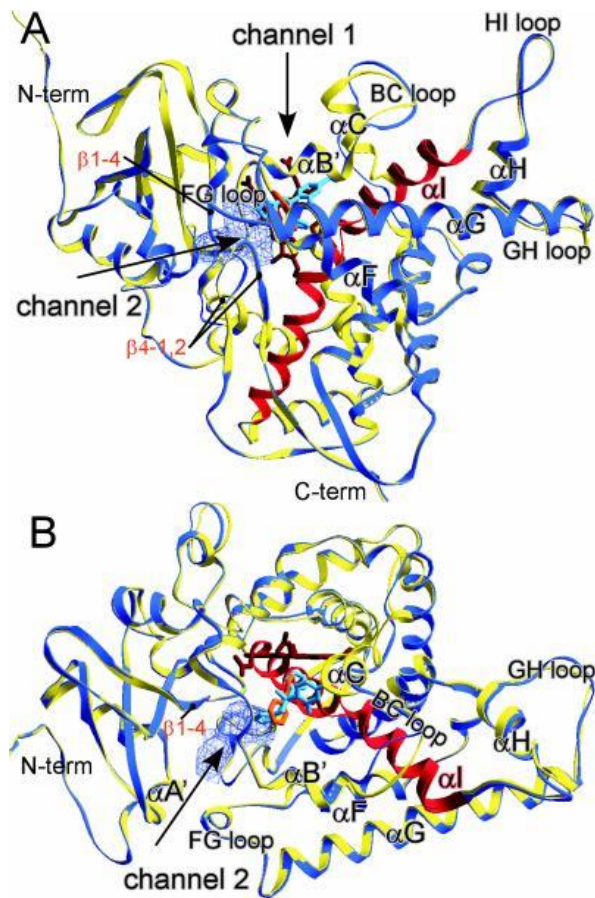


Figure 9. Model of CYP51 proteins. a MtCYP51 from *M. tuberculosis*, Ribbon representation of the MTCYP51 structures with the inhibitors bound. Front (A) and top (B) views of the of 4-PI- (yellow) and FLU- (blue) bound MTCYP51 superimposed with an rms deviation of 0.45 Å. . The second view results in better fitting of the most homologous regions and further divergence of less homologous regions. Heme, red; 4-PI, orange; FLU, light-blue. The I helix which takes part in the open conformation of channel 1, is marked in green. A big cavity of 2,600 Å³, shown in blue, occurs from the active site to the molecular surface with the protein domain interface (channel 2). Structural components largely deviating among P450 structures are marked in black, and β-sheets that are component of the putative substrate-binding site are shown in red. (Podust et al., 2001).

From the middle fraction of the I helix, the different structures begin to deviate largely from each other is well preserved in P450s, including residues A256 and G257, due to the huge repositioning of the I helix, adjacent regions, namely the H and G helices and loops in between, As a consequence, various significant topological variations exhibit among

P450s. Thus, this helix is considered to be the most dominant N-terminal displacement, which leads in bowing of the I helix away from the heme that releases BC.

The channel 2 of prokaryotic MtCYP51 is located at the same position to that of eukaryotic channel, but with difference that substrates and ligands enter there. In eukaryotic CYP51s, the entry channel and the heme pocket are filled by residues of the α -helix B', B'/C loop, helix C, helix I, K / β 1-4 loop and β -strand 1-4. Likewise to the results obtained for MtCYP51. These differences between CYP51 in both the prokaryotic and eukaryotic CYP51s reveal the limitations of the models for fungal proteins that were only based on MtCYP51.

2.5.1.2 CYP51-azole resistance in plant pathogens

CYP51 sterol 14 α -demethylation is a fundamental element of the sterol biosynthetic pathway in eukaryotes. It was firstly purified from *Saccharomyces cerevisiae* in 1984 (Liu et al., 2011). It is located on the outer membrane of the endoplasmic reticulum and catalyses a key step in the biosynthesis of ergosterol, a sterol specifically occurring in fungal membranes, that mediates the process of the permeability and fluidity in the fungal cell (Daum et al., 1998). DMI fungicides inhibit the biosynthesis of ergosterol in fungi due to sterol 14 α -demethylase (CYP51) binding; consequently, deleterious 14 α -methylated sterols may accumulate as a result of an inhibition of CYP51 (Kelly et al., 1995) and, owing to that, the rigidity of fungal membranes decreases (Abe et al., 2009). Because of their importance as the molecular targets of DMI fungicides, CYP51 proteins received much scientific attention (Becher and Wirsal, 2012).

The extensive usage of DMI fungicides in agriculture for decades resulted in the emergence of strains which decreased susceptibilities or even resistances in the fungal populations, including *Blumeriella jaappi* (Ma et al., 2006), *Erysiphe graminis* (Delye et al., 1998) *Monilinia fructicola* (Ma and Michailides, 2004; Lou and Schnabel, 2007), *Podosphaera xanthii* (McGrath and Shishkoff, 2001), *Penicillium digitatum* (Hamamoto et al., 2000) *Venturia inaequalis* (Schnabel and Jones, 2001), *Cercospora beticola* (Becher and Wirsal, 2012) and *Mycosphaerella graminicola*, (Leroux et al., 2007).

Molecular mechanisms causing DMI resistance have been investigated in various important fungal plant pathogens. It was reported that mutations in CYP51 genes are often causing factor

for such effects; in addition, some changes for instance substitutions, insertions and duplications in promoters of CYP51 (Hamamoto et al., 2000) or also gain-of-function mutations in genes encoding CYP51 (Dunkel et al., 2008) frequently cause overexpression of these genes, as well as altering in azole susceptibility, and overexpression of plasma membrane-bound efflux transporters can lead to azole resistance. These mechanisms can combine and the levels of the resistance are frequently defined by combinations of CYP51 alterations, CYP51 gene overexpression and /or increased efflux pump (Cools et al., 2013).

In azole- resistant strains of *Cercospora beticola*, causing sugar beet leaf spot disease, three CYP51 substitutions in resistant strains were found. However, there is no obvious correlation between amino acid changes and resistance; on the contrary, it was suggested that overexpression of CYP51 is more likely correlated with resistance. In resistant strains of *Puccinia triticina*, causing wheat leaf rust, the Y134F substitution in CYP51 is found 4.5 % of European fields but no sensitivity levels were identified towards DMI fungicides indicating that the other mechanisms might play a role in the resistance (Becher and Wirsal, 2012).

For *Mycosphaerella graminicola* (Fuckel) Schröter in Cohn (anamorph *Septoria tritici*), it was suggested that the declining sensitivity for azoles increased, and they are correlated with gradual accumulation of several CYP51 amino acid substitutions (Leroux et al., 2007; Brunner et al., 2008; Cools et al. 2011). Brunner et al. (2008) concluded that the wild-type alleles were the most wide spread until the early 1990s, but presently, they are rare in various European regions. The application intensity and the particular azoles affect the diverse subsequent mutations, and consequently, resistant levels. Some of the MgCYP51 alterations in *M. graminicola* populations developing resistance to azoles are similar to CYP51 alterations in highly resistant strains of the human pathogen *Candida albicans* (Cools et al., 2011).

Up to now, 22 different amino acid alterations (substitutions and deletions) in the MgCYP51 protein have been described in *M. graminicola* populations of Western Europe (Zahn et al., 2006; Leroux et al., 2007; Cools and Fraaije, 2008). However, low resistance levels occur in case of strains with single substitutions for most or all DMI fungicides whereas some strains are resistant only against a subset of azoles or just a single compound (Becher and Wirsal, 2012).

The alteration in MgCYP51 genes may occur because of substitutions and also deletions of amino acids, especially both Y459 and G460 that are typically deleted together. Such strains (Y459/G460) were early found in the UK and Denmark and consequently spread by wind-borne ascospores. On other hand, strains with substitutions as L50S, S188N, or N513K were widely spread in European and New Zealand field isolates associated with reduced azole sensitivity. It was concluded that the overexpression of this gene may lead to acquired azole resistance in *M. graminicola* (Mullins et al., 2011, Becher and Wirsal, 2012).

Regarding the alterations in MgCYP51, it was reported that changes at some positions (459, 460, 461) were associated with low resistance levels against DMI fungicides, while the amino acid exchange I381V (a valine instead of an isoleucine) in combination with the mentioned changes, revealed the highest resistance levels to some DMI fungicides. However, mutations in codons 316 and 317 were also exhibited in some isolates as low resistance factors towards most DMI fungicides (Leroux et al., 2007).

In the same regard, Kiesner (2012) concluded that MgCYP51 mutations at positions 50, 136, 137, 188, 379, 381, 459, 460, 461, 510, and 513 are correlated with variable sensitivity levels but without a direct conclusion on fungicide sensitivity from MgCYP51 gene because of presence a high variability in the assays.

DMI fungicides often lead to abnormal cell wall thickening and numerous branching of fungi, consequently, causing the inhibition of both spore germination and the initial cell growth (Ramirez et al., 2004).

2.5.1.3 CYP51-azole resistance in *Fusarium graminearum*

Presently, DMI fungicides include several commercially used compounds, and around 34 compounds have been used in agriculture since the 1970s. Tebuconazole, metconazole and prothioconazole are particular used to control FHB and reduce the content of the harmful trichothecene mycotoxin deoxynivalenol (DON) in wheat grains. They are considered to be the preferred fungicides to control FHB in North America and Europe.

Recently, due to the intensive usage of DMI fungicides for decades, declining efficacies of these fungicides were reported with cross-resistance in the azole class often being incomplete

(Yin et al., 2009; Fan et al., 2013). Also DMI-resistant isolates have been reported and detected from wheat fields (Liu et al., 2011).

In the same regard, it was interpreted that the increasing usage of DMI fungicides in controlling FHB has caused the selection of less sensitive isolates of *F. graminearum* in Asia and Europe (Klix et al., 2007; Yin et al., 2009; Becher et al., 2010). Up to date, little is known about the resistance mechanism in *Fusarium* species.

In the case of *F. graminearum*, the existence of three paralogous CYP 51 genes (CYP51A, CYP51B, and CYP51C) was revealed. However, up to date their precise functions remain unknown (Liu et al., 2011; Becher and Wirsal, 2012). With regard to DMI resistance in population of FHB pathogens, Klix et al. (2007) suggested that declining sensitivity to DMI in FHB pathogens has not lead to a problem in the field. Recently, Yin et al. (2009) found three out of 159 *F. asiaticum* and *F. graminearum* isolates, which showed resistance to tebuconazole and metconazole in China; however, they did not find mutations associated with DMI resistance by analysing the amino acid sequence of CYP51A and CYP51B.

The same observation was mentioned by Liu et al. (2011), who observed no changes in DMI sensitivity for CYP51B deletion mutants, but they found that the sensitivity of CYP51C and CYP51A deletion mutants to some DMI fungicides was raised compared to the parent strain. However, these CYP51B and CYP51C mutations did not influence the mycelial growth indicating that none of these genes is fundamental for this growth stage.

In the same regard, Talas and McDonald. (2014) noted that the non-synonymous substitutions in CYP51 were not associated with fungicide sensitivity in *F.graminearum*. Additionally, their findings suggested that genes outside of the CYP51 family may seriously contribute to DMI resistance. Also, Tateishi et al. (2010) suggested that the differences in sensitivity of the Japanese *F. graminearum* species complex to metconazole were not associated with substitution of amino acids in CYP51, because the minimum concentration of inhibition ranged from 0.20 to 6.25 mg/l while the 50% growth inhibition was lower than 0.1 mg/l in about 80% of tested isolates.

The DMI resistance in plant pathogenic fungi may be caused by three different mechanisms. These are: 1- point mutations in the target CYP51 gene leading to decreased affinity for

inhibitors, 2- overexpression of the target CYP51 gene which are often caused by insertions in the predicted promoter areas, and 3- overexpression of ATP-binding cassette (ABC) membrane transporters (Becher and Wirsal, 2012).

In reference to DMI resistance in population of FHB pathogens, Spolti et al. (2014) documented the emergence of the first tebuconazole-resistant field isolate of *F. graminearum* identified in the Americas. It had the lowest sensitivity to tebuconazole ($EC_{50} = 8.09$ mg/litter) reported for this species. In another study, Talas and McDonald (2014) suggested that the intensive usage of DMI fungicides in cereals has selected to resistance. They tested sensitivity of 224 isolates of *F. graminearum* from 13 different German field populations, 22 isolates from the UK and some reference isolates from Italy and the USA to propiconazole.

It was revealed that EC_{50} values ranging from 5.4 to 62.2 mg/L, indicating a noticeable potential for selection of resistant isolates. The authors recommended that it is fundamental to incorporate effective anti-resistance strategies in management of FHB with DIM fungicides.

On the other hand, it was reported that in contrast to other single-site fungicides, DMI resistance levels are usually low and the cross-resistance between their members are incomplete, despite of their long-term usage (Cools et al., 2013). However the monitoring of pathogen population for fungicide sensitivity over the time is still an efficient way to determine whether the resistance is developing.

2.5.2 Strobilurin fungicides

Strobilurin fungicides, which belong to the group of QoI (quinone outside inhibitors), were tested at the end of 1990s for efficacy against *F. graminearum*. They are known to have relatively lower fungitoxicity to FHB, and thus they are commonly used in commercial mixtures together with triazoles to broaden the spectrum of protection against multiple leaf diseases of wheat. Their mode of action is depending on inhibiting mitochondrial electron transport by binding the quinol oxidation site (QoI) of cytochrome bc1 complex, which is one of the major components of the mitochondrial complex III. By binding bc1, they lead to disruption of the energy cycle and consequently of ATP synthesis. These compounds are most effective at spore germination and early germ tube growth stages which have high energy states of progress (Audenaert et al., 2010).

This treatment eventually leads to higher yields compared to triazoles alone, especially under high disease pressure (Blandino et al., 2006; Ransom and McMullen, 2008). Spolti et al. (2013), who used a similar mixture of triazoles and strobilurins, suggested that the mixture of metconazole and pyraclostrobin had no significant effect on DON content but could be effective and led to high increase in yield when used in two sequential sprays. Integrated use of pyraclostrobin and epoxiconazole provided high control efficacy of FHB at two locations in China (Chen et al., 2012) only when used in two sequential sprays but this study did not analyse DON content.

Most studies on the use of strobilurins for FHB control have focused on azoxystrobin and the results varied from no effect on yield or DON to an increase in DON accumulation over the non-treated control (Haidukowski et al., 2005; Gilbert and Haber, 2013). Simpson et al. (2001) observed that application of azoxystrobin increased the DON content in infected grains. Also Pirgozliev et al. (2002) found a strong relationship between the amount of *Fusarium* present and the DON concentrations, and noted that azoxystrobin was significantly less effective against *Fusarium* than the triazole fungicide used for comparison, metconazole. The small effect of strobilurins was interpreted by Menniti et al. (2003) that strobilurin do not control *F. graminearum*.

Audenaert et al. (2011) also reported that strobilurins often result in increased DON levels and attributed this observation to the activity spectrum of strobilurins, which mainly target *Microdochium nivale* while being less effective against *F. graminearum*. It is thus possible that the niches that are no longer occupied by *M. nivale* are taken by *F. graminearum* and that this consequently leads to increased DON content. On the contrary, in a study made by Nakajima (2010), the efficacy of azoxystrobin was 40%; however, it was not as effective as triazole fungicides.

Strobilurin efficacy against *F. graminearum* varied, ranging from no effect on yield or DON accumulation (Haidukowski et al., 2005) to an increase in DON accumulation over the non-treated control (Hollingsworth and Motteberg, 2004). Therefore, this conflict in the use of fungicides to control FHB needs to be clarified.

2.5.3 Benzimidazole fungicides

Benzimidazole fungicides (such as carbendazim, benomyl, thiabendazole), which have systemic activity against a wide range of fungi, have been used extensively during the last few decades for the control of FHB. They are specific inhibitors of microtubule assembly. Their mode of action is depending on binding to microtubules and damaging cellular processes, such as cytoskeleton creation and rearrangement, cell division, and, as a result of interference, they inhibit microtubule assembly and hyphal growth of fungi. Microtubules are fundamental for different cellular activities and tubulin is the main element of the microtubules, which is one of several members of a small family of globular proteins. Tubulins are the subunit protein of microtubules, responsible for many cellular processes, such as cell division, ciliar or flagellar motility, and intracellular transport in eukaryotic organisms (Chen et al., 2009; Zhao et al., 2014).

Methyl benzimidazole carbamate (MBC) fungicides, especially carbendazim were used against FHB with β -tubulins as their target. The tubulin genes in fungi are much fewer than in animals. However, it was documented that some fungal species such as *Aspergillus nidulans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *F. graminearum* have two α - or two β -tubulin genes. Two β -tubulin genes (Fig. 10) contribute to various functions in hyphal growth and fungicide resistance and have been identified in the wheat scab fungus *F. graminearum*.

Zhao et al. (2014) attributed that β 1-tubulin (FG09530.1) has a fundamental role in sexual development of *F. graminearum*. In contrast, β 2-tubulin (FG06611.1) is more influential for vegetative stage of fungal growth, proving that functional divergence emerged between both of β -tubulin genes in *F. graminearum*. With regard to the correlation between mutations in both β -tubulin genes in *F. graminearum* and resistance occurrence, mutations in β 2-tubulin genes but not in β 1-tubulin genes have caused resistance to MBC fungicides. The same study suggested that β 1-tubulin genes undergo a purifying selection, while the other tubulin is subject to divergent selective pressure.

In *F. graminearum*, four substitutions in the tubulins structure occurred at amino acid residues that are at or adjacent to the GTP (guanosin triphosphate) binding sites or the interface between α - and β -tubulins (Fig. 10A). These alterations may play a role in the interactions

between FgTub2 (β 2-tubulin, *F. graminearum*) and α -tubulin monomers. In particular, under type II variations, the four residues A153, A291, M331, and N424 are determined on the surface of FgTub1 (β 1-tubulin, *F. graminearum*). Their counterparts in FgTub2, S153, H291, T331, and H424 (Fig 10B) are still on the protein surface but undergo dramatic changes in amino acid properties (Zhao et al., 2014).

It was reported that carbendazim effectively reduced the levels of *F. avenaceum*, *F. culmorum* and *F. graminearum* to about 70% in wheat heads compared to the control treatment (Hutcheon and Jordan, 1990). In another study, carbendazim revealed relatively good control of FHB; however, it was not as effective as tebuconazole, on the contrary, benomyl showed similar level of control to tebuconazole (Jones, 2000). Cromey (2001) suggested that carbendazim considerably decreases both of FHB symptoms as well as mycotoxin content in grain. In a recent study, carbendazim caused 83% inhibition of the mean growth of *F. graminearum* isolates at a concentration of 1mg/l while this rate decreased to 50% for the same concentration of tebuconazole (Ivic et al., 2011).

However, from the 70s of the 20th century, carbendazim was generally used in management of FHB and because of the heavy application of MBC fungicides, their effectiveness dramatically declined and was threatened by the emergence of resistant pathogen population in the field (Chen and Zhou, 2009).

Resistance to MBC fungicides has been identified in many fungi, e. g. in *Botrytis cinerea*, *Cladobotryum dendroides*, *Helminthosporium solani*, *Monilinia fructicola*, *M. laxa*, *Tapesia yallundae*, *Venturia inaequalis* and *Fusarium* species (Liu et al., 2010). Generally, the

In the genome of *G. zeae*, two homologous β -tubulin genes exist. However, it was reported that β 1-tubulin (FG09530.1) in both resistant and sensitive isolates of *G. zeae* had high amino acid identity. In the same regard, it was found that amino acid sequence of this gene from benzimidazole resistant and sensitive strains in *G. pulicaris* (*F. sambucinum*) were identical. The resistance in *G. zeae* to MBC is generally caused by a single point mutation at codons 167 or 200 in β 2-tubulin (FG06611.1) that encodes the target at which the fungicide binds (Zhao et al., 2014).

Additionally, it was mentioned that the substitutions at codons 73 and 198 are responsible for high resistance levels only when double mutation are present, but the existence of this genotype is often low in field populations.

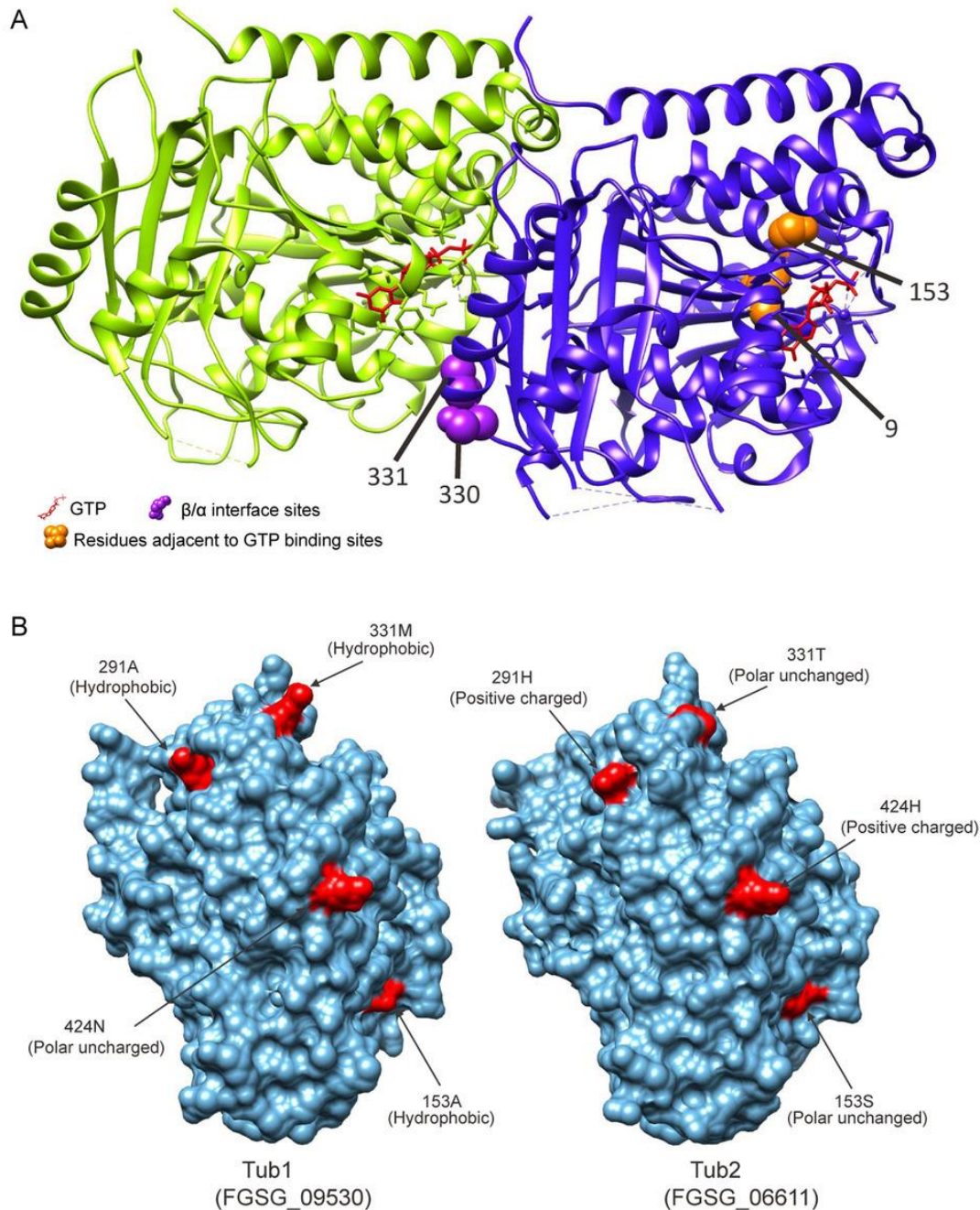


Figure 10. The 3D-structures of tubulins in *Fusarium graminearum* showing amino acid residues contributing to the type II functional divergence. (A) Type II sites directly related to or adjacent to GTP binding or α/β connection. (B) Distribution of the four type II sites (highlighted in red) on the surface of tubulins in *Fusarium graminearum* (Zhao et al., 2014).

Overall, fungicide applications are used besides other strategies, for instance tillage, which buries infested residues and consequently declines the exposure to the infestation resources, crop rotation, i.e. sowing of wheat and barley after crops that are not host of *Fusarium* species, as well as the use of resistant cultivars. All the mentioned strategies are recommended to provide an efficient control with higher yield and low infection.

2.6 Pathogenicity and aggressiveness of *Fusarium graminearum*

FHB has a great economic impact on wheat all over the world (Kotowicz et al., 2014), its harmful effect consists of deterioration of the quality and quantity of yield and production of mycotoxins, especially of the trichothecenes group including deoxynivalenol (DON) and nivalenol (NIV) (Pereira et al., 2014). Aggressiveness and pathogenicity are two important traits of *F. graminearum* (Von der Ohe et al., 2010). According to Thomas and Elkinton. (2004), pathogenicity is defined as the ability of an organism to infect a host causing disease, while aggressiveness is the ability of the pathogen to invade and establish within the host. This trait is identified by the quantity of disease induced by a pathogenic isolate on a susceptible host in a system (Van der Plank, 1968).

Pariaud et al. (2009) suggested that aggressiveness is often separated into elementary quantitative characteristics of the pathogen life cycle and usually assessed through disease severity, which is a compound variable resulting from the integrated effect of infection efficiency and lesion size. Thus, aggressiveness is a basic aspect, which is considered crucial to understand the host-pathogen interaction in the FHB-wheat system. There is a large genetic variation in aggressiveness among *F. graminearum* isolates sampled from various parts of the world (Miedaner et al., 2001), within a country or within a state (Walker et al., 2001).

In addition to being an important pathogen on wheat and barley, *F. graminearum* also infects economic crops such as rice and maize (Goswami and Kistler, 2004) and can cause disease in several plant parts (Purahong et al., 2013). This makes its aggressiveness variable, and for that reason, this pathogen has received an overwhelming attention over the time and considered one of the most intensively studied fungal plant pathogens.

Many assays have been developed to quantify the aggressiveness, including the inoculation of floret tissues, stems, seedlings, coleoptiles and head under field condition (Snijders and Perkowski, 1990; Simpson et al., 2000; Urban et al., 2002; Wu et al., 2005; Toth et al., 2008; Alvarez et al., 2010; Imathiu et al., 2014.), in addition to detached leaf assay (Diamond and Cook, 1999) and Petri-dish test (Purahong et al., 2012).

However, most inoculation methods are conducted at anthesis, with the disadvantages of being labor intensive and expensive, needing a long processing cycle. There are only few studies that utilize more than one test to assess aggressiveness of the fungal isolates (Wu et al., 2005).

Purahong et al. (2012) suggested that the Petri-dish test is an in vitro aggressiveness method for *F. graminearum* in wheat, which is easy and has a high correlation with the floret inoculation method, in addition to being consistent in different cultivars. They showed that the new parameter “Petri-dish aggressiveness index” revealed a good correlation with floret inoculation both in control and field conditions.

In a second study, Purahong et al. (2013) assessed aggressiveness of 32 *F. graminearum* isolates, which belong to different chemotypes (15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, or nivalenol producer) and found a highly significant correlation of the standardized area under disease progress curve $AUDPC_{\text{standard}}$ (Petri-dish test) to mean disease severity. However, there was no significant difference in aggressiveness among the three different chemotypes. The role of trichothecenes in plant disease is not clear; several studies noted a positive correlation between aggressiveness and DON production by *F. graminearum* (Gang et al., 1998).

However, up to now there are few reports that compare the aggressiveness of *F. graminearum* isolates to DNA production.

2.7 Maize

Corn (or maize, as it's known throughout much of the world), a major source of food for both humans and animals. Maize is among the three most important cereal crops throughout the world. The global maize production for 2010–2011 was 817 million metric tons (Mt), representing 32% of the 2.15 billion Mt of world cereal grain production (United State Department of Agriculture, 2011). Maize is classified under division: Magnoliophyta, class: Liliopsida, order: Cyperales, family: Poaceae. genus: *Zea* and species *Zea mays*. Maize is,

after wheat, the second most important cereal crop in human and animal diets worldwide (FAO, 2011).

Maize has a large range of uses for instance; varieties of maize are traditionally used for bakery in various countries. They are also useful owing to their potential value as functional foods and different food commodities for instance corn meal, vegetable oil, high fructose corn syrup, etc. Furthermore, maize varieties are gradually demanded particularly for organic agriculture. Also besides being food for humans and animals, maize has been used for industrial purposes such as bioenergy crop especially as source of ethanol. In addition, several countries build their economies on marketing this cereal, for instance, USA (54%), Argentina (16%) and Brazil (9%) are the major exporting nations of this crop (United State Department of Agriculture, 2011; Solomon et al., 2014).

Maize is globally used as energy crop as an alternatives to fossil fuels which contribute in reducing CO₂ emission. In Germany, maize contributes to 79% of the biomass used for this purpose, consequently the maize area has increased to 2.6 million ha from 1.5 million ha in 2002. Also the maize production was used for both humans and animals (Federal Statistical Office, German Maize-Committee, 2013).

Grain maize and silage maize consider the major important animal feed sources all over the world. Different parts of maize plant can be attacked by *Fusarium* species including the remaining crop, maize kernels, the whole plant used as silage, as well as stalk pieces (Munkvold, 2003). *Fusarium* species lead to huge yield losses and additionally, they produce a large range of mycotoxins before and after harvest, ultimately causing animal health problems because of contaminated feed. As a consequence, attention is paid to this pathogen of the maize plants, because only healthy plants and ears can guarantee high yields and qualities.

Furthermore, contaminated goods are rejected leading to further economic losses (Logrieco et al., 2002; Desjardins, 2006). DON, ZEN and FUM are the major mycotoxins produced by *Fusarium* species. In this regard, the allowed limits of mycotoxins levels were laid down by regulations in the European Union in order to protect consumers from health risks associated with these mycotoxins.

2.7.1 *Fusarium* species in maize

Maize is used as a food for people as well as for feeding of livestock, additionally for biogas production. However, maize is also considered as one of the main host plants of *Fusarium* species. As a consequence of increasing demands for energy use, animal feed and human food, the production of the world maize production is increasing permanently (Shiferaw et al., 2011). *Fusarium* infections do not only result in yield reduction, but also cause mycotoxin production and thus contamination of maize products. In 1995, symptoms of mycotoxin contamination occurred in India which was associated with the consumption of sorghum and maize contaminated with fumonisin B1 (Bhat et al., 1997).

In another study, it was reported that esophageal cancer was associated with trichothecenes and fumonisins in wheat and maize in China and South Africa (Rheeder et al., 1992; Yoshizawa et al., 1994). In the same regard, it was reported that *Fusarium* mycotoxins affected health and productivity of hens, pigs and cattle (Bristol and Djurickovic, 1971; Pestka, 2007).

In Germany, various investigations have been carried out in order to understand and determine the biodiversity of *Fusarium* species in many cereal-producing areas. For instance, Görtz et al. (2008) found 13 *Fusarium* spp. in kernels with an incidence ranging from 0.7 to 99.7 %. The prevalent *Fusarium* species were different between years in a two year survey. Whereas *F. verticillioides*, *F. graminearum* and *F. proliferatum* were predominant in 2006, mainly *F. graminearum* was isolated in 2007. In a recent study, seven *Fusarium* species were found in maize silage, with *F. graminearum* being the dominant species, followed by *F. culmorum*, *F. avenaceum* and *F. poae*. *F. tricinctum*, *F. langsethiae*, and *F. equiseti* were detected with only small amounts (Birr, 2013).

Fusarium species are among the major fungal pathogens in maize, causing several diseases for instance root rot, stalk rot, stalk lodging, ear rot, leading to significant yield losses and potential risk of mycotoxin contamination. Upon infection with *Fusarium* species, the size and weight of the kernels are mainly reduced during the period of colonization of the maize plant. However, most important is the production of toxic metabolites. These substances are causing adverse effects upon ingestion by humans and animals. The infection of different *Fusarium* species on maize in Europe is depending on the environmental conditions. In the dry and hot regions of Southern Europe, *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg are very prevalent while in Northern and Central

Europe, the most widespread *Fusarium* species on maize is *F. graminearum*. This pathogen is predominant in years characterized by low temperature and permanent precipitation during both summer and autumn. In Germany, *F. graminearum* was the most prevalent *Fusarium* species on naturally infected maize ears in 2006 and in 2007 (Görtz et al., 2010).

F. graminearum and *F. culmorum* are considered to be the most important trichothecene producing *Fusarium* species. In contrast, *F. verticillioides* and *F. proliferatum* are the major fumonisin producing species (Parry et al., 1995; Glenn, 2007).

ZEN was firstly identified followed by deoxynivalenol (DON) in maize, both of them being produced by *F. graminearum*, while the *F. verticillioides* mycotoxin, fumonisin B1, was identified in 1988 (Mirocha 1974, Mesterházy et al., 2012). DON levels are significantly reduced during the baking process; on the contrary, fumonisins are fairly heat-stable, and consequently their degradation is very low during fermentation. The maximum allowed limit for fumonisins is 4.00 mg/kg while this rate is 1.75 mg/kg for DON in non-processed maize according to the regulations of the European Union (EU, 2007).

Fusarium ear rot (FER) is usually caused by *F. verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* J. Sheld., sexual stage: *G. moniliformis* Wineland), *F. subglutinans* and *F. proliferatum*, which reduces maize production by destroying the grains and causing mycotoxin contamination. FER is identified by appearance of a white or light pink mould and usually appears on random kernels, groups of kernels or on kernels which are physically injured (Logrieco et al., 2002).

Bisby and Bailey (1923) in Canada firstly reported about this disease. In contrast, *Gibberella* ear rot (GER) is caused by the sexual state of *F. graminearum* (*G. zaeae*), which can cause a stalk rot that results in stalk breakage and lodging, therefore making harvest difficult and accordingly reducing yield (Mesterházy et al., 2012). GER (or red ear rot) is characterized by the colour of fungal mycelium growing on an infected ear of corn. The diagnostic red or pink mould typically starts appearing from the tip of the maize ear covering the ear extensively with mould and continues downwards (Fig. 11). Oldenburg and Ellner (2015) suggested that the *Fusarium* infection on the rachis spreads in the upper part by giving brownish or pink discoloration of the pith.

Fusarium spp. cause maize ear rots which have been differentiated into two separate diseases called pink ear rot which is also called *Fusarium* ear rot and red ear rot which is called *Gibberella* ear rot (Logrieco et al., 2002).



Figure 11. Symptoms of *Fusarium graminearum* on artificially infected maize ears.

The diagnostic red or pink mould typically starts appearing from the tip of the maize ear covering the ear extensively with mould and usually does not infect the entire ear. Due to that, the husks stick to the kernels and may be difficult to remove. The teleomorph *G. zeae* can form blue–black perithecia which are typically observed on infected husks and ear shanks (Munkvold, 2003a; Mesterházy et al., 2012).

In a different study, it was reported that *F. graminearum*, *F. culmorum*, *F. cerealis*, and *F. avenaceum* are the most dominant species causing red ear rot in maize, while the other *Fusarium* species are less important and frequent such as *F. equiseti*, *F. poae*, *F. sporotrichioides*, *F. acuminatum*, *F. semitectum*, *F. solani*, and *F. oxysporum* (Logrieco et al., 2003). In the same study, 90-95% of all isolated *Fusarium* strains causing red ear rot of maize are *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. avenaceum*, and *F. subglutinans*.

Various factors affect the spread and the predominance of *Fusarium* spp. including geographical zone, environmental conditions, climatic factors (in particular temperature and moisture), host genotype and agriculture approaches. Generally, GER is found in cooler regions or those which have high precipitation during the growing season. In contrast, FER predominates under warm and dry conditions before or during the stage of ear filling (Logrieco et al., 2002; Munkvold, 2003a; Polisenka et al., 2008).

Overall, the levels of mycotoxins in infected parts of maize plants are varying considerably according to maize cultivars, being typically higher in susceptible than in more resistant cultivars.

2.7.2 *Fusarium* spp. infection of maize

The genus *Fusarium* belongs to the phylum Ascomycota (tubular fungi) representing around 30 % of all known fungal species and contains a wide range of toxigenic fungi, which are considered to cause concern in many maize growing regions all over the world.

Occurrence of different *Fusarium* species on maize in Europe is largely dependent on the environmental conditions. The residues of crops are the major source of infection of maize kernels. Recent studies reported that maize residue is a more important source of *F. graminearum* inoculum than wheat residue (Logrieco et al., 2002; Munkvold, 2003a; Munkvold, 2003b; Polisenka et al., 2008).

Fusarium species can also survive in senescent tissues of other crops. It was documented that *F. graminearum* can produce chlamydospores, whereas *F. verticillioides* (reported as *F. moniliforme*) can produce thickened hypha. Additionally, *F. graminearum* shapes fruiting structures called perithecia which forcibly discharge ascospores into the air. The optimal temperature for perithecial development is about 28 °C; on the other hand, the favourable temperature for ascospore release is about 16°C. Besides to ascospores, *F. graminearum* produces large asexual spores known as macroconidia. They have various shapes and form in sporodochia. Chlamydospores may also be present; however, their development is slow, if at all, they are round spores serving as long-term survival structures for the fungus (Logrieco et al., 2002; Munkvold, 2003a; Leslie and Summerell, 2006).

On the whole, it seems that the ascospores are the major source of infection. Ascospores are spread by wind, while macroconidia are spread by splashing and wind driven rain.

F. verticillioides and other *Fusarium* species causing *Fusarium* ear rot do not as frequently produce perithecia as *F. graminearum*. In general, both ascospores and macroconidia can spread long distances reaching kilometres from any known inoculum source (Parry et al., 1995; Munkvold, 2003a).

The silks of maize kernels are the main infection pathway, which are susceptible during the first 6 days after silk emergence. The infection occurs after spores reached the silks by water splashes, wind dispersal or insect vectors. The infection via silks is caused by spore germination on the silks, consequently followed by growth of hyphae, and subsequently the hyphae penetrate the cob of maize (Sutton, 1982). In the case of *F. graminearum*, the infection typically starts from the tip towards the base of the ear and may reach the peduncle of an ear during a diverse epidemic. Consequently, the perithecia and spores form when *G. zae* matures leading to spread with succeeding cycles of disease among fields. While *Fusarium* species associated with *G. fujikuroi* enter the corn ears through wounds, in particular those caused by insects, and also mechanical ear wounds caused by birds or hail can lead to infections (Munkvold et al., 1997; Munkvold, 2003a). It was documented that *F. verticillioides* infection in the kernels occurs when the grain moisture reaches 28% (Desjardins, 2003; Munkvold, 2003b).

In general, the infection of *F. graminearum* needs temperature reaching $29^{\circ}\text{C} > 48$ h while the required temperature of the mycelial growth is (10-30°C), however the optimum temperature for *F. graminearum* mycelial growth is 25°C. on the other hand, the temperature *F. culmorum* infection ranged (10-30°C) but the optimum temperature is (20-25°C), while the required temperature of the infection is 26.5°C for 72 h. regarding *F. poae* and *avenaceum*, the temperatures rang (20-25°C). (Rossi et al., 2001; Brennan et al., 2003; Dufault et al., 2006)

In other researches, the optimum temperature for *F. graminearum* has been documented as 24-26°C; on the other hand, for *F. verticillioides*, the optimum temperature is documented as about 30 °C. The infection of *Gibberella* ear rot is facilitated by high levels of moisture around silking, followed by reasonable temperatures and high rainfall during the maturation stage (Munkvold, 2003a).

Environmental conditions have serious effects on the development of these diseases; for that reason, the management of these diseases during the earliest stages of disease progress is considered to be the most effective way of disease control.

2.7.3 Systemic infection of *Fusarium* species in maize

Maize (*Zea mays*) is considered to be the most important crop grown in the world. It is commonly infected by *Fusarium* species which can colonize all parts of maize plants including the root, leaf sheath, stalk and cob (Polisenka et al., 2008).

It is known that *Fusarium* species infect wheat, barley and sorghum and can overwinter in crop residues. Some researchers reported that *Fusarium* species can survive for more than 1 year on residue on the soil (Munkvold, 2003b; Polisenka et al., 2008).

Fusarium infections may lead to the appearance of symptoms but can also cause symptomless infection. Researchers have evaluated colonization of *Fusarium* in maize using molecular methods, such as PCR, which can be used to effectively detect and quantify the pathogen.

It was mentioned that some *Fusarium* species, among them *F. graminearum* and *F. culmorum* can produce B trichothecenes, such as deoxynivalenol and acetyldeoxynivalenol, while other species cannot. Whereas the DNA polymorphism in the tubulin gene is considered the basis to distinguish between these two types of *Fusarium* species (producers and no producers), they can additionally be distinguished, in the large ribosomal subunit of beta-tubulin. (Bakanl et al., 2002).

Regarding trichothecene production, some *Fusarium* species, for instance, *F. graminearum* and *F. culmorum*, have been split into two chemotypes: (i) the nivalenol chemotype including isolates producing nivalenol and fusarenone X, and (ii) the deoxynivalenol chemotype including isolates producing deoxynivalenol and acetyldeoxynivalenol (Bakanl et al., 2002; Desjardins, 2003).

In the same regard, it was reported that *Fusarium* species grown the same culture conditions largely vary in their ability of toxin production, in other words, some strains produce huge amounts of trichothecenes while others produce small or undetectable values (Miller et al., 1991; Desjardins, 2003).

The entry of *Fusarium* into maize ears can result from the growth of mycelium and the airborne conidia on the silks, or from the wounds by insects and birds. *F. graminearum* infection needs silk tissue which are less 8-10 days old. Commonly, only a small percentage

of the infected kernels are symptomatic. Infection by *Fusarium* species also can occur through the seed, where is systemically.

The systemic infection can begin from both conidia and mycelium which can find either on the surface seed or inside the seeds (Foley. 1962; Munkvold et al., 1997a; Munkvold et al., 1997b., Oren et al., 2003).

Regard with the movement of *F. verticillioides* from seeds to stalk and kernels, Kedera et al. (1997) tracked specific strains using vegetative compatibility groups as a marker, thus, found an evidence that the fungus moves from the infected seeds to the kernels by recovering the isolate inoculated from approximately 10% of the kernels.

Similar results were reported in another studies (Leslie et al., 1990; Munkvold et al., 1997a; Munkvold et al., 1997b; Oren et al., 2003), these studies reported that infected seeds may lead to the infection of the kernel, whereas the inoculum surviving in crop residues and in the soil may cause to the systemic infection.

It was documented that the systemic infection of *F. verticillioides* from seeds to roots and stalks can cause rotting in all plant parts, whereas it is considered a cause of stalk root, but its importance appears to be low in kernel infection. Also the movement of the fungus from the seedling crown to the stalk tissue is considered the rate-limiting cause in fungal infection from infected seeds to the upper organs of the plant (Oren et al., 2003). In other words; the seed-transmitted strains do not progress to aboveground parts after pollination.

Regarding the systemic infection of *F. graminearum*, Young and Miller. (1984) suggested that both deoxynivalenol and ergosterol were differently found inside the stalk and considerably appear in the sections above the ear-attachment point. In the same line, it was reported that the movement of fungus from seed to kernel has four steps including (i) seed to seedling transmission, (ii) the fungus colonize in the stalk tissue, (iii) transmission into the air, (iv) spread into the ear, but on the whole, it was mentioned that limited transmission to higher internodes in the stalk occurred.

There are limited information about the systemic infection of *Fusarium* species within maize organs, but it worthwhile to mention that the plant genetic background, as well as, for the pathogen are important factors in disease development.

3. Chapter II

Materials and Methods

The present work consists of six different experiments. For that reason, the materials and methods are presented separately for each one.

3.1 Sensitivity of *Fusarium* species isolates to metconazole and epoxiconazole and comparison of CYP51 sequence of *Fusarium* species with CYP51 of *Septoria tritici*

3.1.1 Fungal isolates

The isolates of *Fusarium* spp. used in this study were provided by the Institute of Phytopathology, University of Kiel, and they are listed in Table 6 (page 53).

In addition, CYP51 sequences of mutant and wild strains of *Septoria tritici* were obtained from a previous study in our institute (Kiesner, 2012), which used them as reference to address the correlation of *in vitro* sensitivity to DMI fungicides and CYP51 mutations at positions 50, 136, 137, 188, 379,381 , 459, 460 , 461 , 510 , 513.

3.1.2 Fungicides

Two commercially available fungicides, Caramba (60 g/l metconazole) and Opus (125 g/l epoxiconazole) were used *in vitro* tests, were pure technical grade, and kindly supplied by BASF (Ludwigshafen, Germany). Stock solutions of both fungicides were made in sterilized water.

3.1.3 Measurement of the sensitivity of the isolates to metconazole and epoxiconazole

The fungicides were dissolved in sterilized water, and added to carrot agar medium after sterilization to produce final concentrations of 0.01, 0.001, 0.0001, 0.00001, 0.000001 and 0.0000001 mol/l. Fungicide sensitivity of *Fusarium* species isolates was assessed by inoculating carrot agar medium plates with *Fusarium* spp. suspension inoculum consisting of 5 µl which was taken from 7-10 day old cultures maintained on SNA (Synthetic Nutrient Poor Agar, Carl Roth, Karlsruhe, Germany) medium. The spore suspension was placed on the center of carrot agar medium plate at each of the above concentrations; four replicate plates for each concentration were used for each isolate. Control plates without fungicides received

an equivalent amount of distilled water. After incubation at 25°C for 4 days in the dark, colony diameter in each plate was measured in two perpendicular directions. The effect of the fungicides was determined by using means of the colony diameter in order to calculate the percentage of growth inhibition of mycelium in relation to the control on the basis of the following equation (Filoda, 2008):

$$S = \frac{A-B}{A} \times 100$$

S- % of the mycelium growth inhibition

A- Mean diameter of fungal colony on a medium without fungicide

B- Mean diameter of fungal colony on a medium with fungicide.

The experiment was repeated twice.

3.1.4 DNA extraction

The mycelium was peeled from 4-7d old PDA (Potato Dextrose Agar, Carl Roth) cultures and manually ground in 1.5 ml of micro-tubes (VWR, West Chester, Pennsylvania, USA) with micro pestle by adding 500 µl of extraction TS- buffer (100 mM Tris, pH 8, 2% SDS) and a small amount of quartz sand, followed by microwave treatment at 800 W for 15s, and incubation at 60°C in a water bath (Lauda E100,-Königshofen, Germany) for 30 min. Then, 300 µl of 3M sodium acetate (pH 5.2) (Carl Roth) was added and the micro-tubes were incubated at -20°C for 30 min.

The micro-tubes were then centrifuged (Eppendorf, Hamburg, Germany) at 1500 x g for 10 min at 4°C. Subsequently, the supernatant was transferred into fresh micro-tubes, 700 µl of isopropanol (Carl Roth) was added, and the micro-tubes were centrifuged at 1500x g for 20 min at 4°C. The whole liquid was thrown away. Micro-tubes were washed with 120 µl ethanol (70%) (Carl Roth), thereafter again centrifuged as above but only for 5 min. The resulting DNA pellet was dried and dissolved in 80 µl of water and the solution stored at -20 °C for further PCR reaction (Aviso, Jena, Germany).

Table 6. Fungal species used in this study.

No	species	origin
1	<i>F. graminearum</i> DSMZ 1095	USA
2	<i>F. culmorum</i> VII2FP 2011	Germany
3	<i>F. langsethiae</i> 9955	Denmark
4	<i>F. langsethiae</i> 8081	Denmark
5	<i>F. sporotrichioides</i> DSMZ62425	Germany
6	<i>F. poae</i> DSMZ 62376	Germany
7	<i>F. poae</i> III FP2011	Germany
8	<i>F. culmorum</i> 8	Germany
9	<i>F. verticillioides</i> BASF1169	Germany
10	<i>F. venenatum</i> DSMZ 21739	Germany
11	<i>F. graminearum</i> DSMZ 1094	Germany
12	<i>F. tricinctum</i> II	Germany
13	<i>F. graminearum</i> III7	Germany
14	<i>F. graminearum</i> AF15	Germany
15	<i>F. graminearum</i> ck3	Germany
16	<i>F. graminearum</i> ck8	Germany
17	<i>F. graminearum</i> Dr8	Germany
18	<i>F. graminearum</i> Hs6	Germany
19	<i>F. graminearum</i> To3	Germany
20	<i>F. graminearum</i> To5	Germany
21	<i>F. graminearum</i> Wtg	Germany
22	<i>F. subglutinans</i> He7	Germany
23	<i>F. avenaceum</i> VI4	Germany
24	<i>F. crookwellense</i> II6	Germany
25	<i>F. sporotrichioides</i> IIFP	Germany
26	<i>F. tricinctum</i> III1	Germany
27	<i>F. tricinctum</i> III3	Germany
28	<i>F. tricinctum</i> VIII3	Germany
29	<i>F. tricinctum</i> AJ13	Germany
30	<i>F. culmorum</i> I4	Germany
31	<i>F. culmorum</i> II4	Germany
32	<i>F. culmorum</i> III6	Germany
33	<i>F. culmorum</i> kr8	Germany
34	<i>F. culmorum</i> FU13	Germany
35	<i>F. culmorum</i> Sc9	Germany
36	<i>F. culmorum</i> wt2	Germany
37	<i>F. graminearum</i> Dr15	Germany
38	<i>F. crookwellense</i> FU10	Germany
39	<i>F. graminearum</i> os12	Germany

Letters and numbers followed by *Fusarium* species refer to different species calculating from various locations.

3.1.5 Analysis of DNA sequence of CYP51 genes from *Fusarium* species isolates with different sensitivities to DMI fungicides

The search for CYP51 sequence in the *Fusarium* comparative database constructed by the Broad Institute, USA, revealed three putative CYP51 genes encoding 14 sterol demethylases. The following three sequences from *F. graminearum* were designated as CYP51A, CYP51B and CYP51C under accession numbers FGSG11024.2, FGSG 01000.3 and FGSG04092.3, respectively (available online). The expressions of these genes in 11 *Fusarium* species isolates were investigated which had been selected according to their covering the range of DMI sensitivities and their importance in Germany.

Four primers were chosen based on the sequences deposited on the genome database of *F. graminearum* at Broad Institute. Additionally, these primers were informative in previous studies (Liu et al., 2008; Kiesner, 2014). The primers were used to amplify the full sequence of CYP51 (Table 7).

PCR amplifications were carried out under the following conditions: initial denaturation at 95°C for 5 min followed by 40 cycles at 94°C for 30s, 58°C for 30s, 72°C for 1.5 min, with a final extension at 72°C for 7 min.

The PCR reaction mixtures consisted of 2 µl of template DNA (30 ng/ µl), 0.5 µl from each primer (Eurofins MWG Operon, Ebersberg, Germany), 0.25 µl Dream Taq-Polymerase, 1 µl dNTPs (2 mM), 2.5 µl of Dream Taq buffer (all mentioned components were from Fermentas Life Sciences, St. Leon-Rot, Germany) and 18.25 µl sterile water (VWR, West Chester, Pennsylvania, USA).

Each isolate was amplified twice. The amplified DNA fragments were firstly electrophoresed on a horizontal agarose gel and visualized under UV light (Bio-Rad Laboratories, Hercules, California, USA), and then using a clean, sharp scalpel, the DNA fragments were cut from the gel and placed in a microcentrifuge tube.

The QIA quick gel extraction kit (Qiagen, Hilden, Germany) was used to extract DNA fragments from the agarose gel according to the manufacturer`s recommendations. Briefly, the gel slice was weighed and three volumes of binding buffer QG were added to one volume of gel and incubated at 50°C (VWR® Digital Dry Block Heater, VWR) for 10 min (or until

the gel slice has completely dissolved). During the incubation, the tubes were inverted every 2-3 min to enhance the efficiency of the extraction and the DNA yield and help the dissolving. After complete dissolving, one gel volume of isopropanol (100%) was added to the sample and mixed well. Subsequently, the sample was transferred into the QIA quick column placed in 2 ml collection tube to bind DNA. Then, the column was centrifuged for 1 min at 11.000 x g at room temperature. The flow-through was discarded, afterward, the QIA quick column was placed back in the same collection tube and 500 µl of buffer QG was added to QIA quick column to remove all traces of agarose.

The column was centrifuged for 1 min and the flow-through was discarded. Subsequently, 750 µl of washing buffer PE was added to QIA quick column and the solution was centrifuged for 1 min. The flow-through was discarded and centrifuged for an additional minute to ensure complete removal of the buffer. The QIA quick column was then placed into a clean sterile (1.5 ml) microcentrifuge tube. Then 50 µl of elution buffer (EB) was added in order to elute the purified DNA, thereafter, the column was centrifuged for 1 min at 11.000 x g. Afterward, the isolated DNA was evaporated to dryness on a vacuum centrifuge (Vacuum centrifuge S Concentrator BAVC- 300H, Saur, Reutlingen, Germany) and then redissolved in 15 ml water. Finally, the samples were sent to Eurofins MWG Operon in order to get the DNA sequence of CYP51.

The experiments were performed twice independently for each isolate to avoid mismatch sequence during PCR amplification and sequencing. Finally, in order to determine differences in expression of CYP51 genes between *Fusarium* isolates and *Septoria tritici*, CYP51 isolates were compared in both wild and mutant strains at the mentioned positions.

The deduced amino acid sequences as deposited in the data base were aligned with the consensus sequence of this gene obtained from different isolates using the program clustalW1.82 (<http://www.ebi.ac.uk/Tools/msa/clustalw/> ; European Bioinformatics Institute, Cambridge, UK)

Table 7. Primers used in this study to determine the sequence of CYP51 (Liu et al., 2008; Kiesner, 2014).

Name of primer	Sequence (5'-3')
CYP51-V1a	ATG GGT CTC CTC CAA GAA C
CYP51-V1b	ATG GGT CTC CTT CAA GAA C
CYP51-V2	CT GGC CAG CAC TCT TCT TC
CYP51-H1a	CAG TGA ATG TTG GCG GGC
CYP51-H1b	CAG TGG ATG TTG GCG GG

3.2 *In vitro* sensitivity of isolates of *Fusarium* species to carbendazim and investigation of the contribution of β -tubulin to its sensitivity

All *F. graminearum* isolates (mentioned in table 7) were used to determine their sensitivities to Sarfun 500 SC (500 g/l carbendazim, producer: ZCH Organika Sarzyna SA, Nowa Sarzyna, Poland). The procedure of sensitivity measurement of fungal strains is similar to the procedure described above for metconazole and epoxiconazole.

The polymerase chain reaction (PCR) was performed with the following parameters: initial denaturation at 95°C for 3 min followed by 35 cycles at 94°C for 40s, 55°C for 40s, 72°C for 1.5 min, with a final extension at 72°C for 5 min using primer pair (forward: AAACGCACACACACACACA, reward: AGATGGCGGAACAA) and primer pair (forward: CGCAACATGAAGACTTTCCA; reward: ACGGAAGTTTGGACGTTGTT) which were informative in previous studies (Chen et al., 2009). These primer were used in order to amplify β -tubulin gene. After purifying PCR products, they were sequenced and DNA fragments were submitted to *G. zeae* genome database under accession number FGSG 06611.

3.3 Application timing and dose rate of triazoles alone or in mixture with pyraclostrobin for the control of *Fusarium graminearum* in wheat and maize

3.3.1 Experimental design

This experiment was aimed to evaluate the effect of protective and curative application and dose rate on the efficacy of metconazole, single applications of epoxiconazole and pyraclostrobin fungicides or in mixture (pyraclostrobin and epoxiconazole) in reducing *Fusarium* occurrences in maize and wheat. Additionally, to compare the effect of the mixture on *Fusarium* development and DON accumulation in wheat and maize grain.

The experiment was conducted in the greenhouse of the University of Kiel, Germany for 2 years on wheat and 1 on maize. Seeds of untreated winter wheat (cultivar Ritmo, highly susceptible to FHB, Beyer et al., 2006) were vernalized in sterile sand for 6 weeks at 4°C. The vernalized seedlings were transferred to the greenhouse and grown plastic pots (size 9x9 cm) under controlled conditions (temperature: 20°C, humidity: 60 %, photoperiod: 14h, photon

flow density: 270-280 mol/m²s). Fungicides were applied around wheat anthesis to generate different levels of *Fusarium* damage; using 30 heads per treatment (three replicates).

Regarding maize, seeds (NK Nekta, untreated) were sown in plastic pots (size 18x18 cm). Plants were irrigated as needed. NPK fertilizer (8-8-6) was used once per month. The experiment was conducted with three replications for each treatment. In addition, two control groups were not treated with fungicide, one of these was inoculated, and the other was sprayed with distilled water.

3.3.2 Fungicide application

Three commercially available fungicides (metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany) were used in the experimental work in the first year on wheat and treatment of pyraclostrobin (F500, BASF, Germany) was added for the second year on wheat and maize. The fungicides were applied in two different doses (100%, 50%) for wheat and one dose (100%) for maize, the first dose agreeing with the official recommendation for commercial field use, and the another using a sublethal dose (lower than recommended). All fungicide treatments were applied using a sprayer (1.75 bar and 400 l/ha) and at several time points around the inoculation. Five one-time application times were tested: 3 and 1 day either prior or post inoculation and during inoculation. The information about fungicides used are given in Table 8.

Table 8. Fungicides, active ingredients, use doses, application times used in fungicides trials in greenhouse.

Fungicide trademark	Dose	Active ingredients	Application time for each fungicide
Caramba ¹	(100%,50%)	Metconazole	One application at 3d pre-inoculation
Opus ²	(100%,50%)	Epoxiconazole	One application at 1d pre- inoculation
F500 ³	(100%,50%)	Pyraclostrobin	One application during inoculation
Retengo ⁴	(100%,50%)	Pyraclostrobin + epoxiconazole	One application at 1d post- inoculation One application at 3d post- inoculation

1, 2, 3, 4 all fungicides were provided by BASF, Germany.

3.3.3 Fungal inoculum

A mixture of seven isolates of *Fusarium graminearum* (os12, ck3, to5, DSMZ 1095, III7, IV5, Hs6), being six from Germany, isolated from wheat and maize in 2011, and one from United States, isolated from wheat in 2010 were used.

During the first trial, the isolates were propagated in liquid cultures in potato dextrose broth (PDB, Potato Dextrose Bouillon, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 3 weeks at room temperature, and then the suspension was homogenized. Wheat heads were inoculated at Zadoks growth stage (GS) 65 (Zadoks et al., 1974) by overhead application of *F. graminearum* mycelial suspension of the above mentioned seven isolates. Flowering heads were sprayed from each side with the inoculum (ca 2 ml for each), using a hand held bottle sprayer. In order to create conditions conducive for infection by the pathogen, all inoculated ears were immediately covered with polythene bag (22 °C, 90-100 % humidity) which were removed 48 h later. The un-inoculated control spikes were sprayed with distilled water and bagged in the same way.

In the second year, to produce the inoculum for wheat and maize, the fungi were grown on SNA medium in Petri dishes (Synthetic Nutrient Poor Agar, Carl Roth GmbH & Co. KG) for 2 weeks; subsequently, the conidia were washed off from these Petri dishes using sterile distilled water. The resulting conidial suspension was adjusted to a concentration of 1×10^5 spores ml^{-1} by adding sterile distilled water (after removing mycelium by straining the suspension through two layers of cheesecloth). The macroconidial suspension was quantified using a hemocytometer.

Maize inoculation was conducted by injecting 2 ml of inoculum per ear into the silk channel just below the tip where the silk emerges following the method of Chungu et al. (1996). Inoculation was done 6-7 days after mid silk emergence. The inoculated ears were covered after inoculation with plastic bags for 48 h to maintain high humidity (Fig. 12) in order to create conditions conducive for infection by the pathogen. The un-inoculated control treatment was sprayed with water and also covered with bags for 2 days.



Figure 12. The incubation of inoculated maize ears.

Maize ears were manually harvested at 20 % of grain moisture, then shelled and subsequently dried. Grains were thoroughly mixed in order to obtain a random distribution of kernels and a 50 g subsample was taken from each treatment for further analyses.

3.3.4 Disease evaluation in wheat

FHB incidence (proportion of diseased spikes) and conditional severity (proportion of disease spikelets per disease spike) were evaluated at 7, 14, and 21 days post-inoculation using a diagrammatic scale (Stack and McMullen, 1998). These two variables were used to calculate the FHB index, (Paul et al., 2010).

Disease incidence was defined as the percentage of spikes infected in the plot; which ranged from 0%- 100% (no infection- all spikes examined were infected). While disease severity was defined as the average percentage of each infected spike; also from 0% to 100%.

Disease incidence and Disease severity were used to calculate FHB index using the following formula: $\text{FHB index} = (\text{per cent disease incidence} \times \text{per cent disease severity})/100$

After maturity, spikes was harvested manually (grain moisture was 13-14 %), and the grains of each group were placed in separate bags, then threshed. The grain was weighed and 5 g random seed sample was selected from each plot to estimate the percentage of *Fusarium*-damaged kernels (FDK). A FDK was considered to be any seed that was shriveled, had mycelial growth or with characteristic chalky white or pink discoloration (Parry et al., 1995). FDK index was calculated using the following formula:

FDK index = (the number of shrivelled and chalky white kernels / the total number of kernels in a sample) \times 100.

3.3.5 DON analysis

Dried grain samples were milled in a laboratory mill (Variable-speed rotor mill pullverisette14, Fritsch, Idar-Oberstein, Germany). The sample material (10 g), additionally, a recovery sample containing 10 g material + 50 nmol DON were extracted using 30 ml of Acetonitrile + water (75+25) (v/v) in Erlenmeyer flasks and shaken for 1.5 h at room temperature (shaker, Edmund Bühler, Hechingen, Germany). Extracts (20 ml) were filtered (filter MN 615 ¼, Macherey- Nagel, Düren, Germany) and 8 ml of filtered extract were cleaned up using Mycosep 226 trich + columns (Coring System Diagnostix, Gernsheim, Germany). Four ml of cleaned extract were transferred to tubes (15 ml) and evaporated to dryness (Vacuum centrifuge S Concentrator BAVC- 300H, Saur, Reutlingen, Germany).

The residues were re-dissolved in 50 μ l acetonitrile (VWR, West Chester, Pennsylvania, USA) and transferred to glass vials (VWR) and stored at -70 °C until use. DON contents (for samples of the first year) and both DON of the extracts were determined by LC/MS analysis (LC/MS500- MS IT Mass Spectrometer Mass Workstation, Varian Inc., Palo Alto, California, USA). All parameters related to LC/MS are shown in table 9. The recovery sample was subjected to LC/MS under the same conditions described above to create the standard curve: Briefly, the LC/MS analysis was separated on a Polaris C18- A column (150x 3.0 mm; particle size distribution 5 μ m; stationary phase) (Varian Inc.). The column was kept at room temperature. The amount of injection volume was 10 μ l for each sample. Eluent A consisted of water/ 0.1% Formic acid and 2mM Ammonium format, while eluent B was acetonitrile.

The gradient elution began at 97% mobile phase A and at 3% mobile phase B. Then within 8 min, mobile phase B started at 97% and continued to 5min (13 min). After that, the mobile phase B decreased to 3%. The mass spectrometer was performed in the electrospray ionization (ESI) mode. Nitrogen was used as spray gas; temperature was set at 350 °C. While helium was damping gas. Mycotoxins were analyzed using selected reaction monitoring channels. The detection (quantification) limit was 5 pmol for DON.

Table 9. The parameters of LC/MS used in this study.

Mycotoxin	Retention (min)	Ionization	Adduct	Adduct mass (da)	Fragment (da)	Pointer tension (v)	Capillary tension (v)	RF- filling (%)	Fragmentation tension (v)
DON	7.9	0.70	HCOO ⁻	341	<u>265</u> 295	-4500	45	90	0.70

3.4 Aggressiveness of *Fusarium graminearum* isolates using several assays

3.4.1 Source of fungal isolates and fungal inoculum

The isolates of *F. graminearum* (to5, DSMZ 1095, Egy1) used in this study were provided by the Institute of Phytopathology, University of Kiel, being from Germany, USA, Egypt, respectively, and they are DON producers.

All isolates have been identified by morphological and molecular assessments which showed that all isolates used belonged to *F. graminearum*.

The chemotype (DON or NIV producer) was determined by LC/MS analysis, as described above. The *F. graminearum* isolates were propagated in liquid cultures in potato dextrose broth for 2-3 weeks at room temperature; then, the mixture of macroconidia mycelium suspension was filtered through a double layer of autoclaved cheesecloth. The final macroconidial concentrations of each isolate were adjusted to 1×10^6 conidia/ml for the Petri-dish test and 3×10^4 conidia/ml for the single floret assay in greenhouse.

3.4.2 Floret inoculation and disease severity assessment

The experiment was conducted in the greenhouse of the University of Kiel, Germany. Seeds of winter wheat (cultivar Ritmo, highly susceptible to *Fusarium* species) were vernalized in sterile sand for 6 weeks at 4°C. The vernalized seedlings were transferred to the greenhouse and grown under controlled conditions, using 100 heads per treatment (4 replicates, each one had 25 heads). In addition, control group was not inoculated, but sprayed with distilled water.

Wheat heads were inoculated at Zadoks growth stage (GS) 65 (Zadoks et al., 1974) by injection 20 μ L of each *F. graminearum* suspension (at a concentration of 3×10^4) into two adjacent florets (10 μ l per floret, left and right) at the middle of each spike (Fig. 13 A). In order to create conditions conducive for infection by the pathogen, all inoculated ears were immediately covered with polythene (22 °C, 90-100% humidity) which was removed 72 h later (Fig. 13, B). The un-inoculated control spikes were injected with distilled water and bagged in the same way. The assessment of aggressiveness indices was based on disease severity.

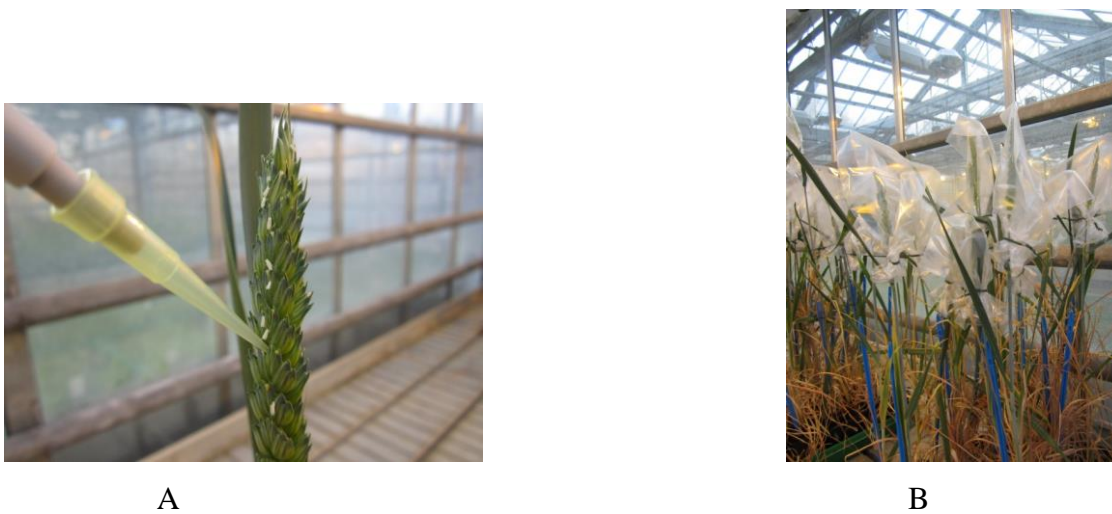


Figure 13. **A:** Floret inoculation method in wheat head. **B:** the incubation of inoculated wheat heads.

Head blight symptoms (disease severity) were evaluated on three dates (7, 14, and 21 days post-inoculation) using a diagrammatic scale (Stack and McMullen, 1998). Disease severity was used to describe the intensity of *Fusarium* damage which was expressed as the average percentage of each spike which was infected in the plot, on a scale of 0-100%. The re-isolation of *F. graminearum* was conducted from infected kernels for each isolate.

3.4.3 Petri-dish test

The Petri-dish test has been carried out according to Purahong et al. (2012). Briefly, Ritmo wheat seeds (highly susceptible to *Fusarium* spp.) were used, as well as the three *F. graminearum* isolates described above. There were four replicates for each isolate (25 healthy seeds/replicate). The seeds were submerged under the fungal inoculum (1×10^6 conidia/ml or

sterilized distilled water for control treatment) in the slanting Petri-dish thereafter, immediately aligned on the filter (Fig. 14). The germinated seeds were counted 2 days after inoculation (DAI) and this value was set at 100% germination, the sound coleoptiles were counted every day from 3 to 6 DAI. The area under healthy tissue progress curve was determined and converted to standardized area under disease progress curve (AUDPC_{standard}) and used as aggressiveness index of the fungal isolate as described by to Purahong et al. (2012).

$$\text{AUHPC} = \frac{B1 + 2B2 + 2B3 + 2B4 + B5}{2}$$

$$\text{AUDPC} = \frac{400 \text{ AUHPC}}{350}$$

AUHPC – area under healthy tissue progress curve.

B1- B5 – percent of healthy coleoptile from 2 to 6 day respectively
(B1 always – 100 %).

AUDPC standard – Standardized area under disease progress curve.



Figure 14. Germinated wheat seeds of Ritmo cultivar. 2 day after inoculation with *Fusarium graminearum* in Petri-dish test.

3.4.4 DNA extraction and quantification

The procedure was performed according to the manufacturer`s instructions with the following modification. Dried grain samples were milled in a laboratory mill. The sample material (100g) was transferred in a 2ml microcentrifuge tube and mixed with 900 µl of Lysis buffer

PL1 and 20 µl of RNase A, after vortexing (it was incubated at 65°C for 30 min, then, 10 µl Lyticase (Sigma-Aldrich, Taufkirchen, Germany) was added and the sample was vortexed again (Vortex-Genie-2mixer, Eydam, Kiel, Germany). The sample was incubated at 37°C, after 40 min, centrifuged at 1.500 ×g for 1 min. The NucleoSpin® plant II column with violet ring (NucleoSpin®plant II, Macherey-Nagel, Düren, Germany) was placed into a 2 ml collection tube and the sample was added and centrifuged until it was drawn through the membrane completely, 1000 µl buffer PC was added after vortexing, The NucleoSpin® plant II column with green ring was placed into a 2 ml collection tube and the sample was added and centrifuged. Then the membrane was washed two times with 500 µl of buffer (PW2, PW1), then were removed completely by performing a centrifugation step. Finally, the NucleoSpin®plant II column was placed in a clean 1.5 ml micro centrifuge tube and DNA was eluted with 50 µl of buffer PE (at 70°C) then centrifuged. DNA extracts were quantified (Nanodrop, General Electric Company, Fairfield, Connecticut, USA) and stored at -20°C. DNA Abundances of the extracts were determined by real-time PCR analysis.

3.4.5 Quantative PCR

QPCR is a quantitative PCR method quantifying the amount of template DNA input which allows the cycle-to-cycle monitoring of the amplified DNA (Higuchi et al., 1992). In this study, the Cyclex iQ™ Real Time PCR Detection System (Bio-Rad, Hercules, California, USA) was used for the quantification of DNA of *Fusarium* spp. in plant samples.

It was carried out in a total of 20 µl consisting of 10µl SYBR Green (SYBR Green Super – mix, Bio-Rad), 2 µl primer (each 1 µl , 10 pmol/1 µl), 2 µl template DNA and 6µl sterilized distilled water filled to 20 µl . Assays for each sample were performed in triplicate. Additionally, negative control containing no template was subjected to the same procedure in order to detect any possible DNA contamination. The primer for *F. graminearum* and maize-wheat plant were used which are shown in Table 10.

The PCR thermal cycling conditions were as follows, 95 C for 10min 40 cycles of 95°C for 15 s, 64°C for 1 min, followed by a melt curve analysis from 65 to 95°C (60 cycles, 5s for each cycle) to determine product specificity.

After the final amplification cycle, a melting curve was built by measuring the fluorescence which was constructed during the annealing stag of each cycle. After amplification, the

melting curves were determined by heating the samples to 95 °C for 1 min, subsequent cooling of samples to 55°C for 1 min; thereafter, the temperature was slowly increasing from 65°C to reach 95°C at the rate of 0.5°C per 10 s, by measuring the fluorescence continuously. To create the standard curve, dilutions (ranging from 0, 0.05, 0.5, 50 ng) of DNA *F. graminearum* which was previously determined, were subjected to qPCR under the same conditions described above. These dilutions can refer to the sensitivity of the real-time assay. The standard curve is defined by a plot of the Ct versus log DNA concentration. A threshold (Ct) value, the amount of fungal DNA was measured using the standard curve. Subsequently these values should be converted from DNA quantity (pg) determined in a PCR tube to DNA quantity in the plant sample (DNA plant material). The value determined by qPCR had to be multiplied with different factors for wheat or maize. Finally, these values were normalized with the DNA estimated amount in plant based on the following formula:

$$\text{Relative quotient} = \frac{E (\text{plant})^{z_{\text{plant}}}}{E (\text{fungi})^{z_{\text{fungi}}}}$$

E: Efficiency

Z: Cycle number

Quantification values were automatically constructed by the IQ™ optical system software version 3.1 (Bio-Rad) and the threshold cycle (Ct) values were then obtained.

3.5 Distribution of *Fusarium* species in maize plant

3.5.1 Experimental design

The silage maize used (NK, Nekta; not dressed) was cultivated on an experimental field at Farm Hohenschulen (University of Kiel, Rendsburg-Eckenförde, Germany). Seeds were sowed with density (105.000 seed/ha) in 20.4. 2013. The preparation for planting was done using stubble processing with grubber, where the land quality index was 50.

Regarding the soil character, it was a mixture of sandy and clayey loam soil while the soil type was para-brown earth. Combined pig slurry (40 m³) and 1.5 dt/ha NP fertilizer (20/20) were applied, the soil cultivating machine was rotary harrow. The maize crop was mono culture since 2009, additionally no fungicides were used during this experiment. Maize samples were collected from the experimental field representing a broad range of environmental conditions.

Ten plants were randomly collected and cut directly above the ground and at several physiological growth stages (stem elongation, heading, flowering, development of fruit, ripening). Single plants were fractionated with a flame-sterilized knife in several sections (node, internode, leaf sheath, leaf blade, rudimentary ears, and matured ears); the number of corresponding organs of each plant was depending on the stage of growth at each sample collection date. Additionally, the matured ear was sampled as spindle and kernels.

The corresponding organs of each plant were pooled, chopped, freeze-dried and homogenised by grinding 1000g of each sample in a laboratory mill in such a way that passed through the appropriate sieve with pore diameter of 0.2 mm. Subsequently, the samples were used for later analyses (DNA extraction, qPCR)

After DNA extraction, the samples were analysed to quantify and identify the accumulation and distribution of *Fusarium* species in different parts of maize plant using qPCR as described above. Additional primers used in this experiment are shown in Table 10.

Table 10. The primers used to determine the distribution of *Fusarium* species in maize plant Nicolaisen et al., 2009).

<i>Fusarium</i> species	Primer	Sequence (5`-3`)
<i>F. culmorum</i>	FculC561 fwd FculC614 rev	CACCGTCATTGGTATGTTGTCCT CGGGAGCGTCTGATAGTCG
<i>F. poae</i>	FpoaeA51 fwd FpoaeA98 rev	ACCGAATCTCAACTCCGCTTT GTCTGTCAAGCATGTTAGCACAAGT
<i>F. avenaceum</i>	Fave574 fwd Fave627 rev	TATGTTGTCCTGTCTCACACCACC AGAGGGATGTTAGCATGATGAAG
<i>F. graminearum</i>	FgB379fwd FgB411rev	CCATTCCCTGGGCGCT CCTATTGACAGGTGGTTAGTGACTGG
Plant-primer (maize-wheat)	Hor1f Hor2r	TCTCTGGGTTTGAGGGTGAC GGCCCTTGTAACCAGTCAAGGT

3.6 Systemic growth of *Fusarium graminearum* and *Fusarium culmorum* in maize plant

3.6.1 Experimental design

The silage maize (Ronaldinho; not dressed) was cultivated on an experimental field at Farm Hohenschulen (University of Kiel, Rendsburg-Eckenförde, Germany). The field plot, planted on 28 April, 2015 has been cropped with maize only since 2009.

The preparation for planting was done using stubble processing with grubber, where the land quality index was 50. The soil was a mixture of sandy and clayey loam soil while the soil type was para-brown earth. The fertilization was done by application of combined pig slurry (40 m³) and 1.5 dt/ha NP fertilizer (20/20), the soil cultivating machine was rotary harrow. Additionally no fungicides were used during this experiment.

Plants were arranged in a completely randomized design with three replicates of 10 plants for each treatment. Plants are randomly collected including the roots at several physiological growth stages (stem elongation, flowering, development of fruit, ripening). Single plants are fractionated in several sections (root, node, internode, rudimentary ears, and matured ears); the number of corresponding organs of each plant is depending on the stage of growth at each sample collection date. Additionally, the nodes and internodes were sampled, arranged and labelled from the first to the last one whereas; the first node was counted from the aerial roots. The same procedure was done for the ears. On other words, at the stem elongation growth stage, the plants had three nodes and two internodes, while the number of nodes and internodes were nine and eight, respectively, at the rest of maize growth stages (Fig. 15).

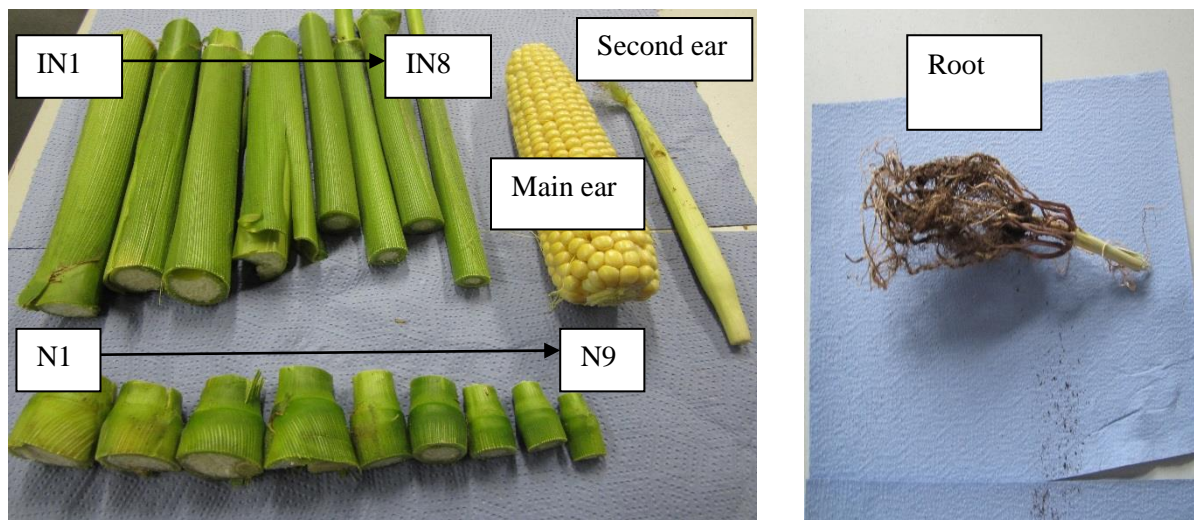


Figure 15. The sampled organs of the maize plant N: nodium, IN: internodium.

The corresponding organs of each plant were pooled, chopped and homogenised by grinding 100g of each sample in a laboratory mill in such a way that it passed through the appropriate sieve with pore diameter of 0.2 mm. Subsequently, the samples were used for later analyses (DNA extraction, qPCR) in order to quantify the concentration of *Fusarium graminearum* and *Fusarium culmorum* in sampled organs of the maize plant.

4. Chapter III

Results

The present work consists of six different experiments. For that reason, the results are presented separately for each one.

4.1 Sensitivity of *Fusarium* species isolates to metconazole and epoxiconazole and comparison of CYP51 sequence of *Fusarium* species with CYP51 of *Septoria tritici*

4.1.1 Sensitivity of *Fusarium* species to DMI fungicides

Sensitivities of isolates towards epoxiconazole and metconazole fungicides were determined by calculating percent growth inhibition at different concentrations (Table 11, Page 71. Table 12, Page 73). In this study, *Fusarium* spp. isolates showed different sensitivity levels to fungicides. Metconazole was the most effective by showing the highest inhibition of both mycelium growth and colony development compared to epoxiconazole. However, both fungicide treatments inhibited or reduced mycelium growth compared to the control at the six concentrations tested. There were significant differences ($P < 0.001$) between the treatments, with metconazole being most effective and epoxiconazole being least effective (Table 13, Page 74), as well as, significant differences ($P < 0.001$) between the concentrations in each fungicide. Only the concentrations 0.01 and 0.001 mol/l did not significantly affect mycelial growth on carrot agar medium amended with metconazole fungicide whereas the percentage of growth inhibition completely inhibited the mycelial growth for all isolates at these concentrations.

The efficacy of metconazole gradually increased with increasing concentration. All isolates did not form any mycelium on carrot agar medium amended with metconazole concentrations of 0.01, 0.001 and 0.0001 mol/l, indicating excellent activity against these isolates; therefore, those isolates were designated as sensitive isolates. Except six isolates named (1, 5, 11, 27, 28, 29), all were able to grow on medium containing the concentration 0.0001 mol/l; among them, the *Fusarium graminearum* isolate 11 showed the slowest growth at 0.0001 mol/l with growth inhibition of 93.4%. Due to this, all six isolates were designated as less sensitive strains. On the other hand, one isolate (*F. culmorum* III6) was able to grow only at concentrations of 0.000001 and 0.0000001 mol/l with percentage of growth inhibition reached 34.2 and 19.9 %, respectively, whereas, the remaining concentrations completely inhibited the mycelium growth for this isolate when added to the growth medium; as a result, it was

Table 11. Percent growth inhibition of *Fusarium* spp. isolates by metconazole fungicide.

<i>Fusarium</i> spp.	Fungicide concentration (mol/l)					
	0.01	0.001	0.0001	0.00001	0.000001	0.0000001
<i>F. graminearum</i> DSMZ 1095 (1)	100	100	86.8	82.9	49.5	11.9
<i>F. graminearum</i> DSMZ 1094 (11)	100	100	93.4	57.1	17.8	1.6
<i>F. graminearum</i> III7 (13)	100	100	100	55.2	19.1	5.4
<i>F. graminearum</i> AF15 (14)	100	100	100	48.6	10.3	0.0
<i>F. graminearum</i> Ck3 (15)	100	100	100	44.6	16.6	3.0
<i>F. graminearum</i> CK8 (16)	100	100	100	46.1	17.4	7.1
<i>F. graminearum</i> Dr8 (17)	100	100	100	45.7	16.3	3.4
<i>F. graminearum</i> Hs6 (18)	100	100	100	44.7	22.2	8.2
<i>F. graminearum</i> To3 (19)	100	100	100	48.6	10.4	0.0
<i>F. graminearum</i> To5 (20)	100	100	100	42.8	17.1	1.7
<i>F. graminearum</i> wtg (21)	100	100	100	40.2	16.5	3.7
<i>F. graminearum</i> Dr15 (37)	100	100	100	41.8	17.3	6.4
<i>F. graminearum</i> os12 (39)	100	100	100	43.7	15.9	4.8
<i>F. culmorum</i> VII2FP 2011 (2)	100	100	100	67.9	38.4	9.7
<i>F. culmorum</i> 8	100	100	100	40.7	13.9	6.8
<i>F. culmorum</i> I4 (30)	100	100	100	64.5	7.3	2.5
<i>F. culmorum</i> II4 (31)	100	100	100	57.6	28.8	15.9
<i>F. culmorum</i> III6 (32)	100	100	100	100	34.2	19.9
<i>F. culmorum</i> kr8 (33)	100	100	100	66.9	23.3	6.2
<i>F. culmorum</i> FU13 (34)	100	100	100	60.6	24.2	4.5
<i>F. culmorum</i> Sc9 (35)	100	100	100	44.8	20.4	10.2
<i>F. culmorum</i> wt2 (36)	100	100	100	66.9	17.6	7.8
<i>F. tricinctum</i> I1 (12)	100	100	100	45.3	15.7	4.8
<i>F. tricinctum</i> II1 (26)	100	100	100	47.4	17.2	6.1
<i>F. tricinctum</i> III3 (27)	100	100	63.7	57.6	21.8	19.5
<i>F. tricinctum</i> VIII3 (28)	100	100	65.4	51.3	23.0	17.1
<i>F. tricinctum</i> AJ13 (29)	100	100	73.8	52.7	26.8	20.1
<i>F. langsethiae</i> 9955 (3)	100	100	100	50.2	42.3	41.1
<i>F. sporotrichioides</i> DSMZ 62425 (5)	100	100	88.4	57.7	38.0	16.6
<i>F. poae</i> II 1 62376 (6)	100	100	100	72.9	29.5	18.7
<i>F. poae</i> III1 FP2011 (7)	100	100	100	49.2	35.2	16.1
<i>F. verticillioides</i> BASF1169 (9)	100	100	100	85.5	4.4	3.6
<i>F. venenatum</i> DSMZ 21739 (10)	100	100	100	43.7	23.8	5.7
<i>F. subglutinans</i> He7 (22)	100	100	100	54.2	28.9	6.9
<i>F. avenaceum</i> VI4 (23)	100	100	100	62.6	29.5	6.0
<i>F. crookwellense</i> II 6 (24)	100	100	100	59.5	26.0	9.0
<i>F. crookwellense</i> FU10 (38)	100	100	100	57.6	24.7	8.5
<i>F. sporotrichioides</i> IIFP (25)	100	100	100	60.8	37.3	9.9
Average for each concentration	100	100	96.7	55.8	23.4	9.8
	a	a	a	b	c	d

Means followed by the same letter are not significantly different.

the most sensitive isolate.

Concerning the sensitivity of *Fusarium* spp. isolates to epoxiconazole, its small efficiency was more pronounced compared to metconazole at similar concentrations. Here, the concentration (0.01 mol/l) was considerably less effective against all isolates compared to the same concentration of metconazole, demonstrating a significant difference ($P < 0.001$), except for isolate *F. graminearum* HS6, which growth was completely inhibited by epoxiconazole in a concentration of 0.01 mol/l.

The value of percent growth inhibition ranged from 79.8 % to 90.6% for the rest of isolates when the same concentration was added to the culture medium. However, the remaining *Fusarium* species isolates formed mycelium at all concentrations and their %growth inhibitions were largely distributed in similar ranges. The efficacy was obviously reduced at lower concentrations (Table 12).

Table 12. Percent growth inhibition of *Fusarium* spp. isolates by epoxiconazole fungicide.

<i>Fusarium</i> spp.	Fungicide concentration (mol/l)					
	0.01	0.001	0.0001	0.00001	0.000001	0.0000001
<i>F. graminearum</i> DSMZ 1095 (1)	80.0	62.0	31.0	27.3	9.0	6.8
<i>F. graminearum</i> DSMZ 1094 (11)	85.2	63.1	30.3	26.2	8.3	5.9
<i>F. graminearum</i> III7 (13)	79.8	61.9	30.1	25.3	9.0	5.8
<i>F. graminearum</i> AF15 (14)	89.3	65.1	47.8	30.3	17.7	14.6
<i>F. graminearum</i> Ck3 (15)	87.3	71.5	50.7	25.7	14.1	13.5
<i>F. graminearum</i> CK8 (16)	90.6	65.6	40.6	22.9	2.6	1.0
<i>F. graminearum</i> Dr8 (17)	81.6	67.0	40.0	20.5	17.3	5.5
<i>F. graminearum</i> Hs6 (18)	100	69.2	54.5	26.9	6.9	0.0
<i>F. graminearum</i> To3 (19)	81.6	67.0	40.0	20.5	17.3	5.5
<i>F. graminearum</i> To5 (20)	87.0	65.2	41.3	23.3	17.1	7.4
<i>F. graminearum</i> wt (21)	79.8	61.9	30.1	25.3	9.0	5.8
<i>F. graminearum</i> Dr15 (37)	88.3	65.1	47.8	30.3	16.7	10.6
<i>F. graminearum</i> os12 (39)	87.3	62.5	50.7	23.7	14.1	13.5
<i>F. culmorum</i> VII2FP 2011 (2)	82.6	66.0	40.0	20.8	17.7	5.5
<i>F. culmorum</i> 8	85.0	63.2	40.3	22.3	15.2	6.4
<i>F. culmorum</i> I4 (30)	87.0	66.2	42.3	22.3	17.1	7.4
<i>F. culmorum</i> II4 (31)	87.5	71.4	44.2	25.0	12.7	8.7
<i>F. culmorum</i> III6 (32)	83.6	67.0	40.3	20.5	17.3	5.4
<i>F. culmorum</i> kr8 (33)	85.5	65.2	41.3	23.3	15.1	7.4
<i>F. culmorum</i> FU13 (34)	87.2	70.2	46.2	25.0	15.7	12.7
<i>F. culmorum</i> Sc9 (35)	81.6	67.0	40.0	20.5	13.3	5.5
<i>F. culmorum</i> wt2 (36)	87.0	65.2	41.3	24.3	16.8	7.4
<i>F. tricinctum</i> II (12)	83.6	66.2	45.3	25.5	14.3	6.4
<i>F. tricinctum</i> III (26)	83.6	67.0	40.3	20.5	17.3	5.4
<i>F. tricinctum</i> III3 (27)	84.6	68.0	42.3	22.5	15.3	7.4
<i>F. tricinctum</i> VIII3 (28)	83.9	65.4	44.3	22.7	14.3	6.4
<i>F. tricinctum</i> AJ13 (29)	85.6	62.8	43.3	24.5	16.3	5.8
<i>F. langsethiae</i> 9955 (3)	83.5	60.7	42.7	23.8	14.2	5.1
<i>F. langsethiae</i> 8081 (4)	85.5	65.2	40.0	20.5	13.6	5.9
<i>F. sporotrichioides</i> DSMZ 62425 (5)	83.6	68.1	43.3	22.5	15.3	6.4
<i>F. poae</i> II 1 62376 (6)	81.6	67.0	40.0	20.5	13.3	8.5
<i>F. poae</i> III1 FP2011 (7)	87.0	64.2	40.3	24.3	15.8	5.4
<i>F. verticillioides</i> BASF1169 (9)	87.0	66.2	42.3	23.3	17.1	7.4
<i>F. venenatum</i> DSMZ 21739 (10)	86.5	67.4	44.2	24.0	12.7	7.7
<i>F. subglutinans</i> He7 (22)	81.8	67.0	40.0	20.8	13.7	4.5
<i>F. avenaceum</i> VI4 (23)	87.0	65.2	42.3	27.3	16.8	6.4
<i>F. crookwellense</i> II 6 (24)	87.8	64.2	41.3	25.3	16.8	5.4
<i>F. crookwellense</i> FU10 (38)	81.1	67.9	40.0	20.5	13.3	5.5
<i>F. sporotrichioides</i> IIFP (25)	85.0	65.2	41.3	24.3	16.8	7.4
Average for each concentration	84.7a	b	c	d	e	f

Means followed by different letter are significantly different.

Table 13. Significant differences between the effect of the concentrations for metconazole and epoxiconazole on the mycelial growth of *Fusarium* species isolates tested. Me: metconazole. Ep: epoxiconazole.

	0.01	0.001	0.0001	0.00001	0.000001	0.0000001
Me	100 a	100 a	96.7 a	55.8 a	23.4 a	9.8 a
Ep	84b	65.6 b	41.4 b	23.4 b	14.3 b	6.7 b

Means followed by different letter in the column are significantly different.

The statistical software R (2014) was used to evaluate the data. The data evaluation started with the definition of an appropriate statistical mixed model. The data were assumed to be approximately normally homoscedastic. These assumptions are based on graphical residual analysis. Statistical model included fungicide (metconazole, epoxiconazole) and concentration (0.01, 0.001, 0.0001, 0.00001, 0.000001 and 0.0000001) as well as their interaction term as fixed factors. Based on this model, an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests in order to compare the several levels of the influence factors.

4.1.2 Nucleotide sequence of CYP51 from *Fusarium* species isolates with different sensitivities to metconazole

Analysis of DNA sequence of CYP51 of *Fusarium* spp. isolates showed that the sequence of CYP51B (FGSG 01003.3) had the highest homology to CYP51 of those isolates tested. Thus, CYP51 gene (FGSG 01003.3) was found to be expressed in the cells of *Fusarium* spp. species isolates but both of the expressions CYP51C (FGSG 11024.3) and CYP51A (FGSG 04092.3) could not be detected.

The sequence of CYP51B (FGSG 01003.3) obtained from genomic DNA was determined by PCR using the previously described primers. The expression of this gene in 11 *Fusarium* species isolates was investigated which had been selected according to their covering the range of DMI sensitivities and their importance in Germany. The deduced sequence of 11 *Fusarium* species isolates were compared with

CYP51B from the *Fusarium* comparative database. Due to that, it was found that the complete length of their cDNA was estimated to be 1578 bp (without stop codon), while the deduced protein was 526 amino acid residues which is in agreement with the sequence in the *Fusarium* comparative database.

The sequences of CYP51B genes of 11 *Fusarium* species selected (1, 5, 6, 7, 14, 15, 16, 18, 19, 20, 32) were similar, except for two isolates. Concerning the first of these two isolates, *Fusarium graminearum* DSMZ1095 (1), six bases were observed to differ among this and the other isolates (C1284T, C1350T, T1365C, C1380T, T1440G, C1467T), and all those differences were silent substitution which did not cause any change in deduced amino acids. With regard to the second isolate, *Fusarium graminearum* Hs6 (18) had 63 bases different from the other isolates. Among these nucleotide substitutions, only seven caused amino acid substitutions (E352D, V364I, V393I, F413Y, K438R, E440D, H500N) and the other differences were silent substitutions. However, all seven point mutations (missense) were not associated with sensitivity levels of DMI. The results including the comparison of the CYP51 nucleotide sequences based on the FGSG 01003.3 sequence and the deduced amino acid sequences are shown in Fig. 16, pages 75-77.

1080

<i>F. gr</i> (1)	-----T-----A-----T---
<i>F. sp</i> (5)	-----T-----A-----T---
<i>F. po</i> (6)	-----T-----A-----T---
<i>F. po</i> (7)	-----T-----A-----T---
<i>F. gr</i> (14)	-----T-----A-----T---
<i>F. gr</i> (15)	-----T-----A-----T---
<i>F. gr</i> (16)	-----T-----A-----T---
<i>F. gr</i> (18)	-----C-----C-----C---
<i>F. gr</i> (19)	-----T-----A-----T---
<i>F. gr</i> (20)	-----T-----A-----T---
<i>F. gr</i> (32)	-----T-----A-----T---
<i>F. gr</i> CYP 51B	-----T-----A-----T---

1140

<i>F. gr</i> (1)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. sp</i> (5)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. po</i> (6)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. po</i> (7)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. gr</i> (14)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. gr</i> (15)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. gr</i> (16)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. gr</i> (18)	----C--A-----T--C--C--T--C-----T----T-----
<i>F. gr</i> (19)	----T--G-----C--T--T--C--T-----C----C-----
<i>F. gr</i> (20)	----T--G-----C--T--T--C--T-----C----C-----

F. gr (32) -----T---G-----C---T---T---C---T-----C---C-----
F.gr CYP 51B -----T---G-----C---T---T---C---T-----C---C-----

1200

F. gr (1) -----C-----C---T---GT---T---C---T-----
F. sp (1) -----C-----C---T---GT---T---C---T-----
F. po (1) -----C-----C---T---GT---T---C---T-----
F. po (1) -----C-----C---T---GT---T---C---T-----
F. gr (14) -----C-----C---T---GT---T---C---T-----
F. gr (15) -----C-----C---T---GT---T---C---T-----
F. gr (16) -----C-----C---T---GT---T---C---T-----
F. gr (18) -----T-----A---C---AC---C---G---C-----
F. gr (19) -----C-----C---T---GT---T---C---T-----
F. gr (20) -----C-----C---T---GT---T---C---T-----
F. gr (32) -----C-----C---T---GT---T---C---T-----
F.gr CYP 51B -----C-----C---T---GT---T---C---T-----

1260

F. gr (1) -----T-----C---T---T-----T-----
F. sp (5) -----T-----C---T---T-----T-----
F. po (6) -----T-----C---T---T-----T-----
F. po (7) -----T-----C---T---T-----T-----
F. gr (14) -----T-----C---T---T-----T-----
F. gr (15) -----T-----C---T---T-----T-----
F. gr (16) -----T-----C---T---T-----T-----
F. gr (17) -----T-----C---T---T-----T-----
F. gr (18) -----C-----T---G---A-----C-----
F. gr (20) -----T-----C---T---T-----T-----
F. gr (32) -----T-----C---T---T-----T-----
F.gr CYP 51B -----T-----C---T---T-----T-----

1320

F. gr (1) --C--T-----T-----C-C-----CAAG---G
F. sp (5) --C--T-----C-----C-C-----CAAG---G
F. po (6) --C--T-----C-----C-C-----CAAG---G
F. po (7) --C--T-----C-----C-C-----CAAG---G
F. gr (14) --C--T-----C-----C-C-----CAAG---G
F. gr (15) --C--T-----C-----C-C-----CAAG---G
F. gr (16) --C--T-----C-----C-C-----CAAG---G
F. gr (18) --T--C-----C-----A-A-----TCGT---T
F. gr (19) --C--T-----C-----C-C-----CAAG---G
F. gr (20) --C--T-----C-----C-C-----CAAG---G
F. gr (32) --C--T-----C-----C-C-----CAAG---G
F.gr CYP 51B --C--T-----C-----C-C-----CAAG---G

1380

F. gr (1) -----G--G-----C---T---T-----C---A---T---C---T
F. sp (5) -----G--G-----C---T---C-----T---A---T---C---C
F. po (6) -----G--G-----C---T---C-----T---A---T---C---C
F. po (7) -----G--G-----C---T---C-----T---A---T---C---C
F. gr (14) -----G--G-----C---T---C-----T---A---T---C---C
F. gr (15) -----G--G-----C---T---C-----T---A---T---C---C
F. gr (16) -----G--G-----C---T---C-----T---A---T---C---C
F. gr (18) -----A--A-----T---C---T-----T---C---C---T---T
F. gr (19) -----G--G-----C---T---C-----T---A---T---C---C
F. gr (20) -----G--G-----C---T---C-----T---A---T---C---C
F. cu (32) -----G--G-----C---T---C-----T---A---T---C---C

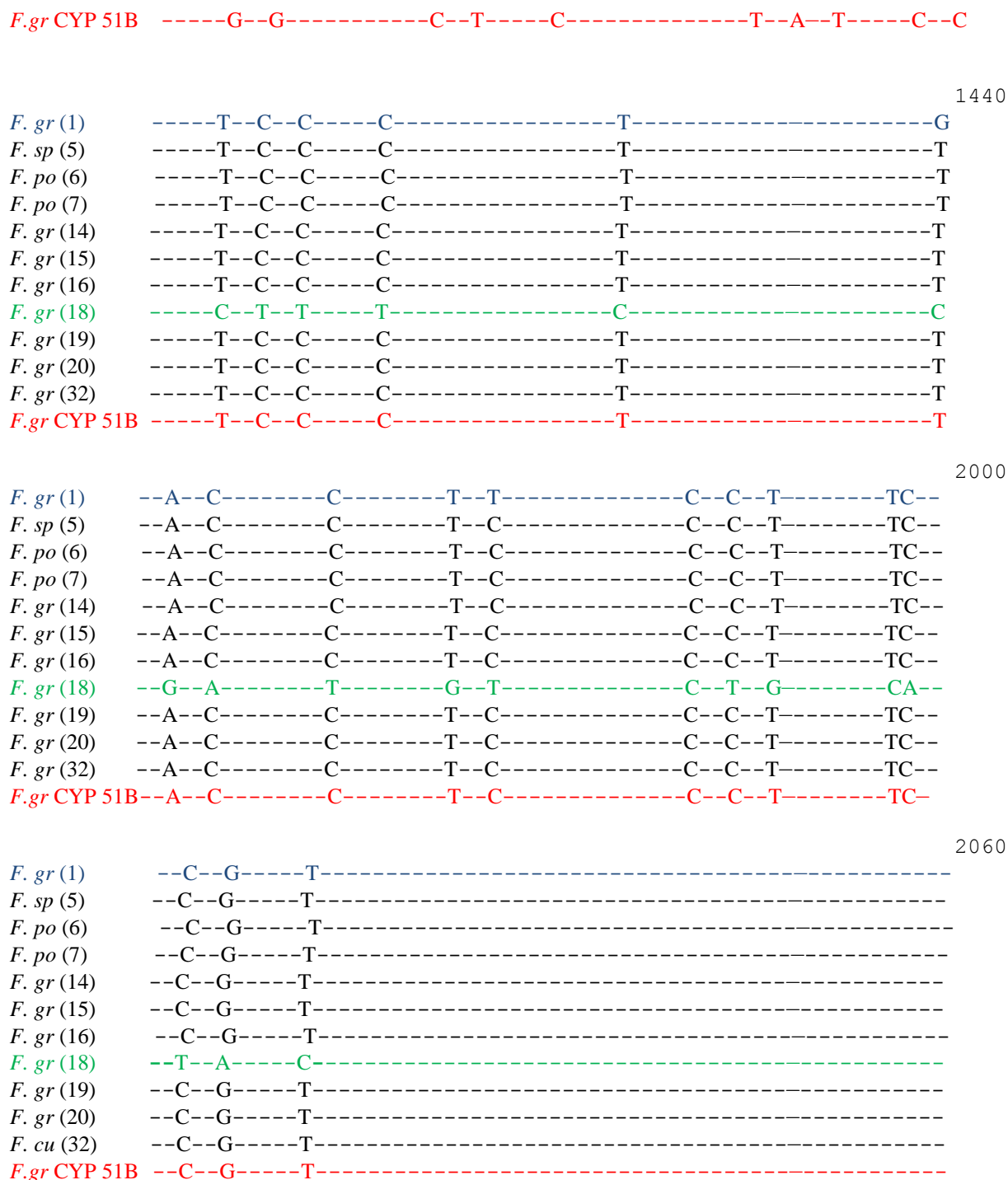


Figure 16. Alignment describes the differences between the nucleotide sequences of the homologues of CYP51gene (FGSG 01003.3) and *Fusarium* spp. isolates. Identical residues are marked by short dash (the values in brackets refer to the number of isolate). *F. graminearum* (*F. gr*). *F. culmorum* (*F. cu*). *F. poae* (*F. po*). *F. sporotrichioides* (*F. sp*). *F. gr* CYP51B (*F. graminearum* (FGSG 01003.3), the Broad Institute, USA).

Regarding *Fusarium graminearum* DSMZ1095 (1), all those differences of DNA sequence were silent substitution which did not cause any change in deduced amino acids. Whereas the deduced amino acid of the homologues of CYP51gene (FGSG 01003.3) and this isolate were

identical at the amino acid positions (352, 364, 393, 413, 438, 440, 550) representing by (glutamic acid, valine, valine, phenylalanine, lysine. glutamic acid, histidine) (Table 14).

Table 14. Differences between the deduced amino acid of the homologues of CYP51 gene (FGSG 01003.3) and of *Fusarium* spp. isolates (the values in brackets refer to the number of the isolate). *F. graminearum* (*F. gr*). *F. culmorum* (*F. cu*). *F. poae* (*F. po*). *F. sporotrichioides* (*F. sp*). *F. gr* CYP51B (*F. graminearum* (FGSG 01003.3), the Broad Institute, USA).

<i>Fusarium</i> spp.	Amino acid position						
	352	364	393	413	438	440	500
<i>F. gr</i> (1)	E	V	V	F	K	E	H
<i>F. sp</i> (5)	E	V	V	F	K	E	H
<i>F. po</i> (6)	E	V	V	F	K	E	H
<i>F. po</i> (7)	E	V	V	F	K	E	H
<i>F. gr</i> (14)	E	V	V	F	K	E	H
<i>F. gr</i> (15)	E	V	V	F	K	E	H
<i>F. gr</i> (16)	E	V	V	F	K	E	H
<i>F. gr</i> (18)	D	I	I	Y	R	D	N
<i>F. gr</i> (19)	E	V	V	F	K	E	H
<i>F. gr</i> (20)	E	V	V	F	K	E	H
<i>F. cu</i> (34)	E	V	V	F	K	E	H
<i>F. gr</i> CYP51B	E	V	V	F	K	E	H

Abbreviation. E: glutamic acid, V: valine, F: phenylalanine, K: lysine. D: aspartic acid, R: arginine, N: asparagine. I: isoleucine, H: histidine, Y: tyrosine.

With regard to the second isolate, *Fusarium graminearum* Hs6 (18) had 63 bases different from the other isolates. Among these nucleotide substitutions, only seven caused amino acid substitutions (E352D, V364I, V393I, F413Y, K438R, E440D, H500N) and the other differences were silent substitutions. However, all seven point mutations (missense) were not associated with sensitivity levels of DMI (Table 15).

Table 15. Comparison of the deduced amino acid CYP51 for different isolates of *Fusarium* and for wild and mutant type of *Septoria tritici* at certain positions. *F. graminearum* (*F. gr.*), *F. culmorum* (*F. cu.*), *F. poae* (*F. po.*), *F. sporotrichioides* (*F. sp.*), *F. gr* CYP51B (*F. graminearum* (FGSG 01003.3), the Broad Institute, USA).

Isolate	Position of amino acid												
	50	134	136	137	188	379	381	459	460	461	462	513	524
<i>F. gr</i> (1)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. sp</i> (5)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. po</i> (6)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. po</i> (7)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. gr</i> (14)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. gr</i> (15)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. gr</i> (16)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. gr</i> (18)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
<i>F. gr</i> (19)	P	D	V	Y	S	A	I	Y	G	Y	G	T	S
Wild type <i>Septoria tritici</i>	L	D	V	Y	S	A	I	Y	G	Y	G	N	S
Resistant <i>Septoria tritici</i>	S	G	A	F	N	G	V	S/ D / N de l	D / de l	S / H / de l	de l	K	T

Abbreviations. V: valine, E: glutamic acid, F: phenylalanine, K: lysine. D: aspartic acid, G: glycine, N: asparagine. I: isoleucine, Y: tyrosine, S: serine. A: alanine, P: proline, L: leucine, T: threonine. Del: deletion mutation.

In terms of the comparison of deduced amino sequence of CYP51 in *Fusarium* spp. isolates with mutant and wild type of *S. tritici*, it was observed that the deduced amino acid sequences

were identical at positions (134, 136, 137, 188, 379, 381, 459, 460, 461, 462, 524) with those in wild type of *S. tritici* representing by aspartic acid, valine, tyrosine, serine, alanine, isoleucine, tyrosine, glycine, tyrosine, glycine, threonine, respectively. Except at two position (50, 513), but at the same time, these changes are not identical with the amino acids in CYP51 mutant *S. tritici*. Whereas the amino acid at both positions of resistant *Septoria tritici* are serine and threonine, respectively, while the amino acid sequence of of CYP51 in *Fusarium* spp. isolates at the same positions were proline and serine, respectively.

As a result, the differences in sensitivity to metconazole and epoxiconazole between the isolates were not associated with substitutions of amino acids in CYP51 at specific positions. In other words, all used isolates have the same amino acid polymorphism in the CYP51 gene of wild type of *S. tritici*, in spite of their various sensitivity levels to DMI fungicides. Consequently, there is no correlation between mutations in this gene and sensitivity levels to DMI fungicides.

4.2 *In vitro* sensitivity of isolates of *Fusarium* species to carbendazim and investigation of the contribution of β -tubulin to its sensitivity

Sensitivities of isolates towards carbendazim were determined by calculating percent growth inhibition at different concentrations (Table16). In this study, percentage growth inhibitions of *F. graminearum* were largely distributed in similar ranges at all concentrations.

All isolates did not form any mycelium on carrot agar medium amended with concentrations of 0.01 and 0.001 mol/l carbendazim, indicating excellent activity against these isolates at these concentrations. However, the efficacy was obviously reduced at lower concentrations, with percentage growth inhibition of 27.2 - 44.2% at the concentration 0.0001 mol/l while the other concentrations (0.00001, 0.000001, 0.0000001 mol/l) did not have any effect on mycelial growth whereas the percentage of growth inhibition was 0% compared to the control. Here the fungal growth was continuously bigger compared to the control.

The statistical software R (2014) was used to evaluate the data as it mentioned above.

Table 16. Percent growth inhibition of *Fusarium graminearum* strains by carbendazim fungicide.

<i>Fusarium</i> spp.	Fungicide concentration (mol/l)					
	0.01	0.001	0.0001	0.00001	0.000001	0.0000001
<i>F. graminearum</i> DSMZ 1095 (1)	100	100	40.2	0	0	0
<i>F. graminearum</i> DSMZ 1094 (11)	100	100	40.7	0	0	0
<i>F. graminearum</i> III7 (13)	100	100	36.7	0	0	0
<i>F. graminearum</i> AF15 (14)	100	100	37.1	0	0	0
<i>F. graminearum</i> Ck3 (15)	100	100	44.2	0	0	0
<i>F. graminearum</i> CK8 (16)	100	100	36.8	0	0	0
<i>F. graminearum</i> Dr8 (17)	100	100	37.6	0	0	0
<i>F. graminearum</i> Hs6 (18)	100	100	35.3	0	0	0
<i>F. graminearum</i> To3 (19)	100	100	35.6	0	0	0
<i>F. graminearum</i> To5 (20)	100	100	38.2	0	0	0
<i>F. graminearum</i> wtg (21)	100	100	37.8	0	0	0
<i>F. graminearum</i> Dr15 (37)	100	100	27.2	0	0	0
<i>F. graminearum</i> os12 (39)	100	100	35.7	0	0	0
<i>F. graminearum</i> (average)	100a	100a	34.6 b	0c	0c	0c

Means followed by different letter are significantly different.

In terms of the comparison of deduced amino acid sequence of β 2-tubulin gene in *Fusarium graminearum* isolates, DNA sequence of β 2-tubulin (Genbank access number FG 06611.1) in four isolates was analysed and determined. Our results showed that a point mutation at position 198 in tested isolates was identified; this mutation results in a single amino acid substitution, where glutamic acid was converted to glutamine in all isolates (Fig. 17).

198

F. gr (Genbank) EEFPDRMMATFSVMPSPKVSDTVVEPYNATLSLNQLVENS D **E** TFCIDNEALYD
F. gr (1) EEFPDRMMATFSVMPSPKVSDTVVEPYNATLSLNQLVENS D **Q** TFCIDNEALYD
F. gr (11) EEFPDRMMATFSVMPSPKVSDTVVEPYNATLSLNQLVENS D **Q** TFCIDNEALYD
F. gr (18) EEFPDRMMATFSVMPSPKVSDTVVEPYNATLSLNQLVENS D **Q** TFCIDNEALYD
F. gr (20) EEFPDRMMATFSVMPSPKVSDTVVEPYNATLSLNQLVENS D **Q** TFCIDNEALYD

Figure 17. Alignment of the deduced amino acid sequence (positions 157 to 209) of the β 2-tubulin (*tub2*) gene from isolates of *Fusarium graminearum*. Abbreviations. E: glutamic acid, V: valine, F: phenylalanine, K: lysine. D: aspartic acid, R: arginine, N: asparagine. I: isoleucine, H: histidine. Y: tyrosine, S: serine. A: alanine, P: Proline. Q: glutamine. A: alanine. M: methionine. T: threonine.

4.3 Application timing and dose rate of triazole alone or in mixture with pyraclostrobin for the control of *Fusarium* head blight and deoxynivalenol in wheat and maize

4.3.1 Visual assessment of FHB

4.3.1.1 First trial

Three commercially available fungicides [metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany)] were used in the experimental work in the first year on wheat.

Effect of fungicide treatments differed, depending on the application form. For applications prior to inoculation and also for treatment during inoculation, we found all fungicides tested to be effective at the full and half recommended dosage. In all cases, the treatments significantly ($P < 0.001$) reduced FHB index compared to the non-treated control, indicating excellent activity against FHB. However, there were significant differences between the treatments with metconazole being most effective and epoxiconazole alone being least effective (Table 17).

Table 17. Significance of mean square values for effect of fungicides (dosage, application time) on the *Fusarium* head blight index (FHB index %) during the first trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin.

Fungicide (dosage)	FHB index				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	1 a	0 a	0 a	8.7 b	10.5 bc
Met (50%)	1.5 a	0.3 a	0 a	24 b	31.6 c
Epox (100%)	7.6 a	1.7 b	0.7 a	12.2 c	12.8 c
Epox (50%)	2.6 a	1.7 a	0.9 a	16.5 b	16 bc
Epox + Pyra (100%)	0.9 a	0.8 a	0 a	16 b	18 bc
Epox + Pyra (50%)	2.6 a	0.8 b	0.3 a	26.7 c	29 cd

Means followed by the same letter in row are not significantly different.

On 3d prior to inoculation, both metconazole and a mixture of epoxiconazole and pyraclostrobin largely reduced FHB index reaching level of 94% compared to the control. On

the other hand, the other treatment showed lower efficiency with reduction of 54% when applied at this time point. On 1 d prior to inoculation, all treatments showed a high efficiency with reduction ranged 89-100% compared to the control. For application during inoculation the efficiency observed were more pronounced, here both metconazole treatment and the mixture treatment completely inhibited FHB index when they applied at this time point. On the other hand, epoxiconazole treatment showed lower efficiency with reduction reached 95% compared to the control.

For spray after inoculation the differences observed were more pronounced. Again, the dose 100% was most effective for metconazole and considerably less effective for epoxiconazole and pyraclostrobin mixture and epoxiconazole alone when applied 1d post-inoculation. On 3d post-inoculation metconazole treatment still resulted in significant reduction of FHB index, while the other two fungicides had no effect at this time point (Fig. 18).

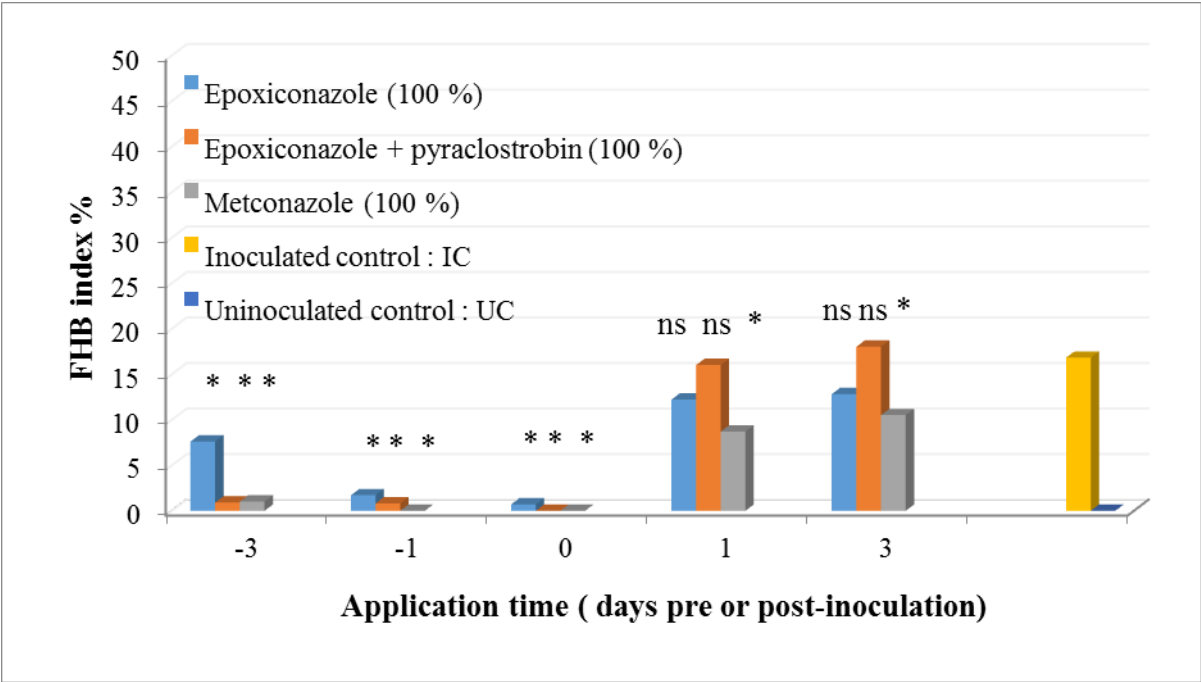


Figure 18. *Fusarium* head blight index (FHB index) on wheat grain during the first trial (dose 100%). *significant, ns not significant (compared with inoculated control).

Regard with dose 50%, the treatments significantly ($P < 0.001$) reduced FHB index compared to the non-treated control when applied prior to inoculation and also for treatment during inoculation, indicating excellent efficiency against FHB at these time points. However, there were significant differences between the applications with metconazole being most effective and epoxiconazole alone being least effective (Table 17). On 3d prior to inoculation, both epoxiconazole alone or in a mixture with pyraclostrobin reduced FHB index reaching level of

84% compared to the control, while this value was 91% for the metconazole treatment when applied at this time point. On the other hand, the efficiency of all treatments increased and ranged 89-98% when they applied on 1d prior to inoculation. For application during inoculation the efficiency observed were more pronounced, here both metconazole treatment and the mixture treatment largely reduced FHB index reaching level of 98% compared to the control, while epoxiconazole treatment showed lower efficiency with reduction reached 94% compared to the control when applied during the inoculation.

The dose 50%, on the other hand, showed no curative activity for any of the tested fungicides, but rather led to an increased FHB index compared to the control, indicating that the lower (sublethal) dose is not effective as a curative treatment. (Fig. 19)

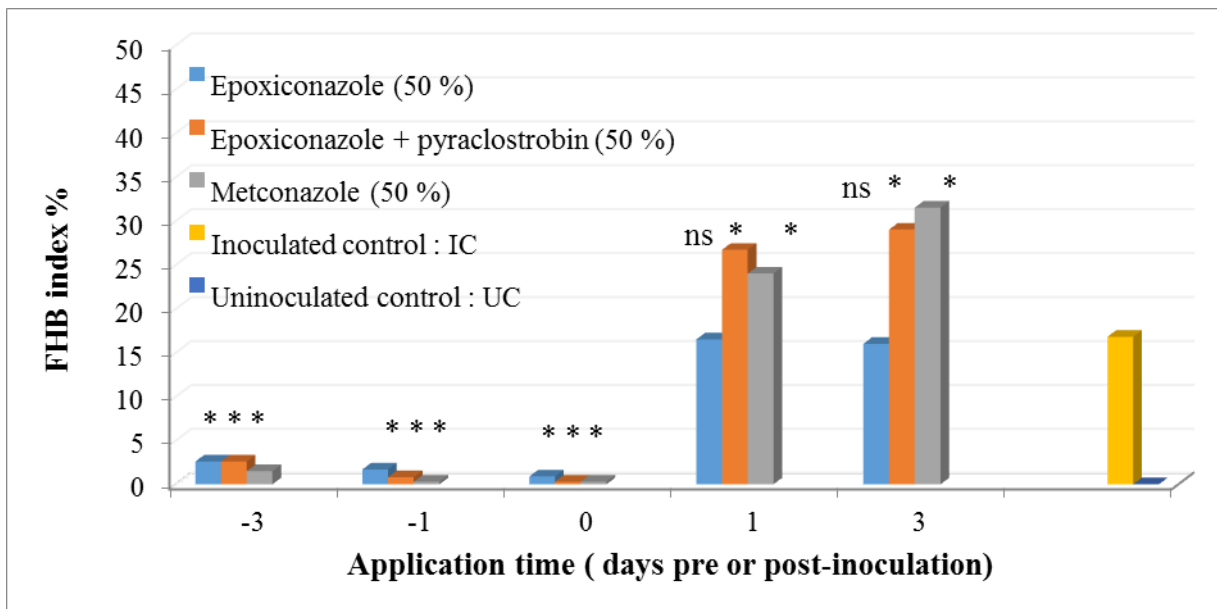


Figure 19. *Fusarium* head blight index (FHB index) on wheat grain during the first trial (dose 50%).*significant, ns not significant (compared with inoculated control).

4.3.1.2 Second trail

Four commercially available fungicides [metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany), pyraclostrobin (F500, BASF, Germany)] were used in the experimental work in the second year on wheat and maize.

The results were roughly similar to the previous year. Here, a new treatment of pyraclostrobin (F500, BASF, Germany) was added on wheat. In this work, the effect of fungicide depended on the application form. With regard to the applications prior to inoculation and during inoculation, all fungicides were effective at the full and half recommended dosage. In all cases, the application of fungicides significantly ($P < 0.001$) reduced FHB index compared to the inoculated control indicating an excellent efficacy against this index. However, we found significant differences between the applications with metconazole being most effective and the epoxiconazole and pyraclostrobin mixture being least effective (Table 18, Fig. 20). On 3d prior to inoculation, all treatments reduced FHB index ranging 93- 96% compared to the control, while this reduction increased and ranged 93-97% for all treatments when applied at 1d prior-inoculation. On the other hand, the efficiency of all treatments was 100% when they applied during- inoculation.

Table 18. Significance of mean square values for effect of fungicides (dosage, application time) on the *Fusarium* head blight index (FHB index %, dose 100%) during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	FHB index				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	1.3a	1a	0a	3.8a	5.3a
Epox (100%)	1.7a	2.3a	0a	27.3bc	25.7bc
Epox + Pyra (100%)	2.2a	3.9b	0a	25c	26.7c
Pyra (100%)	2.3a	2.3a	0a	19.2ac	24.6ac

Means followed by the same letter in column are not significantly different

Regarding sprays after inoculation, the differences observed were more pronounced. Again, the dose 100% was most effective for metconazole and considerably less effective for the epoxiconazole and pyraclostrobin mixture and epoxiconazole alone when applied 1d post-inoculation, whereas metconazole treatment reduced this index by 88.7% compared to the inoculated control while the reduction was 25.8 and 18.9 %, respectively, for the other treatments. On 3d post-inoculation metconazole treatment still resulted in a significant reduction of FHB index reaching a level of 84.3%, while the other fungicides only had a small effect at this time point.

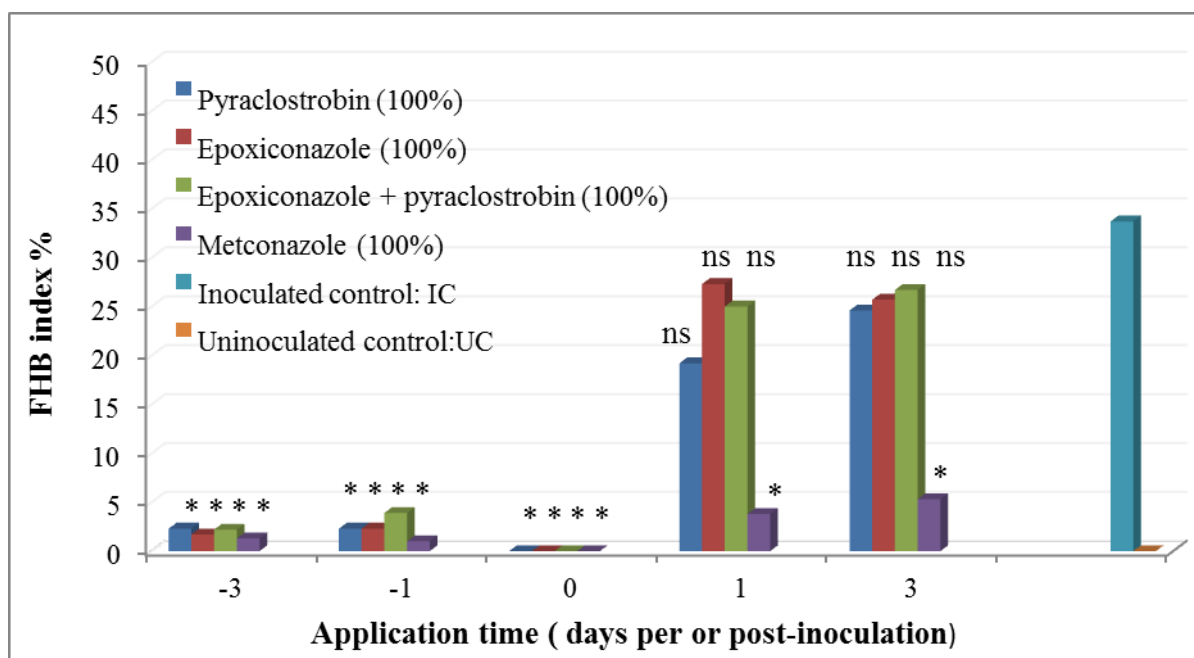


Figure 20. *Fusarium* head blight index (FHB index) on wheat grain during the second trial (dose 100%). *significant, ns not significant (compared with inoculated control).

Regard with dose 50%, the treatments significantly ($P < 0.001$) reduced FHB index compared to the non-treated control when applied prior- inoculation and also for treatment during inoculation, indicating excellent efficiency against FHB at these time points. However, there were significant differences between the applications with metconazole being most effective and epoxiconazole alone being least effective (Table 19).

Table 19. Significance of mean square values for effect of fungicides (dosage, application time) on the *Fusarium* head blight index (FHB index %, dose 50%) during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	FHB index				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (50%)	1,3a	1a	0a	20a	20,4a
Epox (50%)	1,3a	1a	0a	30ab	32,3abc
Epox + Pyra (50%)	0,5a	0,5a	0a	36b	37,7b
Pyra (50%)	0,6a	0,7a	0a	23,3ab	29ac

Means followed by the same letter in column are not significantly different.

On 3d prior-inoculation, both epoxiconazole alone or in a mixture with pyraclostrobin reduced FHB index reaching level of 93 and 97%, respectively, compared to the control, while this value was 93% for the metconazole treatment and 96% for pyraclostrobin alone

when applied at this time point. On the other hand, the efficiency of all treatments increased and ranged 94-97% when they applied on 1d prior to inoculation. For application during-inoculation the efficiency observed were more pronounced, here all treatments were able to completely inhibit FHB index compared to the control at this time point.

The dose 50%, on the other hand, showed no curative activity for any of the tested fungicides, but rather led to an increased FHB index compared to the control for all application, whereas FHB index for all application was 20-36% and 20.4-37.7% when they were sprayed on 1d or 3d post-inoculation, respectively, while this index was 19.3 % for the inoculated control, indicating that the lower (sublethal) dose is not effective as a curative treatment (Table 19, Fig. 21). It is worthwhile to refer that this trial with dose 50% was separately conducted from the dose 100%, due to that, each one has a different control.

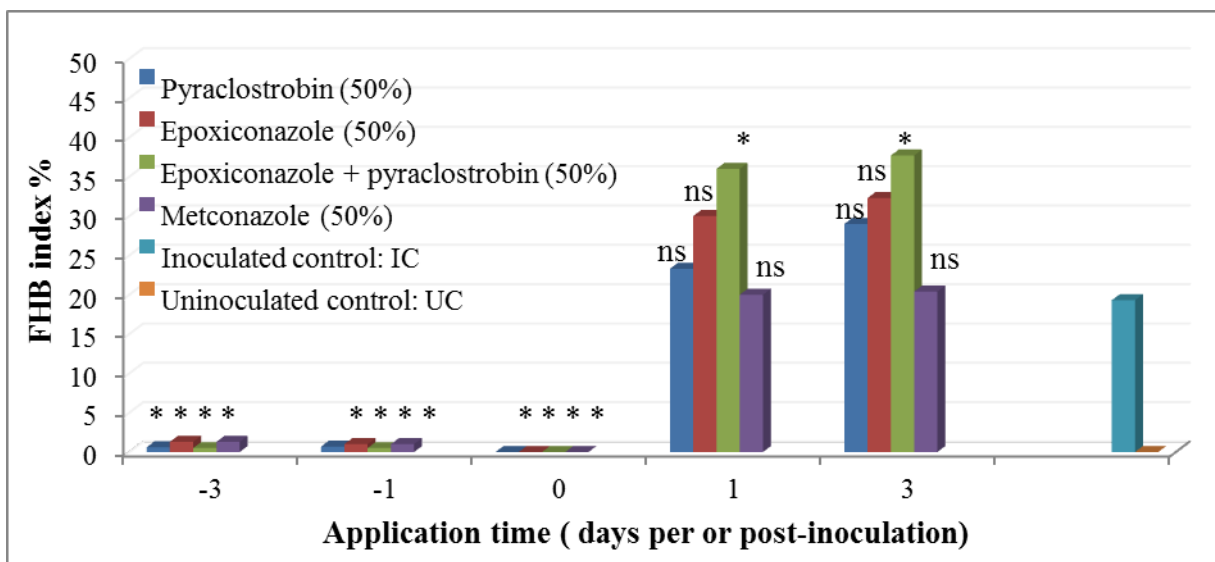


Figure 21. *Fusarium* head blight index (FHB index) on wheat grain during the second trial (dose 50%). *significant, ns not significant (compared with inoculated control).

4.3.2 FDK percentage

4.3.2.1 First trial

Three commercially available fungicides [metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany)] were used in the experimental work during the first year on wheat.

All of the fungicide treatments reduced FDK compared with the control at dose 100%, but there were significant differences among the fungicides (Table 20, Fig. 22). On 3d prior-inoculation, both epoxiconazole + pyraclostrobin mixture and metconazole treatment reduced FDK level reaching level of 94% compared to the control, while epoxiconazole alone showed lower reduction with 82% when applied at this time point.

Table 20. Significance of mean square values for effect of fungicides (dosage, application time) on Percentage of *Fusarium*- damaged kernels (FDK) in wheat during the first trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin.

Fungicide (dosage)	FDK index				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	1 a	0 a	0 a	1 a	1 a
Met (50%)	2 a	1 a	0 a	10 b	15 bc
Epox (100%)	3 a	1 a	1 a	6 a	15 b
Epox (50%)	4 a	1 a	1 a	16 b	38 c
Epox + Pyra (100%)	1 a	0 a	0 a	1 a	10 b
Epox + Pyra (50%)	4 a	1 a	1 a	10 b	32 c

Means followed by the same letter in row are not significantly different.

On the other hand, the efficiency of epoxiconazole + pyraclostrobin mixture and metconazole treatment increased and ranged 100% when they applied on 1d prior to inoculation and during-inoculation, while epoxiconazole provided reduction of FDK level by 94% compared to the control when applied at both time points.

As for the FHB index, metconazole was the most effective among the tested fungicide treatments especially for the treatments post-inoculation. On 1d post-inoculation, both metconazole treatment and the mixture of epoxiconazole and pyraclostrobin showed a good efficiency with reduction 94% compared to the control, while this reduction was 64% for the epoxiconazole treatment at this time point. At 3d post-inoculation, metconazole application still reduced the FDK percentage considerably compared to the non-treated control, reaching level of 94%. On the other hand, the epoxiconazole alone or in a mixture with pyraclostrobin showed little effect when applied this late, reaching level of 11 and 41% (Fig 22).

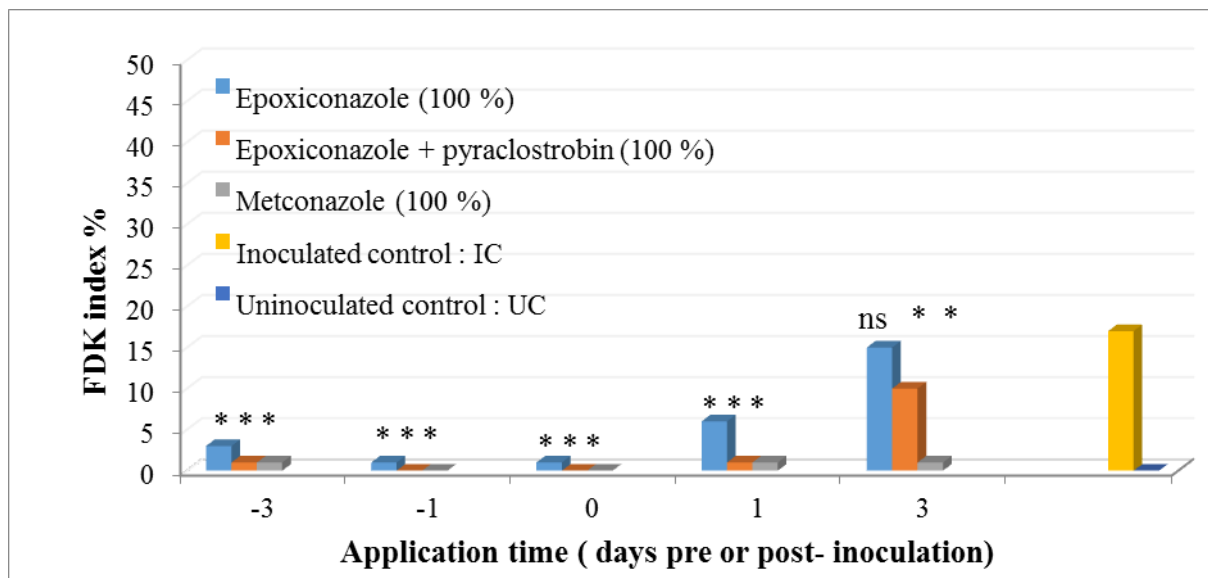


Figure 22. Percentage of *Fusarium* damaged kernels (FDK) in wheat grain during the first trial (dose 100 %). *significant, ns not significant (compared with inoculated control).

At dose 50%, the protective treatment (3d, 1d pre-inoculation) and treatment during inoculation resulted in reduced FDK percentage for all fungicides compared to the non-treated control. On 3d pre-inoculation, metconazole application reduced FDK levels 88% compared to the control while this reduction was 76% for epoxiconazole alone or in a mixture with pyraclostrobin when applied at this time point. On 1d pre-inoculation and also during-inoculation, the reduction reached 94% for all the fungicides used in this study. However, this dose again was not effective as curative treatment. At 1d post-inoculation, metconazole and the mixture of epoxiconazole and pyraclostrobin applied at 50% dose still reduced the FDK levels compared to control by ca. 40%, while epoxiconazole alone did not significantly reduce FDK levels. Treatment with 50% dose at 3d post-inoculation resulted in higher FDK levels than in the control for epoxiconazole + pyraclostrobin mixture and epoxiconazole alone, on the other hand, metconazole treatment showed a little effect with reduction 11% compared to the control when applied at this time point (Fig. 23).

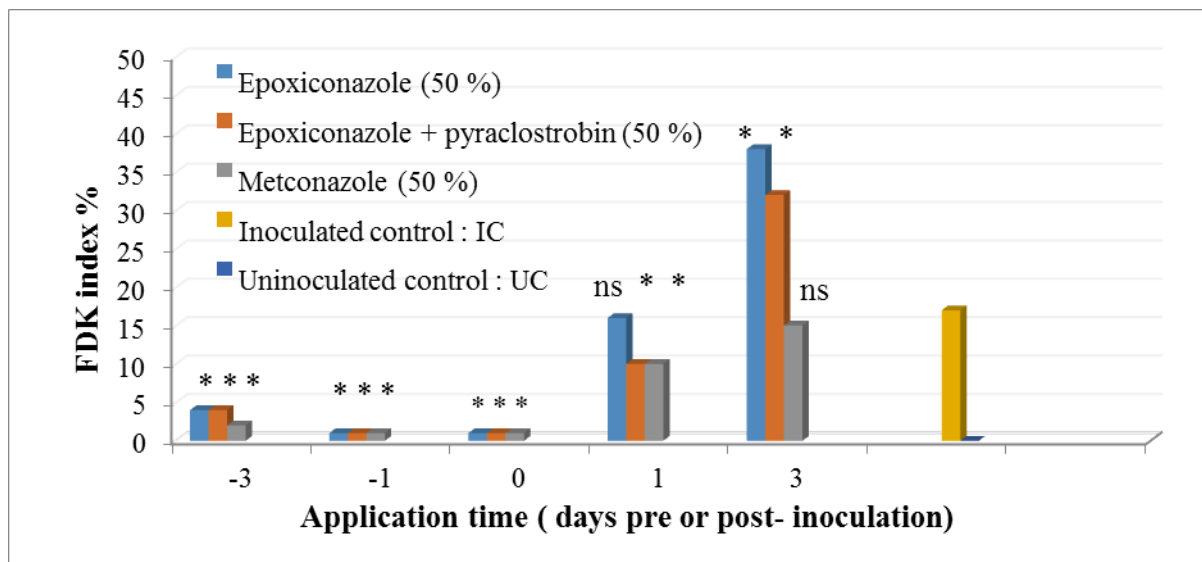


Figure 23. Percentage of *Fusarium* damaged kernels (FDK) in wheat grain during the first trial (dose 50 %). *significant, ns not significant (compared with inoculated control).

4.3.2.2 Second trial

Four commercially available fungicides [metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany), pyraclostrobin (F500, BASF, Germany)] were used in the experimental work in the second year on wheat and maize.

In this work, the effect of fungicide depended on the application form. With regard to the applications prior- inoculation and during- inoculation, all fungicides were effective at the full recommended dosage. In all cases, the application of fungicides significantly ($P < 0.001$) reduced FDK levels compared to the inoculated control indicating an excellent efficacy against this index. However, we found significant differences between the applications with metconazole being most effective and the epoxiconazole alone being least effective (Table 21, Fig. 24). On 3d prior-inoculation, all treatments reduced FDK levels ranging 68-96% compared to the control, while this reduction increased and ranged 92-97% for all treatments when applied at 1d prior-inoculation. On the other hand, the efficiency of all treatments increased ranging 96-100% when applied during-inoculation.

Concerning the application after inoculation, the results were considerably similar to the previous year. Here all of the fungicide treatments reduced FDK compared with the control at dose 100% with metconazole being most effective with reduction of ca 92% and epoxiconazole+ pyraclostrobin mixture being least effective with reduction of 16% compared

to the control. On 3d post-inoculation, metconazole still resulted in high reduction of 88% while the pyraclostrobin treatment showed a little reduction of 8% and the other treatments did not affect on FDK levels when applied at this time point. Significant differences were found among the fungicides (Table 21, Fig. 24).

Table 21. Significance of mean square values for effect of fungicides (dosage, application time) on Percentage of *Fusarium*- damaged kernels (FDK, dose 100%) in wheat during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	FDK index				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	1c	1a	0a	2a	3c
Epox (100%)	3a	2a	1a	16c	25bd
Epox + Pyra (100%)	2b	1a	1a	21d	28a
Pyra (100%)	2abd	2a	1a	19b	23d

Means followed by the same letter in column are not significantly different.

As for the FHB index, metconazole was the most effective among the tested fungicide treatments especially for the treatments post-inoculation with a reduction of 92% 1d post inoculation while this value ranged from 16-36% on 1d post-inoculation for all other treatments.

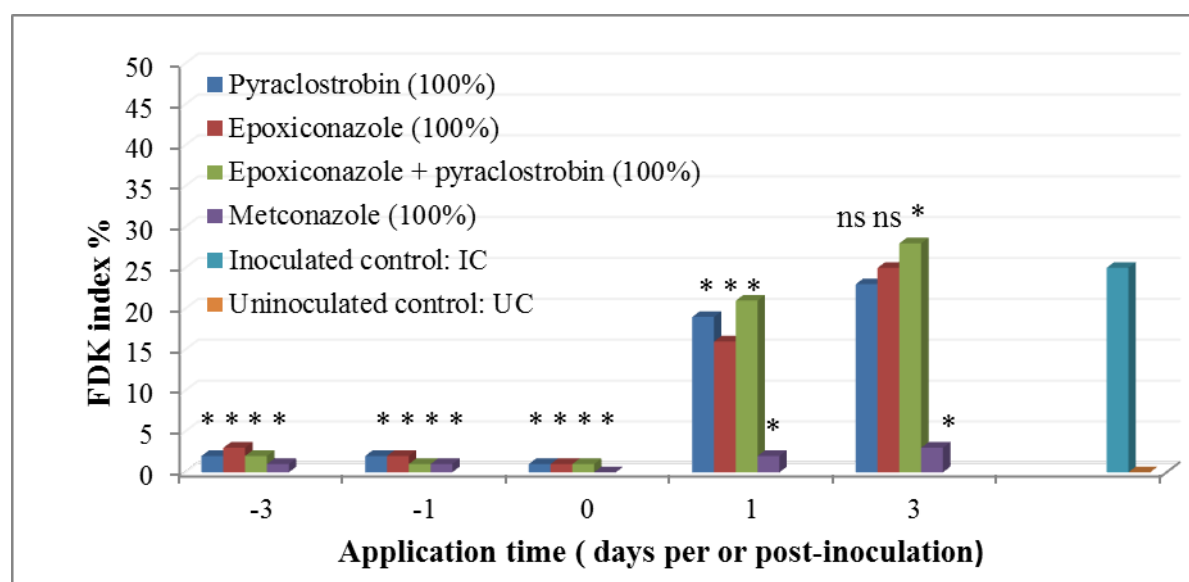


Figure 24. Percentage of *Fusarium* damaged kernels (FDK) in wheat grain during the second trial (dose 100 %). *significant, ns not significant (compared with inoculated control).

At dose 50%, the protective treatment (3d, 1d pre-inoculation) and treatment during inoculation resulted in reduced FDK percentage for all fungicides compared to the non-treated control with a reduction of 78- 95 % when applied on 3d pre-inoculation while this value was 82-95% on 1d pre-inoculation. On the other hand, all treatments provided reduction by 95 % when applied during-inoculation. However, this dose again was not effective as curative treatment (Table 22, Fig. 25).

Table 22. Significance of mean square values for effect of fungicides (dosage, application time) on Percentage of *Fusarium*- damaged kernels (FDK, dose 50%) in wheat during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	FDK index				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (50%)	1a	1a	1a	16cd	18a
Epox (50%)	3abc	2ab	1a	20bc	20b
Epox + Pyra (50%)	5bc	4bd	1a	30a	35c
Pyra (50%)	3c	3bc	1a	22bd	25a

Means followed by the same letter in column are not significantly different.

At 1d post-inoculation, metconazole, epoxiconazole alone and pyraclostrobin alone applied at 50% slightly reduced the FDK levels compared to control with levels reaching, 30, 13 and 4 %, respectively. Treatment with 50% dose at 3d post-inoculation resulted in higher FDK levels than in the control for epoxiconazole + pyraclostrobin mixture and each fungicide alone. On the other hand,, metconazole treatment resulted in a small reduction reaching level of 21.7% compared to the control while epoxiconazole showed a litte effect with reduction did not exceed 13% compared to the control when applied at this time point (Fig. 25). On the wholeThis small effect of sublethal dose indicates that dose again was not effective as curative treatment in reducing FDK levels, likewise, on FHB index as mentioned above.

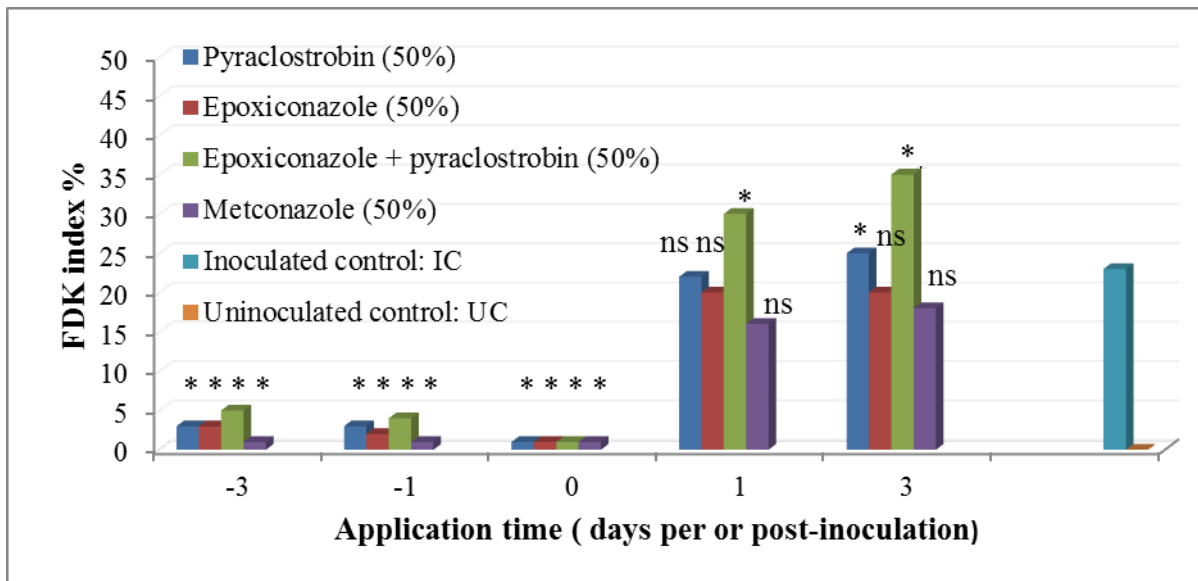


Figure 25. Percentage of *Fusarium* damaged kernels (FDK) in wheat grain during the second trial (dose 50 %). *significant, ns not significant (compared with inoculated control).

4.3.3 Quantification of *Fusarium graminearum* and DON accumulation in wheat grain

4.3.3.1 DON accumulation in wheat grain during the first trial

Three commercially available fungicides [metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany)] were used in the experimental work during the first year on wheat.

Effect of fungicide treatments differed, depending on the application time point. For applications prior to inoculation and also for treatment during inoculation, we found all fungicides tested to be effective at the full and half recommended dosage. For all fungicides tested and at both doses, protective treatment and the during-inoculation treatment reduced DON content to 0 µg/kg, indicating an excellent protective activity against FHB even with sublethal doses. The curative treatment, however, showed dose- and fungicide specific differences. For the dose 100%, again metconazole demonstrated the highest effect in reducing DON content; even when applied 3d post-inoculation, it reduced DON content to 0.3 µg/kg. Epoxiconazole and pyraclostrobin treatment reduced DON content to 1030 µg/kg when applied at one day post-inoculation. However, this reduction only brought the DON content down close to the EU limit value, The dose 100%, on the other hand, showed no curative activity for epoxiconazole and pyraclostrobin treatment and epoxiconazole alone when

applied on 3d post-inoculation, but rather led to an increased DON content compared to the control, whereas DON content was 2600 $\mu\text{g}/\text{kg}$ and 2624 $\mu\text{g}/\text{kg}$, while this content was 2400 $\mu\text{g}/\text{kg}$ for the inoculated control (Fig. 26).

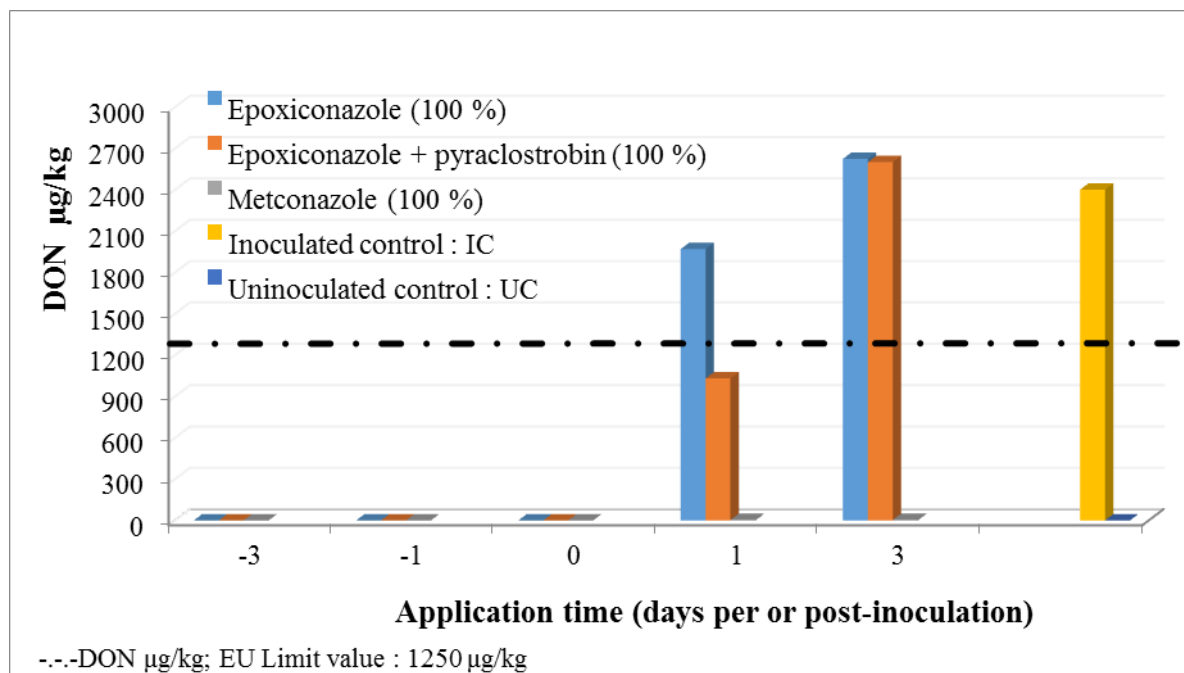


Figure 26. Concentration of deoxynivalenol (DON) in wheat grain during the first trail (dose 100 %).

Concerning the effect of fungicides (dosage, 50%) on DON content, here, the 50% dose did not reduce the DON content, irrespective of fungicide and time of application. All curative treatments with this dose resulted in DON levels above the EU limit value of 1250 $\mu\text{g}/\text{kg}$ (Fig. 28). Whereas the epoxiconazole alone or in a mixture with pyraclostobin resulted in 2435 and 2021 $\mu\text{g}/\text{kg}$ of DON concentration, respectively when applied on 1d post-inoculation while this value reached 2816 and 2520 $\mu\text{g}/\text{kg}$ on 3d post inoculation for both treatments, respectively. On the other hand, metconazole treatment showed a little reduction but this reduction was not able to bring the DON content down to the EU limit value, whereas DON content was 2128 $\mu\text{g}/\text{kg}$ and 2144 $\mu\text{g}/\text{kg}$ when the dose 50% of metconazole applied on 1d and 3d, respectively. As result, irrespective of fungicide and time of application. All curative treatments with this dose resulted in DON levels above the EU limit value of 1250 $\mu\text{g}/\text{kg}$ (Fig. 27).

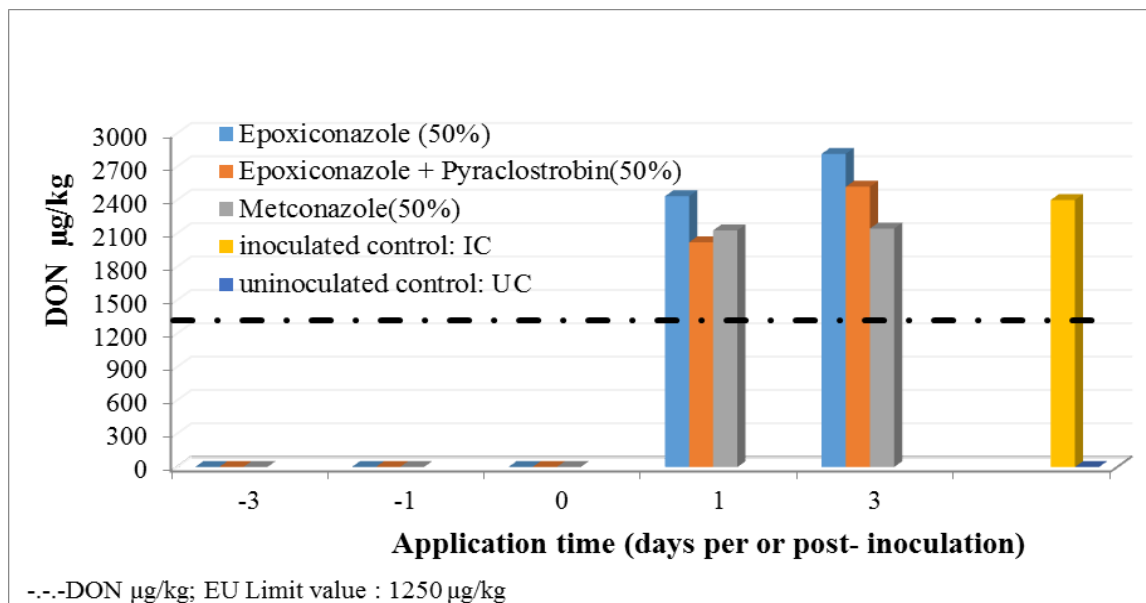


Figure 27. Concentration of deoxynivalenol (DON) in wheat grain during the first trial (dose 50 %).

4.3.3.2 Quantification of *Fusarium graminearum* and DON accumulation in wheat grain during the second trial

Four commercially available fungicides [metconazole (Caramba, BASF, Germany), epoxiconazole (Opus, BASF, Germany), a mixture of epoxiconazole and pyraclostrobin (Retengo, BASF, Germany), pyraclostrobin (F500, BASF, Germany)] were used in the experimental work in the second trial on wheat and maize. The fungicides were applied in one dose on maize and in two different doses (100%, 50%) on wheat, the first dose agreeing with the official recommendation for commercial field use, and the another using a sublethal dose (lower than recommended).

Similar to the results of the first trial, the effect of fungicide treatments depended on the application time point showing a high protective activity for all treatments using both doses on wheat grain. Concerning sprays prior to inoculation and also for treatment during inoculation, we found all fungicides tested to be severely effective at the full recommended dosage with significant differences between the fungicides used (Table 23). whereas DON concentration was 0µg/kg for triazole treatments while low concentration of DON were determined when pyraclostrobin alone or in mixture applied, reaching level of 8 and 8 µg/kg, respectively. on 1d pre-inoculation, all treatments completely inhibited DON accumulation

except for pyraclostrobin treatment which resulted in reduction of 98% compared to the control when applied at this time point.

Table 23. Significance of mean square values for effect of fungicides (dosage, application time) on deoxynivalenol (DON) concentration (dose 100%) in wheat grain during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	<u>DON concentration (µg/kg)</u>				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	0a	0a	0a	10a	10a
Epox (100%)	0a	0a	0a	90a	1062b
Epox + Pyra (100%)	8a	0a	0a	1170b	1449b
Pyra (100%)	81a	11a	20a	130ac	1200bc

Means followed by the same letter in column are not significantly different.

Again, all fungicides with the dose 100% led to completely inhibit DON accumulation in wheat grain when applied during-inoculation. These results indicated an excellent protective activity against this disease.

The curative treatment, however, showed dose-and fungicide specific differences. The dose 100% of metconazole again demonstrated the highest effect in reducing DON content; even when applied 3d post-inoculation, it also reduced DON content to 10 µg/kg on 1d post-inoculation. On the other hand, epoxiconazole demonstrated a good efficacy with reduction, reaching level of 91 % compared to control while pyraclostrobin alone reduced DON by 88% compared to the control when applied at 1d post-inoculation (Fig. 28).

On the other hand, epoxiconazole + pyraclostrobin treatment did not reduce DON level at this time point, but rather led to increase DON concentration when applied on 1d and 3d post-inoculation compared to the control. On 3d post-inoculation, only metconazole treatment largely reduced DON concentration by 99% compared to the control while the other treatments did not reduce DON level except a small reduction of 2 % for epoxiconazole treatment. However, this reduction only brought the DON content of this treatment down close to the EU limit value (1250 µg/kg) while DON level exceed the limited value by epoxiconazole + pyraclostrobin treatment which reached (1449 µg/kg).

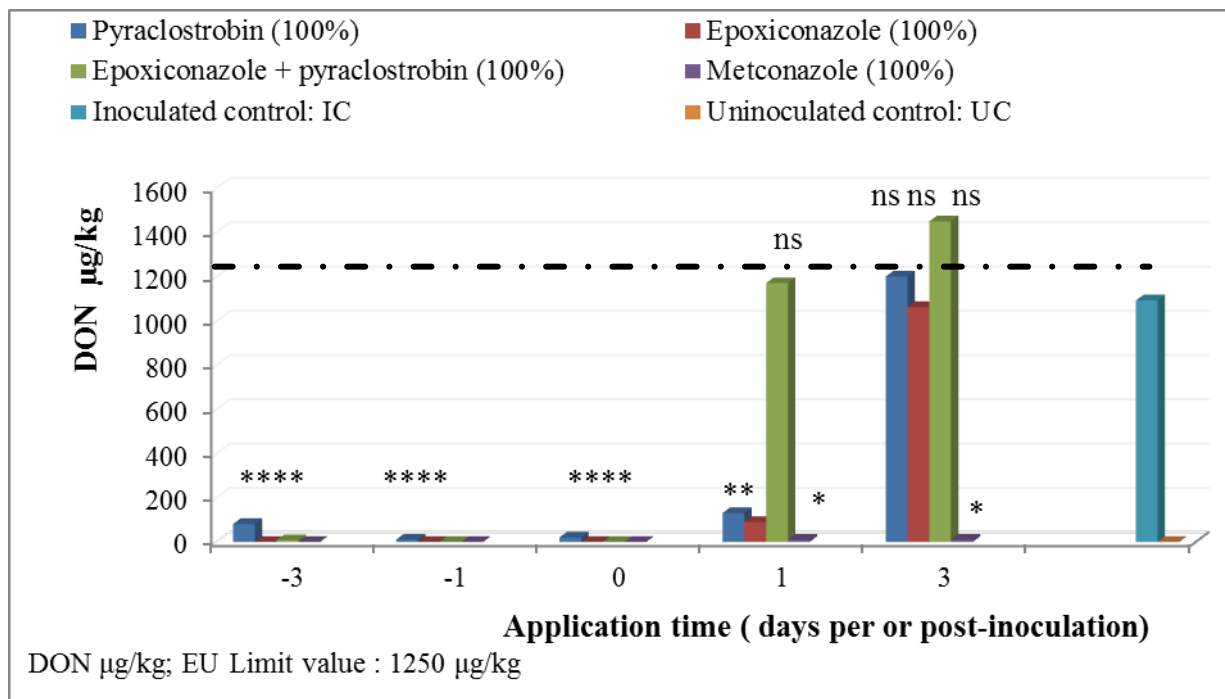


Figure 28. Concentration of deoxynivalenol (DON) in wheat grain during the second trail (dose 100 %).

At dose 50%, the protective treatment (3d, 1d pre-inoculation) and treatment during inoculation reduced or inhibited DON concentration for all fungicides compared to the non-treated control. No significant differences were determined between the fungicides used in this work. On 3d prior-inoculation, all treatments inhibited DON accumulation except a treatment of pyraclostrobin which resulted in reduction of 98% compared to the control. A good efficiency was observed on 1d pre-inoculation and during-inoculation with a reduction of 95-100%.

Table 24. Significance of mean square values for effect of fungicides (dosage, application time) on deoxynivalenol (DON) concentration (dose 50%) in wheat grain during the second year of the trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	<u>DON concentration (µg/kg)</u>				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	0a	11a	0a	459a	485a
Epox (100%)	0a	0a	0a	647a	694a
Epox + Pyra (100%)	0a	11a	28a	648a	911a
Pyra (100%)	9a	14a	11a	825a	918a

Means followed by the same letter in column are not significantly different.

However, this dose again was not effective as curative treatment, but rather led to an increased DON concentration compared to the control for all application except metconazole treatment which resulted in reduction of ca 26% compared to the control when applied on 1d and 3d post-inoculation (Fig. 29).

At 1d and 3d post-inoculation, only metconazole treatment slightly reduced the DON level compared to control with reduction of 26 %. On the other hand, irrespective of fungicide and time of application, pyraclostrobin alone, epoxiconazole alone and the epoxiconazole + pyraclostrobin mixture resulted in DON when applied as curative treatment.

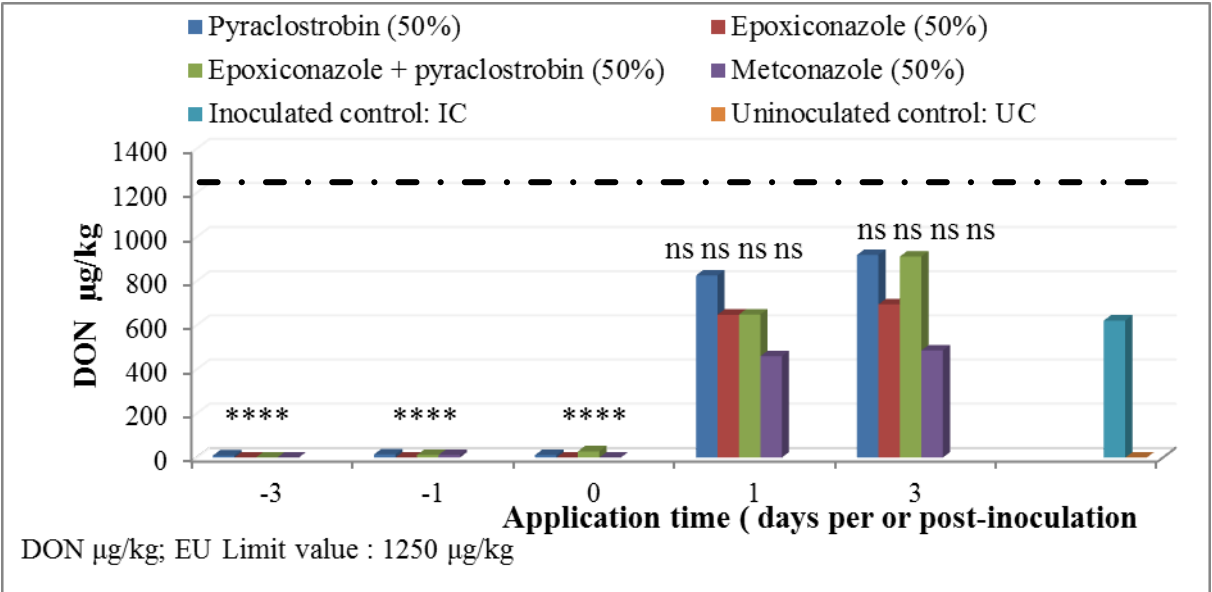


Figure 29. Concentration of deoxynivalenol (DON) in wheat grain during the second trail (dose 50 %).

Regarding DNA in wheat grain; in our present work, the effect of fungicide treatments differed, depending on the application time for all treatments. For applications prior or also during inoculation day, we found all fungicides tested to be effective at the recommended dosage. Fungal DNA was found in low or undetectable concentrations when fungicides used pre- or during inoculation. In all cases, the treatments significantly ($P < 0.001$) reduced DNA content compared to the non-treated control, indicating excellent activity against *F. graminearum* at this time point. However, there were significant differences between the treatments with metconazole being most effective and pyraclostrobin alone or in mixture being least effective (Table 25)

Table 25. Significance of mean square values for effect of fungicides (dosage, application time) on quantification of *Fusarium graminearum* (dose 100%) in wheat during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	<u>DNA fungi/ DNA plant ‰</u>				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	2,2a	1.6b	0a	2.5a	4a
Epox (100%)	3.3b	4.3ac	0.2a	34.8b	129.7b
Epox + Pyra (100%)	4.8c	4.4cd	0.7a	99.7c	113.5c
Pyra (100%)	5.1d	2bd	0.5a	79.3d	103.2d

Means followed by the same letter in column are not significantly different.

For sprays after inoculation the differences observed were more pronounced (Fig. 30). The quantity of DNA fungal increased and distributed in various amounts according to the treatments. Again, this dose was most effective for metconazole and considerably less effective for epoxiconazole and pyraclostrobin mixture and pyraclostrobin alone when applied 1d post inoculation. Whereas metconazole treatment reduced DNA concentration by 98% compared to the inoculated control while the reduction was 18.3 and 35 %, respectively, for the other treatments. On 3d post-inoculation metconazole treatment still resulted in a significant reduction of DNA content reaching a level of 96.7%, while the other fungicides only had a small effect or no effect at this time point (Fig. 30).

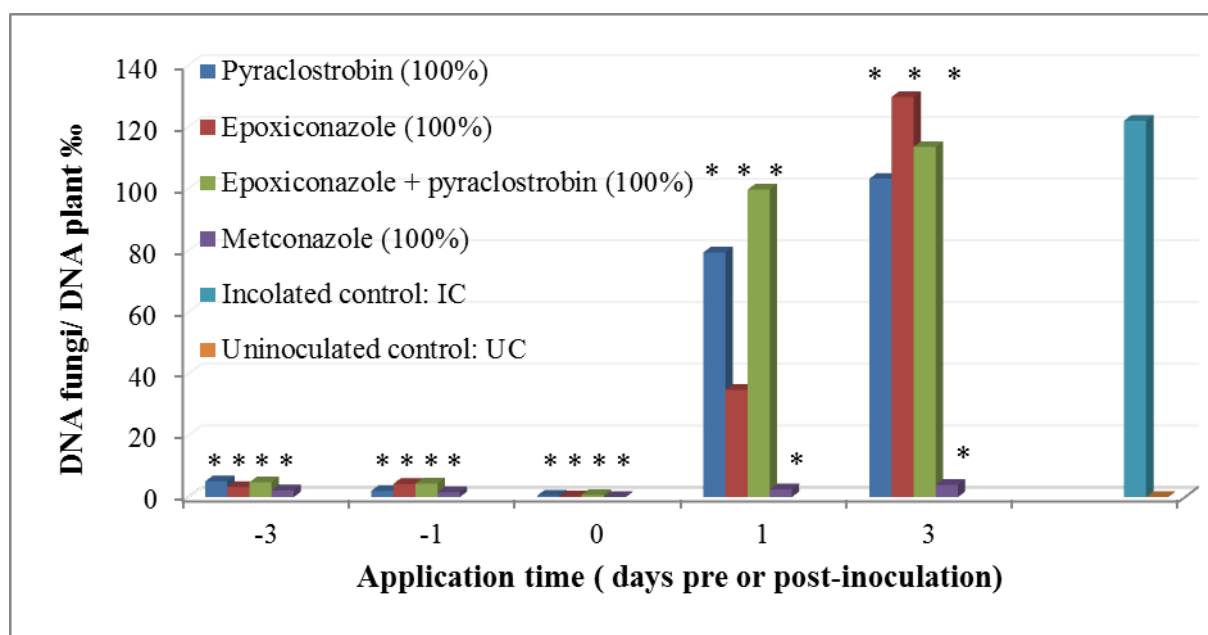


Figure 30. Quantification of *Fusarium graminearum* in wheat grain during the second trail (dose 100%). *significant, ns not significant (compared with inoculated control).

DNA levels of the un-inoculated control group were zero in the work; therefore, these data were not included in the graphics.

At dose 50%, the protective treatment (3d, 1d pre-inoculation) and treatment during inoculation resulted in reduced DNA concentration for all fungicides compared to the non-treated control with a reduction of 95-100%. However, this dose again was not effective as curative treatment, but rather led to an increased DNA concentration compared to the control for most application, significant differences were determined between the application when applied post-inoculatio. (Table 26)

Table 26. Significance of mean square values for effect of fungicides (dosage, application time) on quantification of *Fusarium graminearum* (dose 50%) in wheat during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	DNA fungi/ DNA plant ‰				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (50%)	2,3a	1,6a	0a	87,5a	88,9b
Epox (50%)	3,3a	4,3a	0,2a	94,7b	99,5c
Epox + Pyra (50%)	3,8a	3,9a	0,4a	102,7c	107,4a
Pyra (50%)	2,3a	3,1a	0,3a	89,9a	104,4a

Means followed by the same letter in column are not significantly different.

At 1d post-inoculation, metconazole, pyraclostrobin alone and epoxiconazole alone applied at 50% slightly reduced the DNA levels compared to control with levels reaching, 11.2, 8.7 and 3.9%, respectively. Treatment with the dose 50% at 3d post-inoculation resulted in higher DNA levels than in the control for epoxiconazole + pyraclostrobin mixture and each fungicide alone. However, metconazole treatment resulted in a small reduction which reached 9.7%.

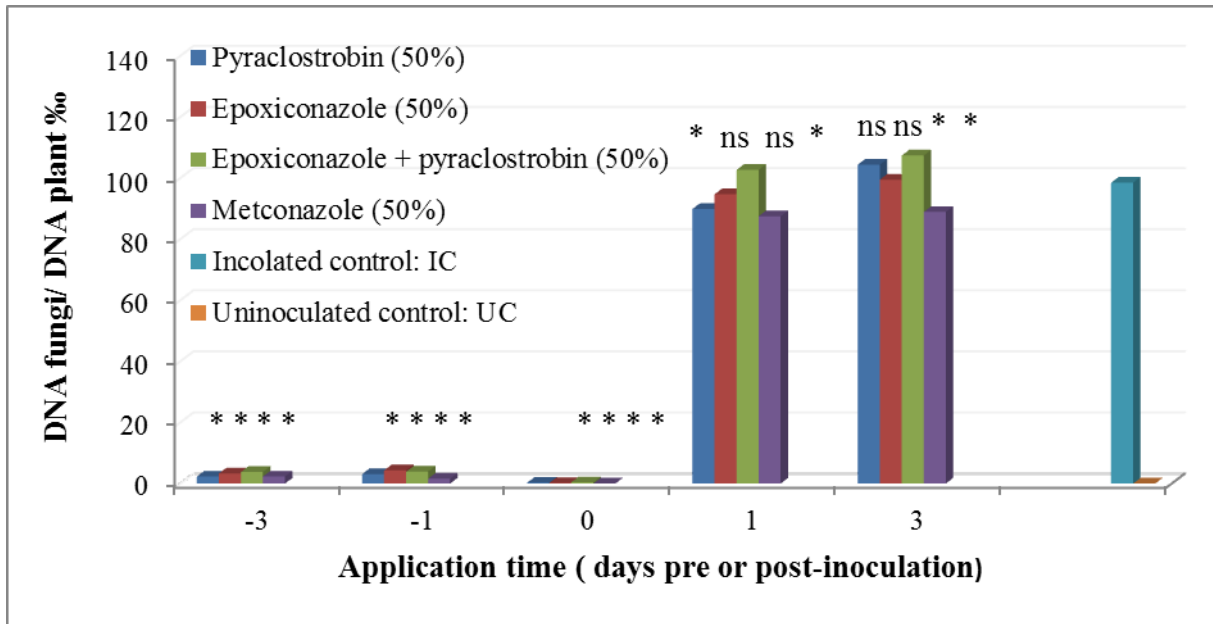


Figure 31. Quantification of *Fusarium graminearum* in wheat grain during the second trail (dose 50%). *significant, ns not significant (compared with inoculated control).

4.3.4 Quantification of *Fusarium graminearum* and DON accumulation in maize grain

4.3.4.1 Quantification of *Fusarium graminearum* in maize grain during the second year

The four fungicides were applied in one doses (100%), agreeing with the official recommendation for commercial field use.

In our present work, the effect of fungicide treatments differed, depending on the application time for all treatments. For applications prior- or also during inoculation day, we found all fungicides tested to be effective at the recommended dosage. Fungal DNA was found in low or undetectable concentrations when fungicides used pre- or during inoculation. In all cases, the treatments largely reduced DNA content compared to the non-treated control, indicating excellent activity against *F. graminearum* at this time point. However, there were no significant differences between the fungicides used at this time point (Table 27, Fig. 32).

For sprays after inoculation the differences observed were more pronounced. The quantity of DNA fungal increased and distributed in various amounts according to the treatments. Again, this dose was most effective for metconazole and considerably less effective for epoxiconazole and pyraclostrobin mixture and pyraclostrobin alone when applied 1d post

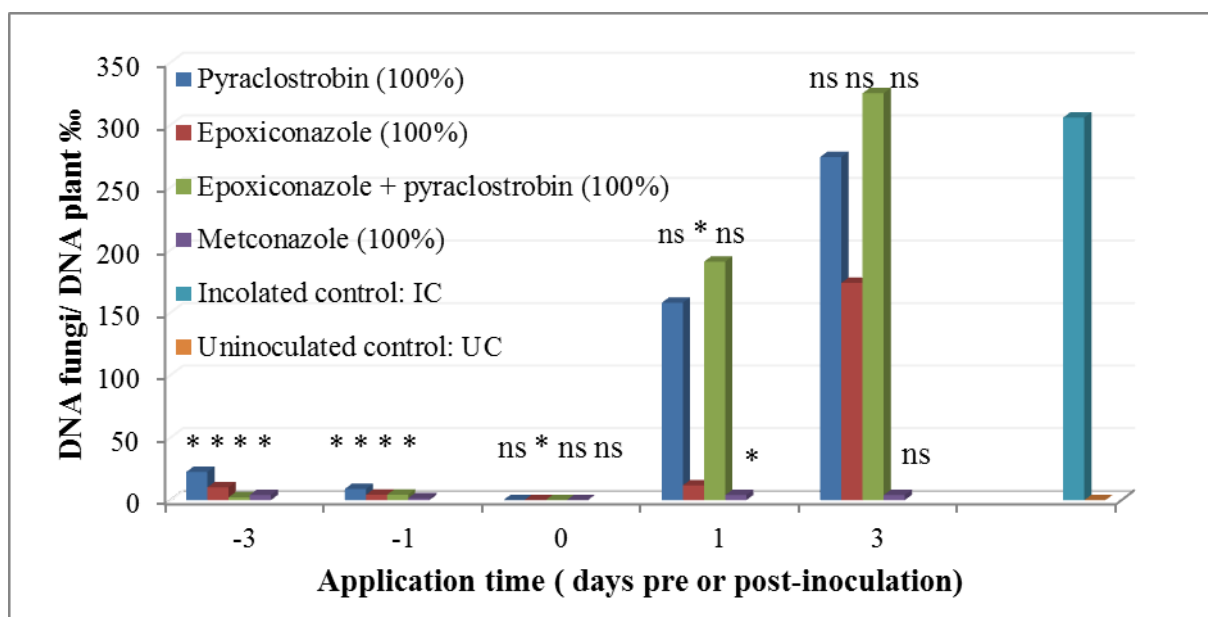


Figure 32. Quantification of *Fusarium graminearum* in maize grain during the second year of the trial (dose 100). *significant, ns not significant (compared with inoculated control).

inoculation, reaching levels of 190.7 and 157.9 DNA fungi/ DNA plant ‰, respectively. On 3d post-inoculation metconazole treatment still resulted in high reduction of DNA content, while only epoxiconazole had a little effect in reducing DNA concentration at this time point. Whereas *F. graminearum* was detected in higher amounts in treatment of epoxiconazole and pyraclostrobin mixture and pyraclostrobin treatment alone, reaching levels of 325.3 and 274.4 DNA fungi/ DNA plant ‰, respectively.

Table 27. Significance of mean square values for effect of fungicides (dosage, application time) on quantification of *Fusarium graminearum* (dose 100%) in maize grain during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	<u>DNA fungi/ DNA plant ‰</u>				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	4.1a	1.6a	0a	4a	4a
Epox (100%)	10.3a	4.3a	0a	11.8a	173.7a
Epox + Pyra (100%)	2.3a	4.4a	0a	190.7a	325.3a
Pyra (100%)	22.6a	9a	0a	157.9a	274.4a

Means followed by the same letter in column are not significantly different.

On other words, metconazole was the most effective among the tested fungicide treatments especially for the treatments post inoculation. At 3d post inoculation, it still reduced DNA

concentration considerably compared to the non-treated control with reduction of 98.7 %, while the others showed little or no effect when applied this late.

DNA levels of the un-inoculated control group were zero in the work; therefore, these data were not included in the graphics.

4.3.4.2 DON accumulation in maize grain during the second trial

The four fungicides were applied in one doses (100%), agreeing with the official recommendation for commercial field use. Similar to the previous results, the effect of fungicide treatments depended on the application time point. Concerning sprays prior to inoculation and also for treatment during inoculation, we found all fungicides tested to be severely effective at the full recommended dosage and significant differences were observed between the fungicides used (Table 28).

Table 28. Significance of mean square values for effect of fungicides (dosage, application time) on deoxynivalenol (DON) concentration (dose 100%) in maize grain during the second trial, (A-3d) 3d prior inoculation, (A+3d) 3d post-inoculation, (A-1d) 1d prior-inoculation, (A+1d) 1d post-inoculation, (A0d) during inoculation. Met: metconazole. Epox: epoxiconazole. Epox + Pyra: epoxiconazole+ pyraclostrobin. Pyra: pyraclostrobin.

Fungicide (dosage)	<u>DON concentration (µg/kg)</u>				
	application time				
	A-3d	A-1d	A0d	A+1	A+3d
Met (100%)	0a	0a	0a	0a	0a
Epox (100%)	0a	0a	0a	265ad	2535bcd
Epox + Pyra (100%)	0a	0a	0a	1870b	2305c
Pyra (100%)	0a	0a	0a	1668bc	2472d

Means followed by the same letter in column are not significantly different.

Whereas DON concentration was 0µg/kg for all protective treatments and the during-inoculation treatments, indicating an excellent protective activity against this disease. The curative treatment, however, showed dose-and fungicide specific differences. The dose 100%, again metconazole demonstrated the highest effect in reducing DON content; even when applied 3d post-inoculation, it reduced DON content to 0 µg/kg. On the other hand, on 1d post-inoculation, epoxiconazole demonstrated a good efficacy with reduction of 93% compared to control while pyraclostrobin alone and epoxiconazole + pyraclostrobin treatment provided reduction of 57%, 62%, respectively when applied at 1d post-inoculation. However,

this reduction only brought the DON content of pyraclostrobin treatment down close to the EU limit value (1750 $\mu\text{g}/\text{kg}$) while exceed the limited value by epoxiconazole + pyraclostrobin treatment.

The dose 100%, on the other hand, were not able to reduce DON content down to the EU limit value, for both epoxiconazole, pyraclostrobin alone and epoxiconazole + pyraclostrobin treatment when applied on 3d post-inoculation, whereas DON content was 2535 ,2472 and 2305 $\mu\text{g}/\text{kg}$, respectively, while this content was 4412 $\mu\text{g}/\text{kg}$ for the inoculated control (Fig. 33).

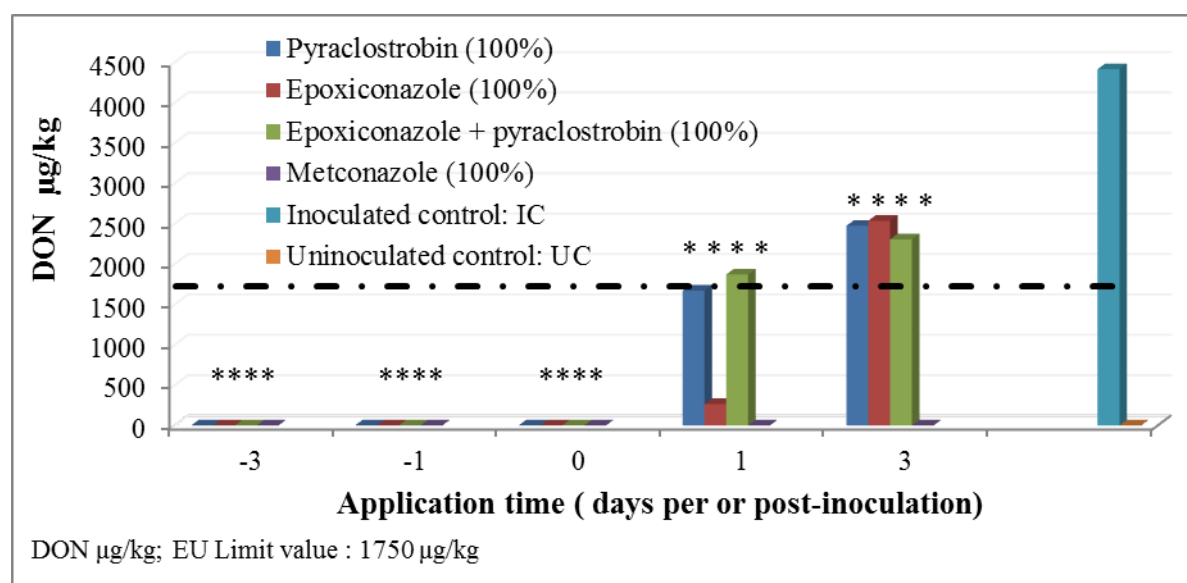


Figure 33. Concentration of deoxynivalenol (DON) in maize grain during the second trail (dose 100%). *significant, ns not significant (compared with inoculated control).

The severity of FHB, ratio of FDK, and DON, DNA levels of the un-inoculated control group of heads were zero in the work, therefore, these data were not included in the analyses. The statistical software R (2014) was used to evaluate the data. First, the appropriate statistical models were based on generalized least squares. The data were assumed to be normally distributed and to be heteroscedastic due to the different days. These assumptions are based on a graphical residual analysis. Two statistical models were used, the first excluding the control group, the second with the control group. The two models included treatment (epoxiconazole, pyraclostrobin, epoxiconazole +pyraclostrobin, metconazole), dose (50%, 100%) and dates (3d, 1d pre-, post-, and during-inoculation), as well as all their interaction terms (two-fold and three-fold) as fixed factors. Based on the first model, an analysis of variances (ANOVA) was conducted mainly to check for significance and interactions between

the influence factors. After this, multiple contrast tests were conducted based on the second model to compare the different levels of the influence factors. A corresponding cell means model was applied. Note that the residual standard deviations of the models correspond to technical replications – not to biological replications. DON content was not statistically evaluated during the first year of the trial, since due to the high cost for DON analysis, no replications were performed.

4.3.5 Correlation among the FHB response variables in wheat grain during the second year of the trial

The association between FHB index, FDK index and DNA concentration in wheat grain for both doses were determined (Fig. 34, 35, 36). Concerning the dose 100%, FDK index was higher correlated with DNA concentration in wheat grain than FHB index, whereas this value reached ($R^2 = 0.9545$, $R^2 = 0.8389$), respectively. Thereby the high levels of correlation index indicate a high correspondence between these variables.

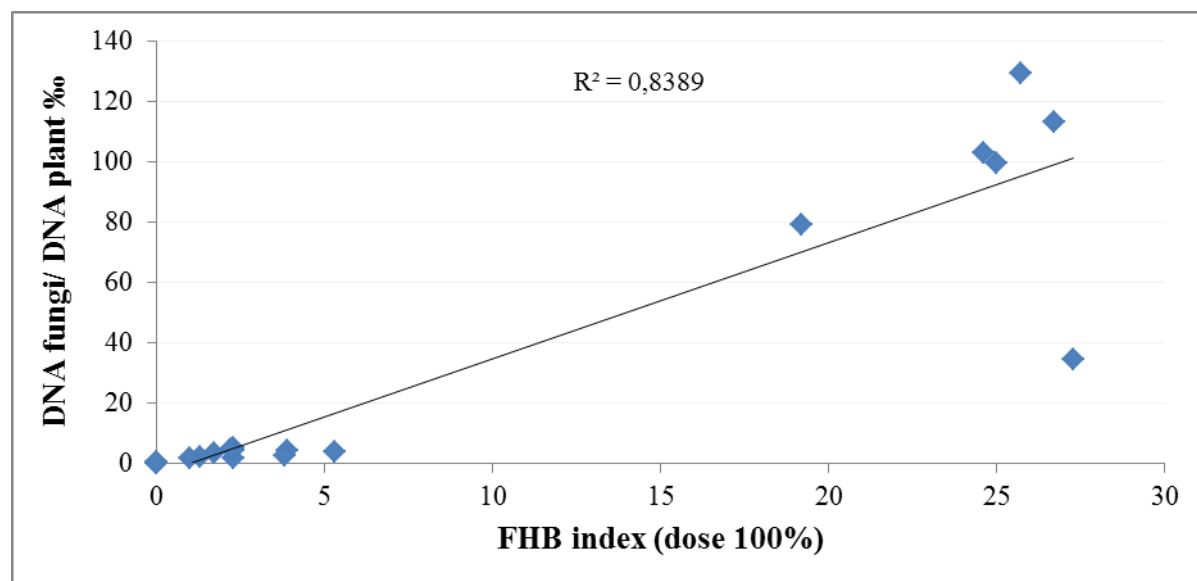


Figure 34. Correlation between FHB index and DNA concentration (dose 100%) in wheat grain.

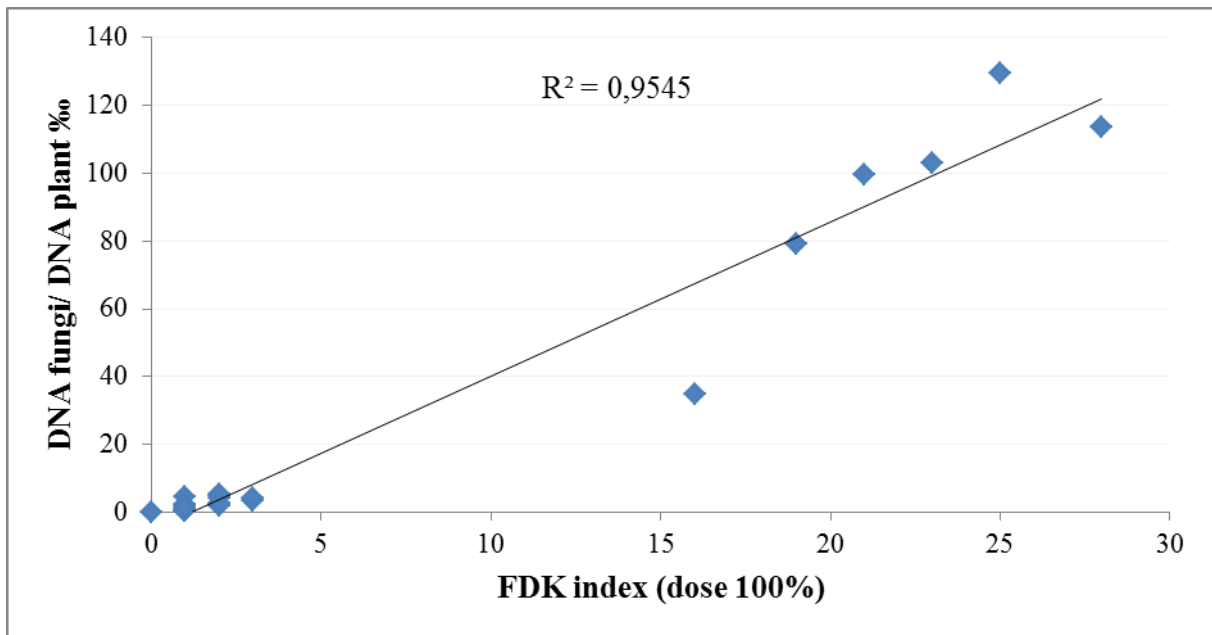


Figure 35. Correlation between FDK index and DNA concentration in wheat grain (dose 100%).

Strong correlation was observed between FHB- FDK index for this dose reaching level of $R^2 = 0.9389$ (Fig. 36) thus the high levels of association index indicate a high correspondence between these variables.

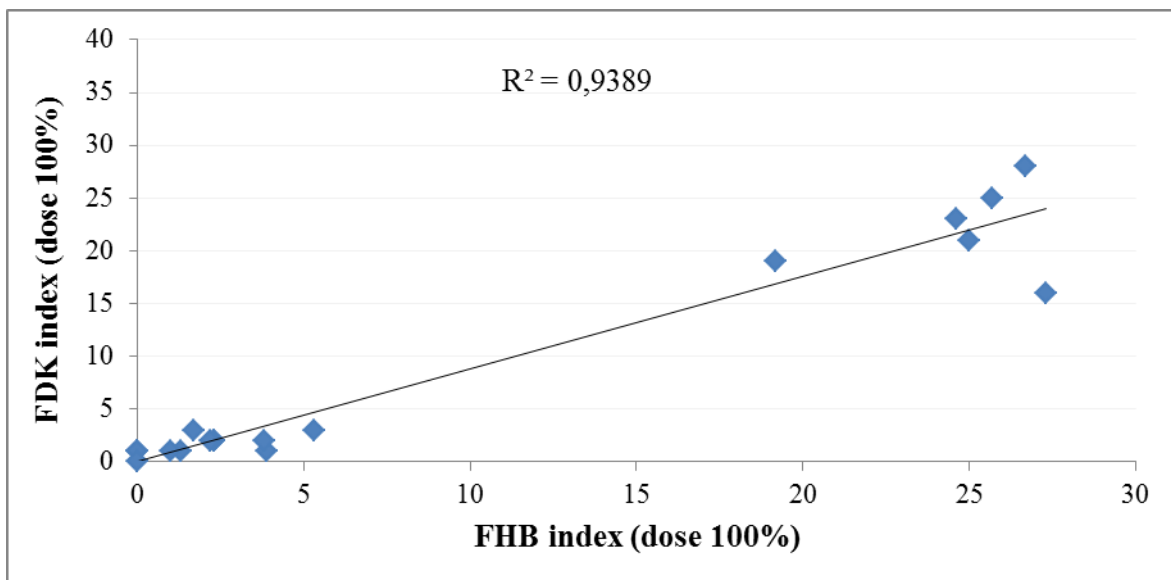


Figure 36. Correlation between FHB index and FDK index in wheat grain (dose 100%).

Regarding the second dose (50%), the relationship between *F. graminearum* quantity in wheat grain and both FHB; FDK index was also strong, whereas the Pearson correlation coefficients were (0.9601, 0.921), respectively, referring a high association between those variables (Fig. 37, 38).

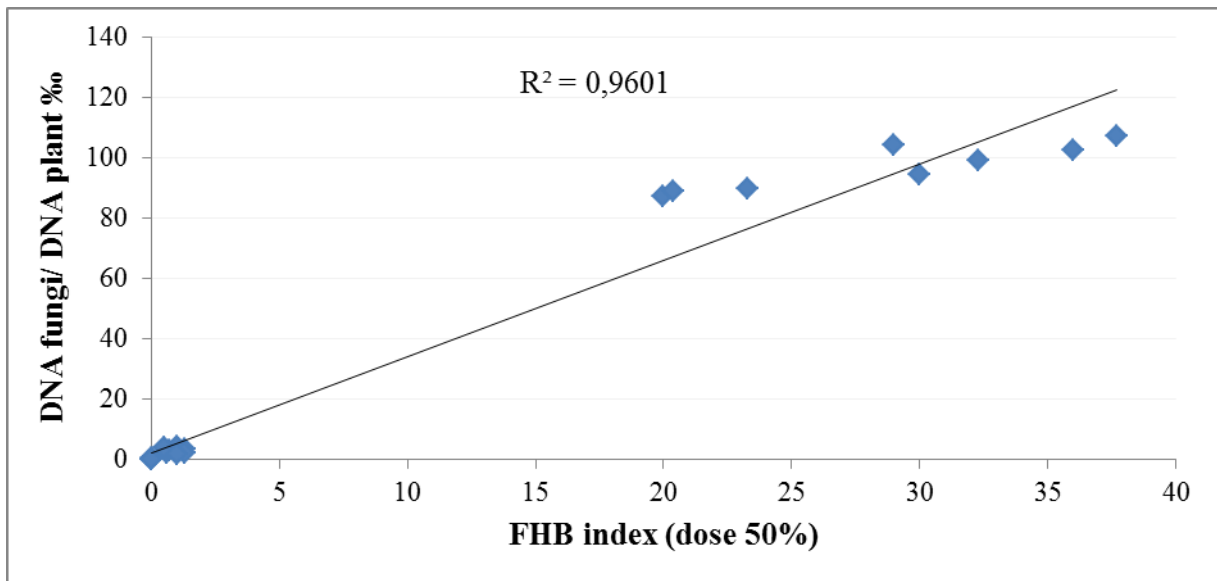


Figure 37. Correlation between FHB index and DNA concentration in wheat grain (dose 50%).

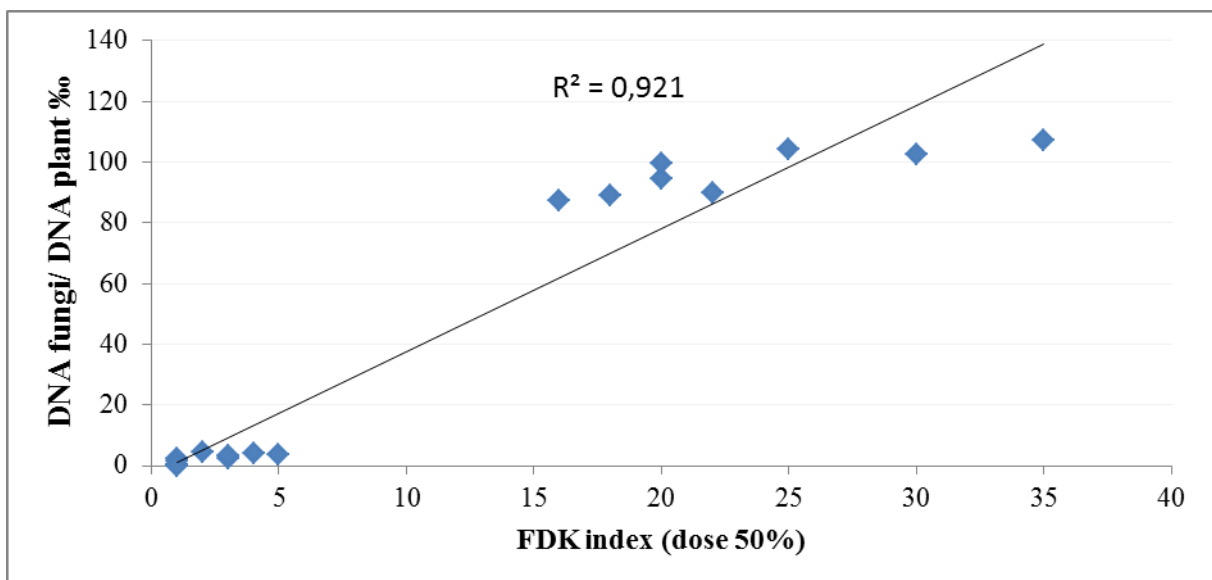


Figure 38. Correlation between FDK index and DNA concentration in wheat grain (dose 50%).

On the other hand, FHB-FDK index were highly associated with level of $R^2 = 0.9541$ for the sublethal dose (Fig. 39), thus the high levels of association index indicate a high correspondence between these variables.

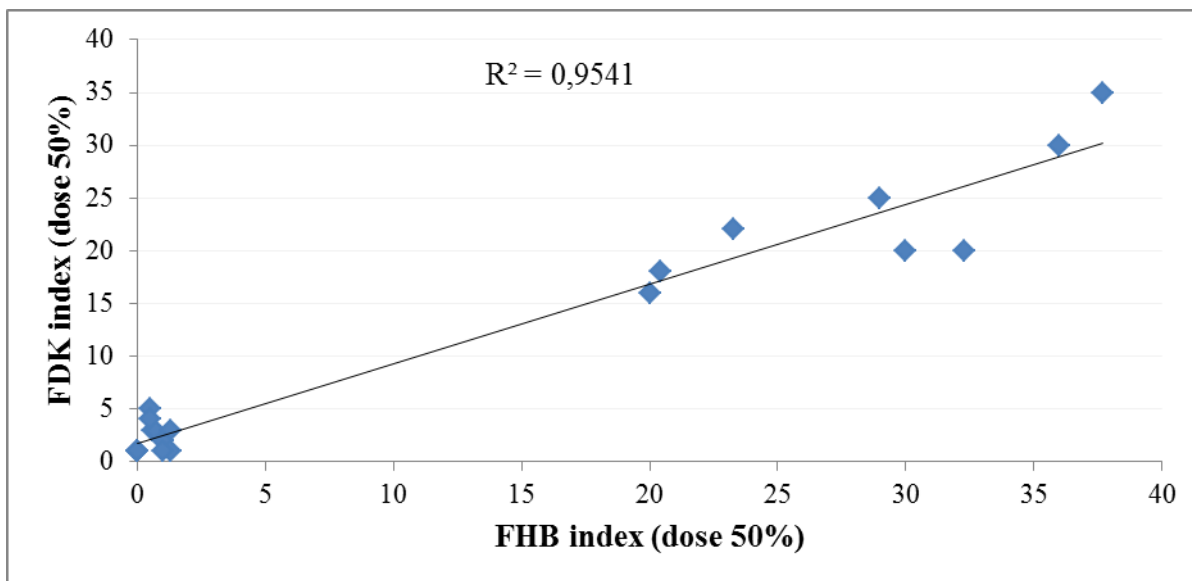


Figure 39. Correlation between FHB index and FDK index in wheat grain (dose 100%).

Concerning the association between DON accumulation and FHB; FDK and DNA quantification of *F. graminearum* for both doses, the results are determined and shown in (Fig. 40, 41, 42, 43, 44, 45). Whereas this association lower between DON concentration of *F.graminearum* and FHB index compared to the DON- FDK association, whereas the Pearson correlation coefficients were 0.6642 and 0.7935, respectively.

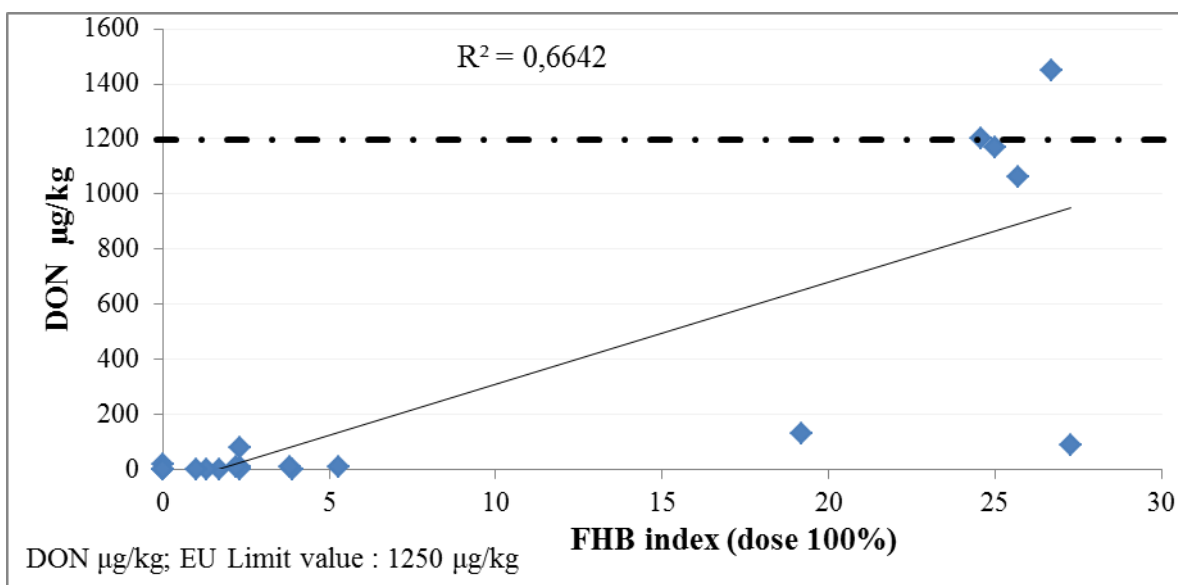


Figure 40. Correlation between FHB index and DON accumulation in wheat grain during the second trial (dose 100%).

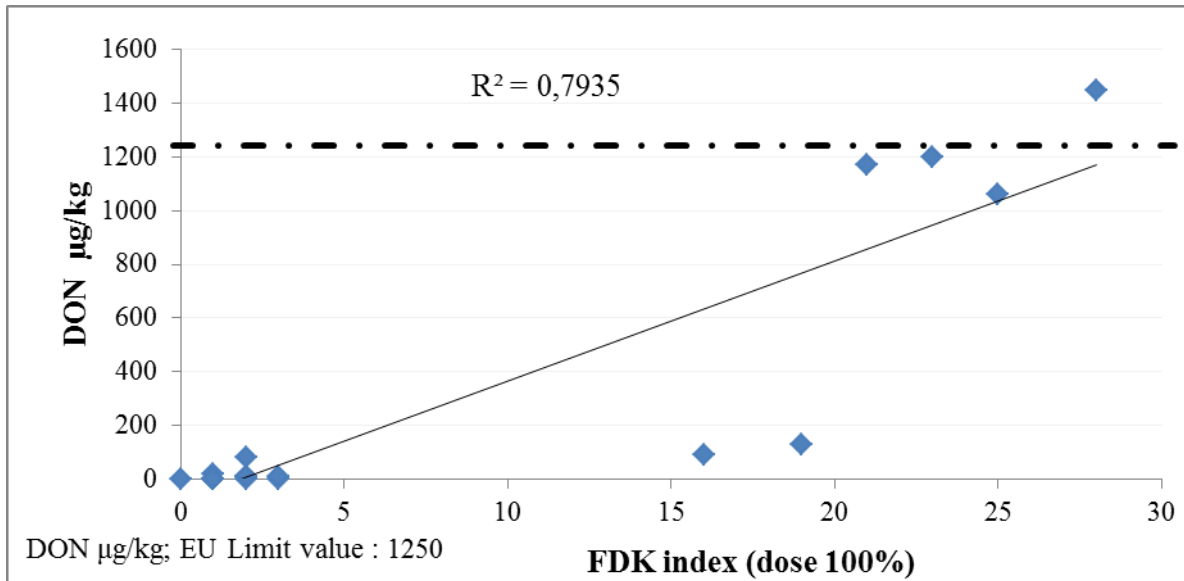


Figure 41. Correlation between FDK index and DON accumulation in wheat grain during the second trial (dose 100%).

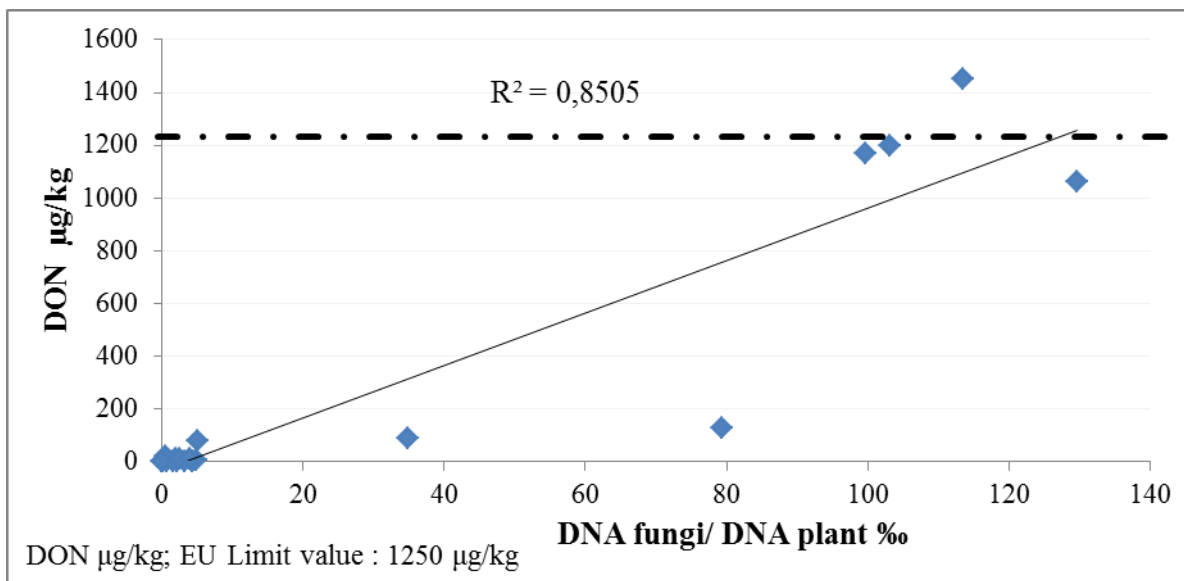


Figure 42. Correlation between DNA concentration and DON accumulation in wheat grain during the second trial (dose 100%).

Regarding the dose 50% in wheat grain, the correlation between FHB index- DON concentration and FDK index- DON concentration were different, it was determined that the first correlation was higher compared to the second. Whereas reached levels of 0.9188 and 0.8927, respectively (Fig. 43, 44).

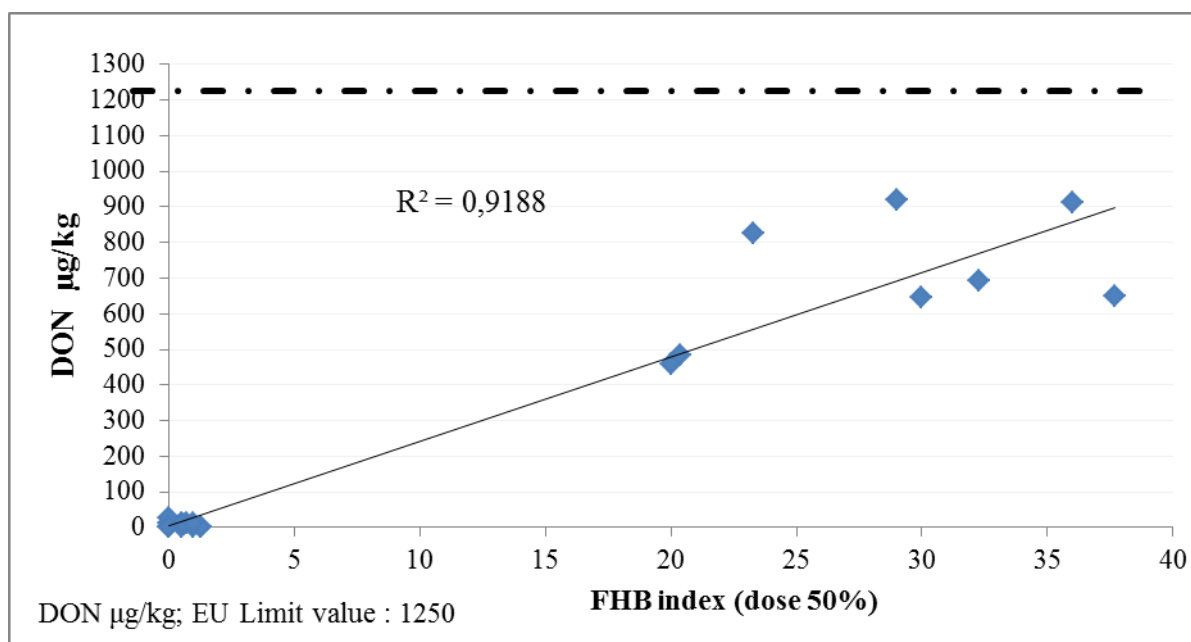


Figure 43. Correlation between FHB index and DON accumulation in wheat grain during the second trial (dose 50%).

On the other hand, the association between FDK levels and DON concentration of *F.graminearum* was determined and reached 0.8927 (Fig. 44), while this association was stronger between the DNA concentration and DON accumulation of *F.graminearum* determined using LC/ MS analysis in wheat grain during the second trial for the same dose 50% (Fig. 45).

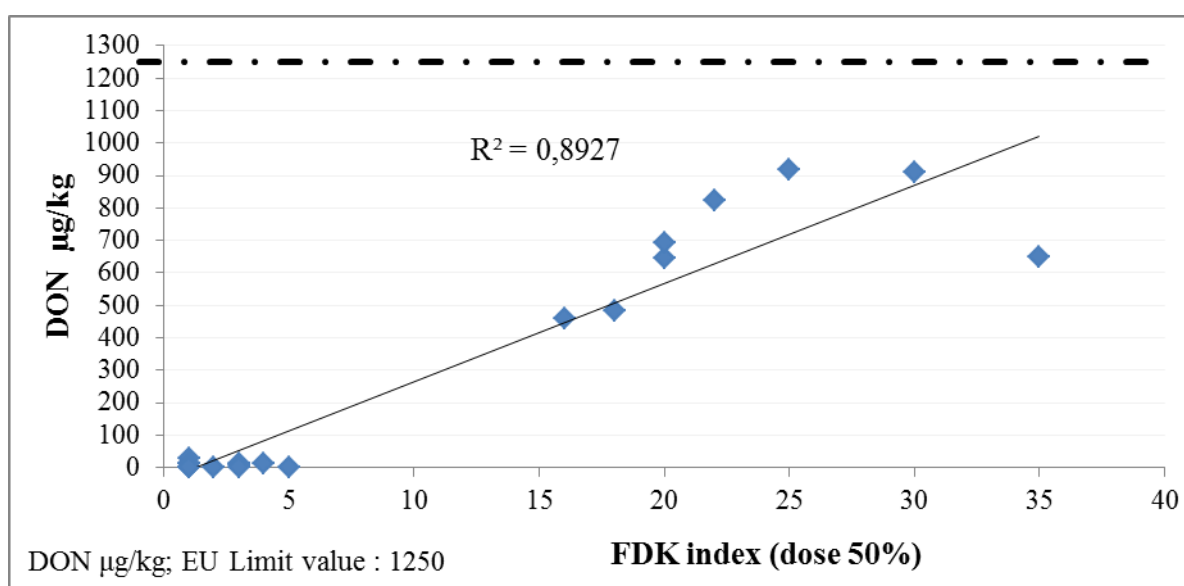


Figure 44. Correlation between FDK index and DON accumulation in wheat grain during the second trial (dose 50%).

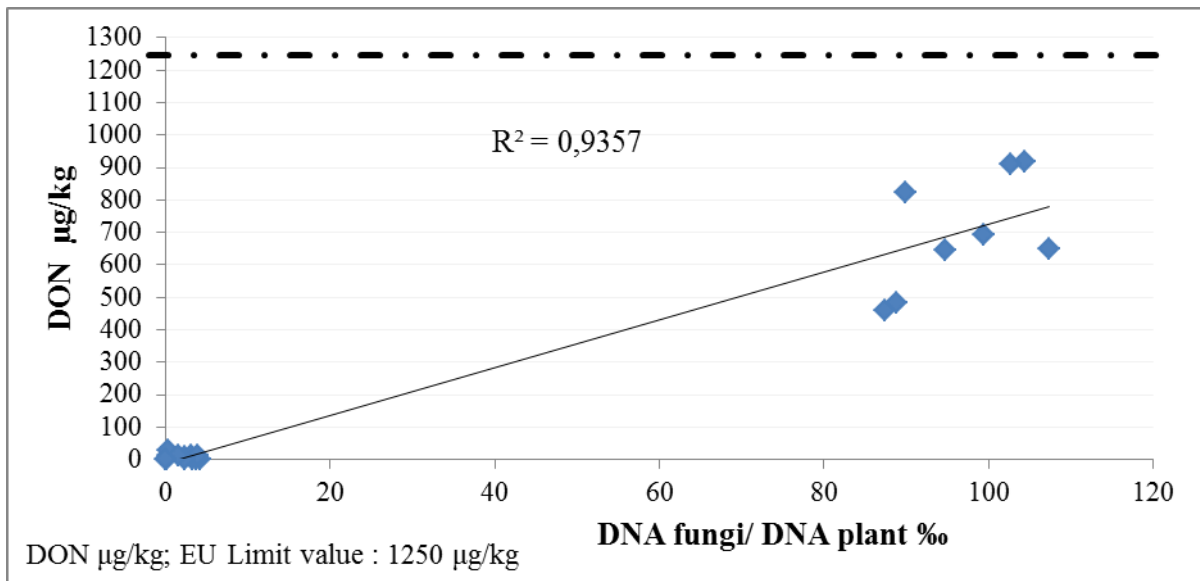


Figure 45. Correlation between DNA concentration and DON accumulation in wheat grain during the second trial (dose 50%).

4.3.6 Correlation between DNA concentration and DON accumulation in maize grain during the second year of the trial

The comparison of DON concentration determined using LC/ MS analysis and the data deriving from molecular analysis of *F. graminearum* quantification (quantative- qPCR analysis) are shown in (Fig. 46.) the relationship between DNA fungal and DON accumulation was high ($R^2 = 0,9167$) thus, the strong correlation indicated a high correspondence between the two variables.

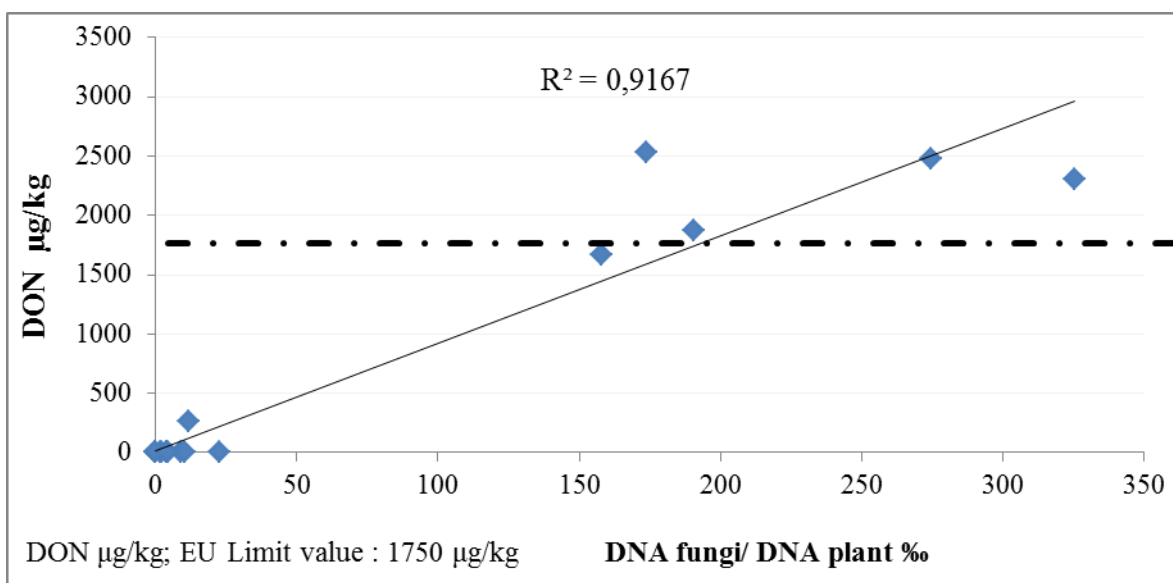


Figure 46. Correlation between DNA concentration and DON accumulation in maize grain during the second trial.

4.4 Aggressiveness of *Fusarium graminearum* isolates using several assays

4.4.1 Pathogenicity of fungal isolates

The three *F. graminearum* isolates tested were shown to be pathogenic in all three assays applied: The single floret inoculation assay showed bleached spikelets at the first assessment (7d post-inoculation) for all isolates. However, the progression of the FHB symptoms was faster for *F. graminearum* isolate DSMZ 1095 than for the two other isolates. The control group did not show any bleached spikelets. In the DNA assay, all wheat grain samples contained fungal DNA in different amounts which confirm that all isolates were pathogenic, whereas the control samples did not contain fungal DNA. In the Petri-dish assay, all three *F. graminearum* isolates caused brown spots on the coleoptiles and/or mycelium that completely or partly covered the seeds of the susceptible wheat cultivar (Ritmo), whereas there were no symptoms of *Fusarium* infection in the control treatment (Fig. 47).

4.4.2 Single floret inoculation

To determine disease severity values, we performed a single floret inoculation assay and arranged the isolates according to their aggressiveness as low, medium and highly aggressive. The tested *F. graminearum* isolates differed significantly in causing FHB symptoms in wheat. Normalized to the most aggressive isolate (*F. graminearum* isolate DSMZ 1095), the disease severity induced determined as 90.5%, closely followed by the *F. graminearum* isolate to5 with 75.1%, whereas the *F. graminearum* isolate Egy1 showed the lowest aggressiveness with disease severity level of 56.9% (Table 29). The FHB severity level of the un-inoculated control group was zero (data not shown).

4.4.3 Petri-dish test

The wheat seeds (Ritmo cultivar) reacted differently to the infection of *F. graminearum* isolates. Obvious symptoms of *Fusarium* infected seedlings were observed from the first assessment and exhibited a substantial variation in aggressiveness as defined by AUDPC

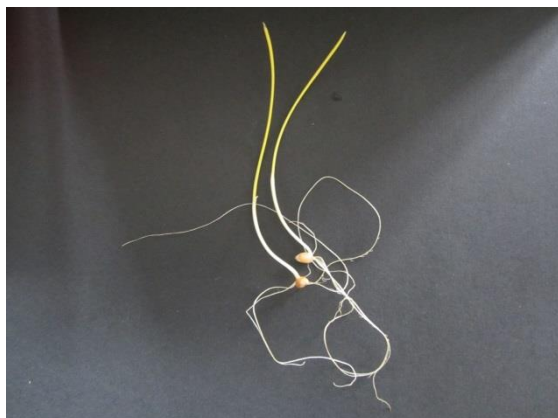
standard ranging from 0.40-0.89 on the AUDPC_{standard} scale, which ranges from 0 (not aggressive) to 1 (very aggressive). Also in this assay, the *F. graminearum* isolate DSMZ 1095_{standard} revealed the most aggressive isolate, with isolate To5 ranging slightly lower and isolate Egy1 revealing the lowest aggressiveness (Table 29). The control (wheat seeds treated with sterilized distilled water) had an AUDPC_{standard} value of 0.0 (data not shown).



F. graminearum isolate to5



F. graminearum isolate DSMZ 1095



Control



F. graminearum isolate Egy1

Figure 47. Symptoms on seedlings of wheat (Ritmo) inoculated with several isolates of *Fusarium graminearum* compared to the control at 6 day in Petri-dish test.

4.4.4 DNA quantification

All three *F. graminearum* isolates were able to produce fungal DNA, but differed significantly in their level of production. We determined the DNA concentration of grain samples of adult plants inoculated with the three different isolates (Table 29). Also here we found the highest

value for the *F. graminearum* isolate DSMZ 1095 (15300 DNA fungi/ DNA plant%), a lower value the *F. graminearum* isolate to5 (8850 DNA fungi/ DNA plant%), and the lowest value for the *F. graminearum* isolate Egy1 (2562 DNA fungi/ DNA plant%). Again, the uninoculated control group did not contain DNA.

Table 29. % disease severity values in Ritmo winter wheat cultivar inoculated with several isolates of *Fusarium graminearum*, the standardized area under disease progress curve (AUDPC_{standard}) for the fungal isolates used within Petri-dish test, DNA concentration in grain wheat plants inoculated with several isolates of *Fusarium graminearum* within floret inoculation test in greenhouse.

Variables	<i>Fusarium graminearum</i> isolates		
	DSMZ 1095	to5	Egy1
% disease severity	90.5 a	75.1 b	56.9 c
DNA concentration (DNA fungi/ DNA plant%)	15324 a	8850 b	2562 c
AUDPC _{standard}	0.89 a	0.56 b	0.40 c

Means followed by different letters in the row are significantly different

The data evaluation was started with the definition of an appropriate statistical model. The data were assumed to be approximately distributed and to be homoscedastic. These assumptions are based on a graphical residual analysis. The statistical model included (disease severity, DNA content, AUDPC_{standard}) as fixed factors. Based on this model, an analysis of variance (ANOVA) was conducted following by multiple contrast tests in order to compare the several level.

4.5 Distribution of some *Fusarium* species in maize plant

Recently, qPCR has been largely used for the evaluation of *Fusarium* spp. in the field by quantifying the fungal biomass in various parts of maize plant. In our present work, for all *Fusarium* species, fungal DNA was found in low or undetectable concentrations in the all tested organs until the flowering stage. Thereafter, the quantity of individual fungal species increased and varied among the samples which distributed in various amounts according to the growth stage.

4.5.1 Quantification of *Fusarium graminearum* in maize plant

In case of *F. graminearum*, DNA concentration was low in all tested organs before the flowering stage (GS 65) (Fig. 48), being more abundant in leaf sheath and leaf blade than in node and internode. In the subsequent course of development of fruit (GS 75), *F. graminearum* was detected in higher amounts in spindle and kernel, reaching levels of 6000 and 6337 DNA fungi/ DNA plant ‰, respectively. Whereas leaf sheath and leaf blade were largely accumulated compared to nodes and internodes. Here, DNA amounts considerably increased in all plant parts,

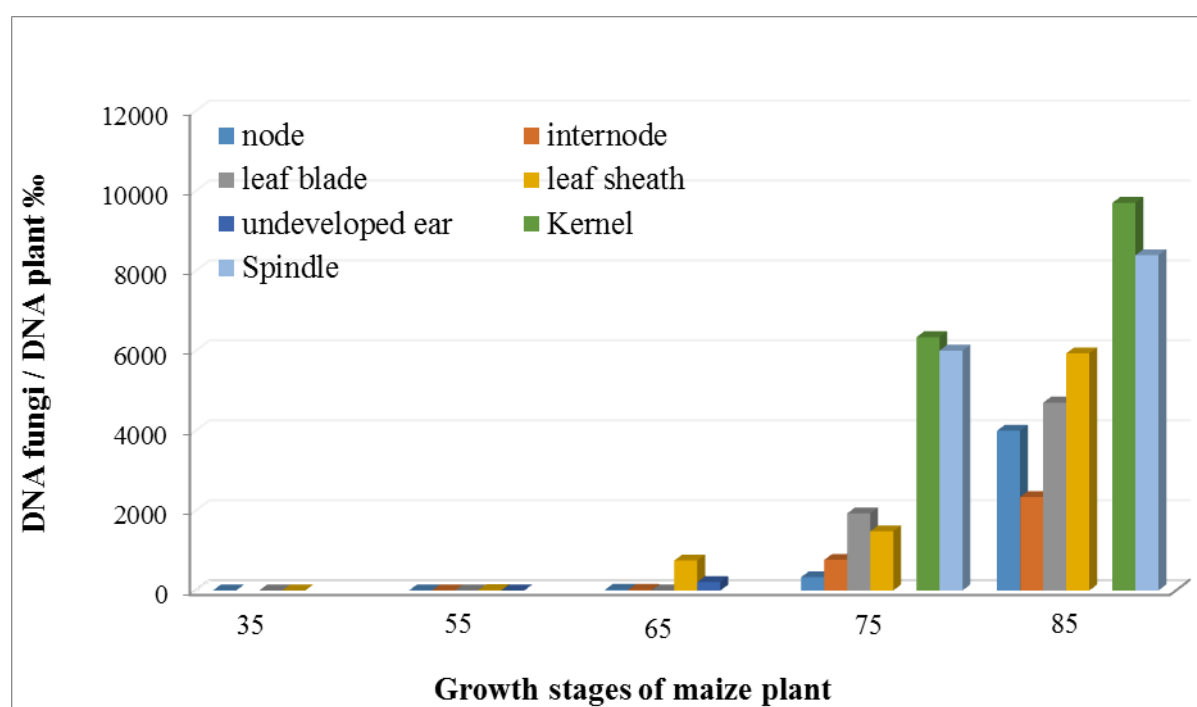


Figure 48. Quantification of *Fusarium graminearum* in maize plant.

particularly in the spindle and kernel, reaching levels of 8388 and 9700 DNA fungi/ DNA plant ‰, respectively. Higher amounts were also determined in the corresponding leaf blades and leaf sheaths (4700 and 5933 DNA fungi/ DNA plant ‰, respectively). On contrast, low amounts of DNA *F. graminearum* were observed in both internodes and nodes (2339 and 4000 DNA fungi/ DNA plant ‰, respectively) which were the smallest detected amounts in the tested organs in maize plant at the ripening stage (GS85).

4.5.2 Quantification of *Fusarium culmorum* in maize plant

Concerning the distribution of *Fusarium culmorum* in maize plant, DNA concentration had broad ranges during the various growth stages (Fig. 49). At the stem elongation (GS35), DNA

was detectable in all tested organs, and additionally, the organs were more contaminated with DNA of *F. culmorum* than *F. graminearum* at this stage. In case of *F. graminearum*, the tested organs were free of DNA at this stage, except small amounts lower than 3.4 DNA fungi/ DNA plant ‰ detected in nodes. In the subsequent course of the heading stage (GS55), it was observed that fungal DNA was present in all parts, being most abundant in leaf sheaths (reaching 32 DNA fungi/ DNA plant ‰). Subsequently, this value reached 1540 DNA fungi/ DNA plant ‰ at the flowering stage which was the most contaminated compared to the rest of the tested organs.

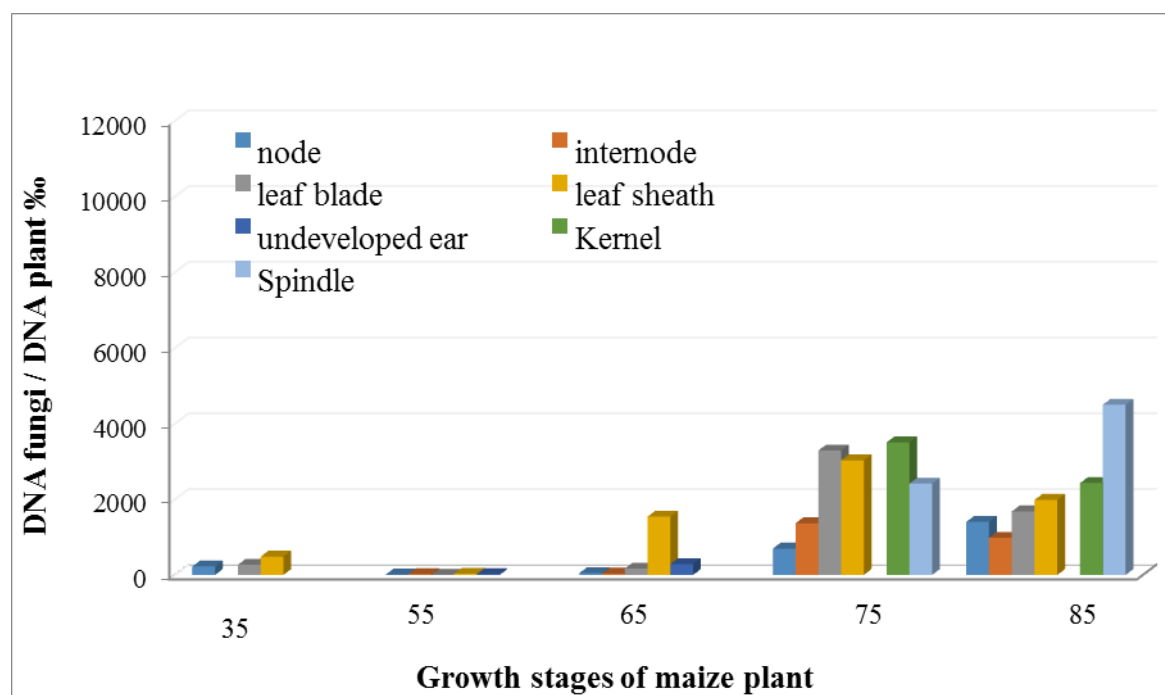


Figure 49. Quantification of *Fusarium culmorum* in maize plant.

At the development of fruit stage, the kernels were largely infected with *F. culmorum* (3501.2 DNA fungi/ DNA plant ‰), followed by the infection of leaf blades (3287 DNA fungi / DNA plant ‰). At the ripening stage, it was observed that DNA concentration gradually increased with the plant age. Here, DNA amounts considerably increased in all plant parts, especially in the spindle which is identical to *F. graminearum* at the same stage of growth. However, the fungal DNA reached 4498 DNA fungi/ DNA plant ‰ in spindles and 2428 DNA fungi/ DNA plant ‰ in kernels, followed by contamination level 1983 DNA fungi/ DNA plant ‰ in the leaf sheaths.

4.5.3 Quantification of *Fusarium avenaceum* in maize plant

F. avenaceum was also detected in maize plant, but in low concentrations compared to the mentioned *Fusarium* species (Fig. 50). Similar to the other fungal species, DNA concentration was low in all tested organs before the flowering stage.

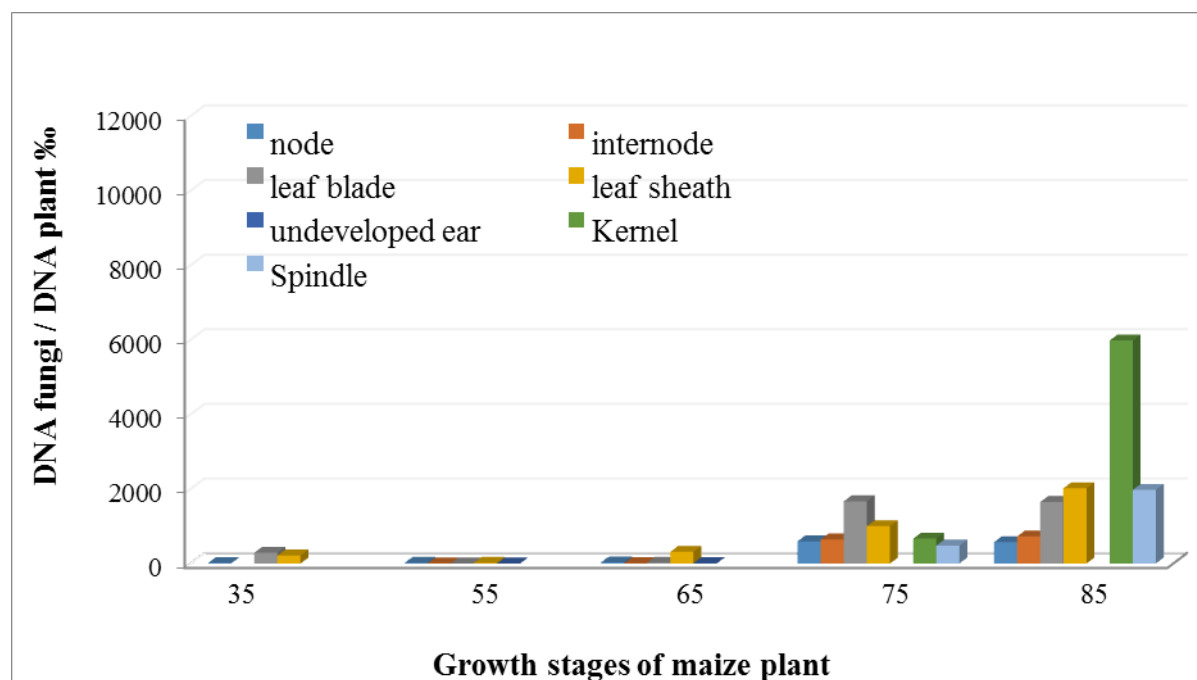


Figure 50. Quantification of *Fusarium avenaceum* in maize plant.

. DNA concentration slightly increased with plant age, the fraction of leaf blades being the most contaminated with amounts 1667 DNA fungi/ DNA plant %, while the remaining fractions showed less accumulation of *F. avenaceum* at the development of fruit stage. At the ripening stage, infection of kernels was substantially higher than in the other various plant fractions and also higher than the contamination with *F.graminearum* at this growth stage.

4.5.4 Quantification of *Fusarium poae* in maize plant

In terms of quantification of *Fusarium poae* in maize plant, small amount of DNA were detected in the corresponding organs over the development of the plant (Fig. 51). Here, the leaf sheaths were the most contaminated with *F. poae* until the development of fruit stage. This value ranged from 68.1 to 2730 DNA fungi/ DNA plant %. On the other hand, it was

observed that the spindles had the highest concentrations of DNA of *F. poae* (3249 DNA fungi/ DNA plant ‰), followed by the infection of leaf sheaths (2730 DNA fungi/ DNA plant ‰) at the ripening stage of maize plant.

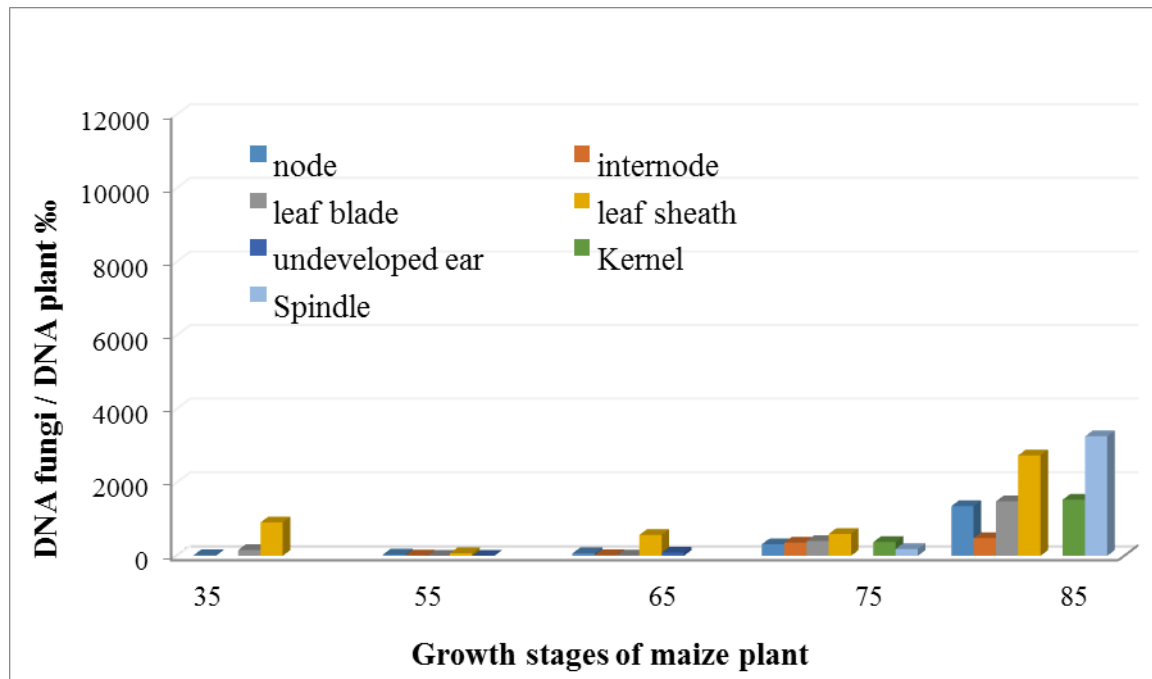


Figure 51. Quantification of *Fusarium poae* in maize plant.

4.5.5 Rainfall and temperature in the experimental field

Concerning the environmental conditions (Fig. 53), it was observed that rain events were low during maize growth, whereas rainfall was rarely recorded during July. On the other hand, low amounts were recorded during August and September.

The flowering stage occurred from 28 July until 11 August with mean temperature reached 20 °C and mean precipitation was 35.8 mm

The average maximum temperature during this stage was 25.5 °C and the average minimum temperature was 15.3 °C. Thus, environmental conditions have serious effects on the development of these fungal species.

Overall, the distribution of *Fusarium* species was considerably different throughout the development of maize plant. Additionally, the environmental conditions including rainfall and temperature influenced this appearance and distribution.

In summary, the predominant *Fusarium* species in the maize plant after flowering stage was *F. graminearum*, followed *F. culmorum*, while *F. poae* was the less prevalent.

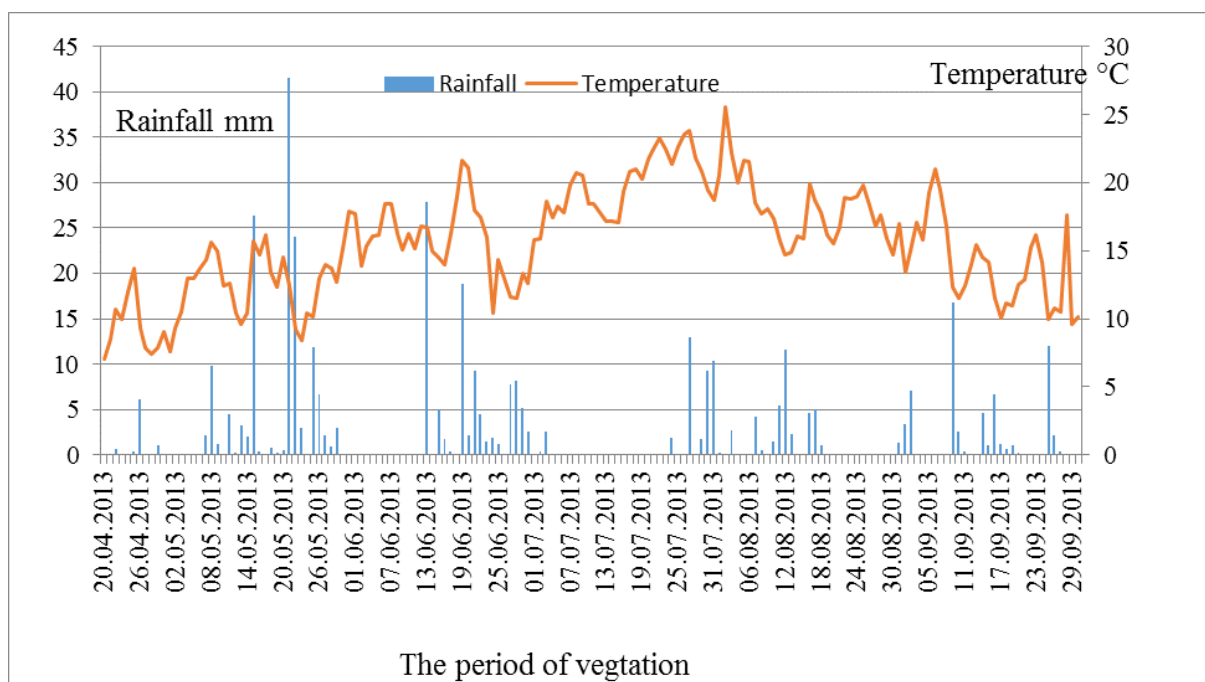


Figure 52. Rainfall and temperature in the experimental field (Hohenschulen) between April and September 2013.

4.6 Systemic growth of *Fusarium graminearum* and *Fusarium culmorum* in maize plant

4.6.1 Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (growth stage 33)

Presently, qPCR is being widely used for the assessment of *Fusarium* spp. in the field by quantifying the fungal biomass in various parts of maize plant. In our present study, for both *F. graminearum* and *F. culmorum*, fungal DNA was found in various concentrations in the all tested organs and this distribution was systemically distributed until the flowering stage. In other words, DNA values of the corresponding organs mostly decreased from the root to the section below the ear. Thereafter, the quantity of individual fungal species from the ear node increased and again was systemically distributed in various amounts according to the growth stage (GS).

At GS 33, in case of *F. graminearum*, DNA concentration largely was more abundant in the root compared to the other organs, reaching levels of 28.4 DNA fungi/ DNA plant %.

Subsequently, DNA concentration decreased from the first node to the fourth node from 2.1 to 1.2 DNA fungi/ DNA plant ‰, respectively. In conclusion, there were no significant differences between DNA amounts in both nodes and internodes (Fig. 53, Table 30).

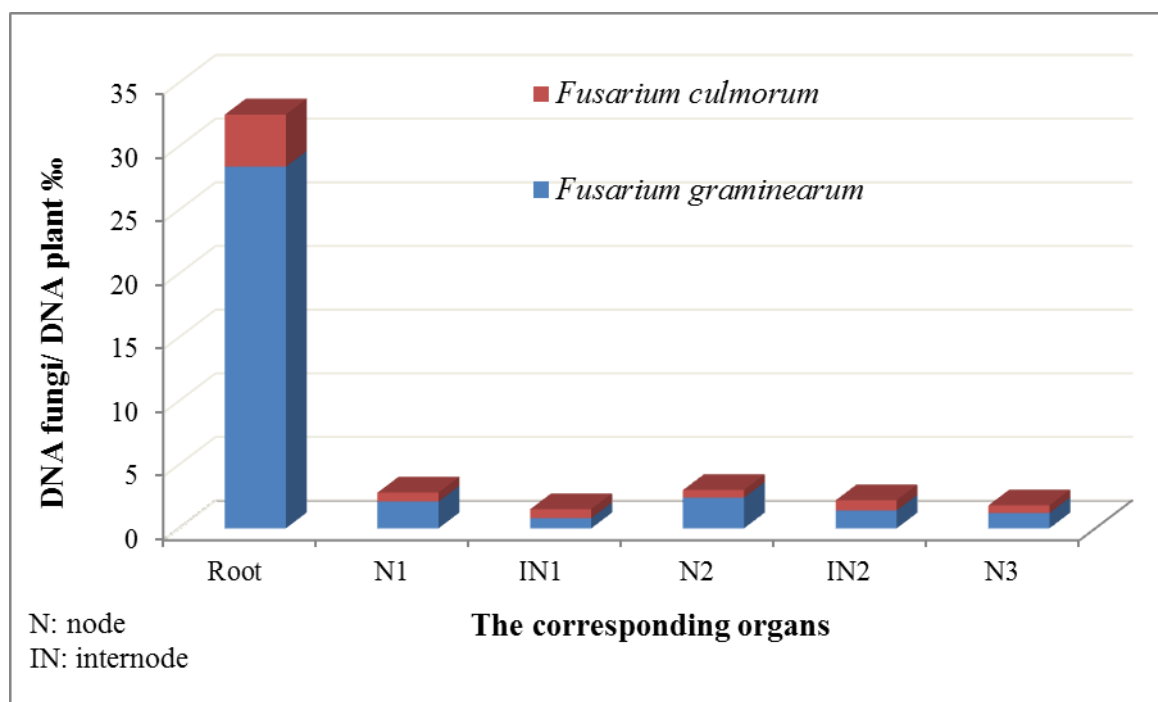


Figure 53. Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (GS 33).

Concerning the distribution of *Fusarium culmorum* in maize plant, DNA was largely detected in the root similar to *F.graminearum* but in lower amounts (4.1 DNA fungi/ DNA plant ‰). On the other hand, DNA was systemically detectable in the other tested organs. Additionally, the organs were more contaminated with DNA of *F. graminearum* than *F. culmorum* at this stage, however we could not find any significant differences (Table 30).

Table 30. Significant differences of quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (GS 33). N: node, IN: internode.

<i>Fusarium</i> spp.	Root	N1	IN1	N2	IN2	N3
<i>F. graminearum</i>	28,4a	2,1a	0,8a	2,4a	1,4a	1,2a
<i>F. culmorum</i>	4,1a	0,7a	0,7a	0,6a	0,8a	0,6a

Means followed by same letters in the row and column are not significantly different

4.6.2 Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (growth stage 65)

At the growth stage of flowering (GS 65), *F. graminearum* was detected in higher amounts in nodes and internodes, reaching levels of 19.9 and 13.4 DNA fungi/ DNA plant ‰, in the first node and internode, respectively, while DNA concentration in the fourth node and internode was lower (5.2 and 6 DNA fungi/ DNA plant ‰), respectively (Fig. 54).

At this stage, the fifth node was rather infected with *F. graminearum* (17 DNA fungi/ DNA plant ‰) compared to other corresponding nodes and internodes except the first node, followed by the sixth node in the section above the main ear (9.6 DNA fungi/ DNA plant ‰), Higher amounts were also determined in the corresponding main ear (58 DNA fungi/ DNA plant ‰). In contrast, a low amount of DNA *F. graminearum* was observed in the second ear, reaching a level of 11.3 DNA fungi/ DNA plant ‰. The smallest amounts in the tested organs in maize plant were determined in the last internode and node, reaching levels of 2 DNA fungi/ DNA plant ‰. Only significant differences were found between DNA fungal of the sixth internode and both the eighth and ninth internodes (Fig. 54, Table 31).

At the same stage, concerning the distribution of *Fusarium culmorum* in maize plant, it was observed that DNA concentration roughly decreased with the arrangement of the organs until the ear node. The root was contaminated with 11.4 DNA fungi/ DNA plant ‰, the first node and internode with 11.3 and 11 DNA fungi/ DNA plant ‰, respectively, while this amount decreased at the fourth node and internode, reaching a level of 4.9 and 6 DNA fungi/ DNA plant ‰, respectively. Thereafter, DNA amounts considerably increased and were systemically distributed until the last node, whereas its concentration was 17 DNA fungi/ DNA plant ‰ in the ear node. Afterwards, this amount gradually decreased, reaching a level of 2 DNA fungi/ DNA plant ‰ in the last node, whereas significantly differed from the fourth and seventh internode (Table 31, Fig. 54).

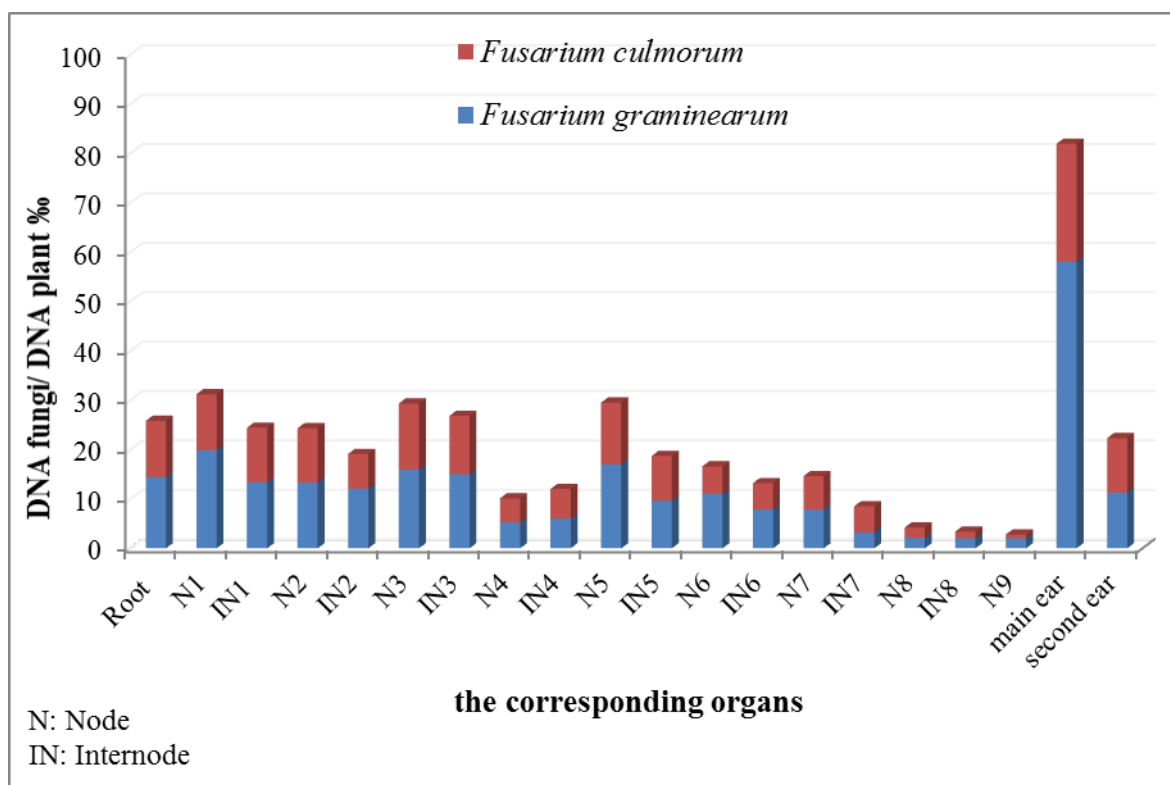


Figure 54. Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize (GS 65).

The main ear was contaminated with the highest amount of fungal DNA, *F. graminearum* being followed by *F. culmorum* (58 and 23.9 DNA fungi/ DNA plant %, respectively). However, this amount was lower in the second ear for both *Fusarium* species with levels of 11.3 and 11 DNA fungi/ DNA plant %, respectively. It is worthwhile to refer that the main ear was occasionally found at the sixth node. Generally, the organs were more contaminated with DNA of *F. graminearum* than *F. culmorum* at this stage, however no significant differences were found between the two species in the corresponding organs.

Table 31. Significant differences of quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (GS 65) N: node, IN: internode, *F. gra*: *F.graminearum*, *F.cul*: *F. culmorum*. Only p values less than 0.1 are shown (p< 0.1).

<i>Fusarium</i> spp.	fraction	Estimated mean difference	P value
<i>F. cul</i>	N9 - IN4	-7.667e+00	0.0564.
<i>F.cul</i>	N9 - IN7	-4.767e+00	0.0190*
<i>F. gra</i>	IN8 - IN6	-5.533e+00	0.0775.
<i>F. gra</i>	N8 - IN6	-5.533e+00	0.0263*
<i>F. gra</i>	N9 - IN6	-5.533e+00	0.0253*

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1. All other hiding means are not significantly different.

4.6.3 Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (growth stage 75)

At the development of fruit stage, *F. graminearum*, DNA concentration was more abundant in the plant compared to *F. culmorum*. DNA concentration considerably varied in the corresponding organs. Being more abundant in internodes than nodes, where it reached 102.8 DNA fungi/ DNA plant ‰ in the first internode while it was 24 DNA fungi/ DNA plant ‰ in the first node. DNA concentration decreased from the root (214 DNA fungi/ DNA plant ‰) through the stalk until the fourth node (6.2 DNA fungi/ DNA plant ‰). After that, DNA was largely available in the fifth node, where the main ear was located, with a level of 64.9 DNA fungi/ DNA plant ‰ (Fig. 55).

At this stage, the fifth node was largely infected with *F. graminearum* compared to other corresponding nodes and internodes, followed by the infection of the sixth node in the same section (50.3 DNA fungi/ DNA plant ‰), which significantly differed from nodes (N4,N8, N9) and internodes (IN5,IN8). Higher amounts were also determined in the corresponding main ear (227.8 DNA fungi/ DNA plant ‰). In contrast, a low amount of *F. graminearum* DNA was determined in the second ear, reaching a level of 22.9 DNA fungi/ DNA plant ‰, however DNA concentration was more abundant in the plant compared to *F. culmorum*. The nodes (N6, N9) and the sixth internode (IN6) were significantly contaminated with *F. graminearum* than *F. culmorum* (Table 32).

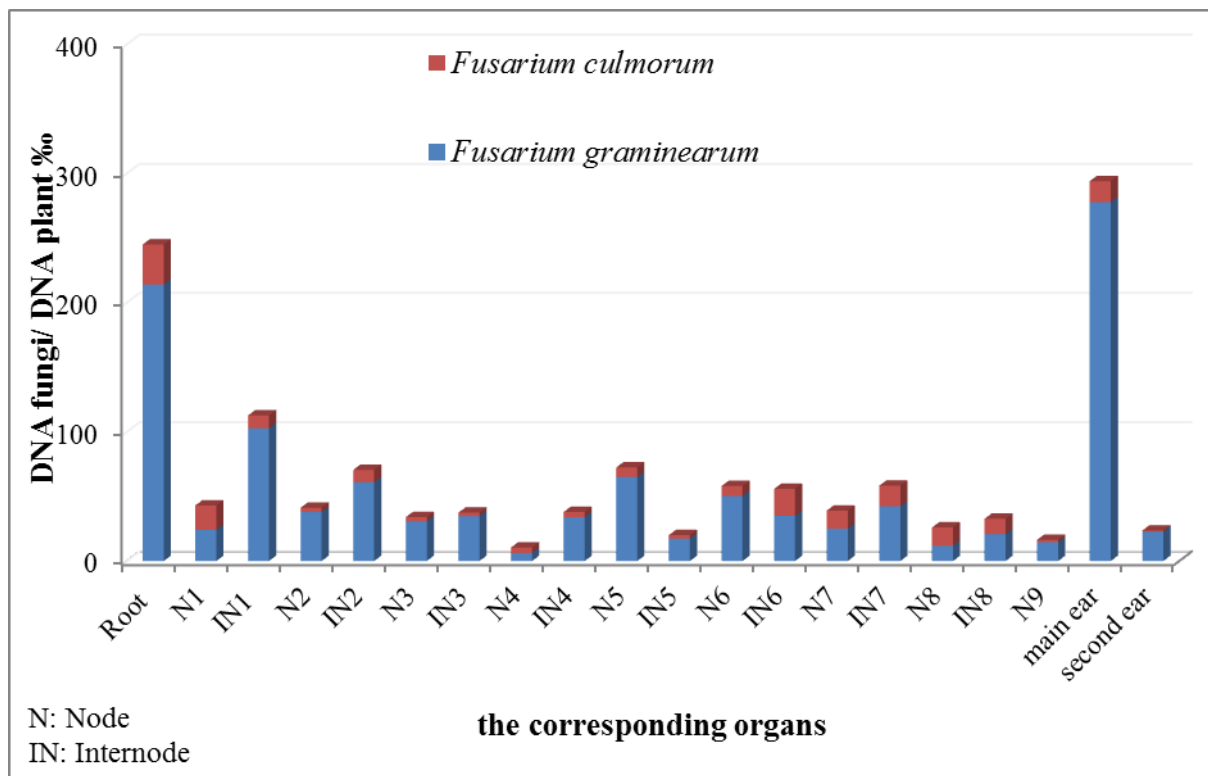


Figure 55. Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize (GS 75).

Regarding the distribution of *Fusarium culmorum* in maize plant, it was found that DNA values roughly decreased with the arrangement of the organs until the ear node. Whereas fungal DNA was 31 DNA fungi/ DNA plant %, the concentration was 19 and 9.8 DNA fungi/ DNA plant % in the first node and internode, respectively, while this amount decreased at the fourth node and internode, reaching levels, of 4.3 and 3.9 DNA fungi/ DNA plant %, respectively. Afterwards, DNA amounts increased and reached 7.5 and 2.7 DNA fungi/ DNA plant % in fifth node and internode, respectively. The last node was contaminated with a small amount of DNA, reaching a level of 1.8 DNA fungi/ DNA plant % similar to *F.graminearum*. The main ear, on the other hand, was infected with higher concentration of DNA compared to the second ear (16.2 and 0.7 DNA fungi/ DNA plant %, respectively), however no significant differences were determined between the content of corresponding organs of *F. culmorum* (Fig. 56). It is worthwhile to refer that the main ear was occasionally found at the sixth node. Generally, the organs were more contaminated with DNA of *F. graminearum* than *F. culmorum* at this stage, (Table 32).

Table 32. Significant differences of quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (GS 75) N: node, IN: internode, *F. gra*: *F.graminearum*, *F.cul*: *F. culmorum*. Only p values less than 0.1 are shown (p< 0.1).

<i>Fuarium</i> spp.	fraction	Estimated mean difference	P value
<i>F. gra</i>	N6 - IN5	3.270e+01	<0.01***
<i>F. gra</i>	N4 - IN6	-2.780e+01	<0.01**
<i>F. gra</i>	N9 - IN6	-2.100e+01	0.0110*
<i>F. gra</i>	N6 - IN8	3.200e+01	<0.01 **
<i>F. gra</i>	N6 - N4	4.380e+01	<0.01 ***
<i>F. gra</i>	N8 - N6	-3.900e+01	0.0137 *
<i>F. gra</i>	N9 - N6	-3.700e+01	<0.01 ***
<i>F. gra – F. cul</i>	IN6	21.600	0.0533.
<i>F. gra – F. cul</i>	N6	42.267	<1e-04 ***
<i>F. gra – F. cul</i>	N9	11.200	<1e-04 ***

Significant codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1. All other hiding means are not significantly different.

4.6.4 Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (growth stage 85)

At ripening growth stage (GS 85), DNA concentration of *F. graminearum* was roughly more abundant in all tested organs above the ear node compared to the previous growth stages. Here, the infection of the fifth node reached 72.4 DNA fungi/ DNA plant ‰ which significantly differed from DNA fungal from the third to the eighth internode and (N6,N8, N9) as well as, the root and the second ear. On the other hand, DNA concentration reached 41 DNA fungi/ DNA plant ‰ in the fifth internode, which significantly was contaminated compared to the nodes from the second one until the ninth one, additionally, the root and second ear. Also, the smallest amounts in the tested organs in maize plant were determined in the last internode and node. DNA concentration was less considerably at the last internode and node compared to other parts, reaching levels of 6 and 0.5 DNA fungi/ DNA plant ‰, respectively. Higher amounts were also determined in the corresponding main ear (233 DNA fungi/ DNA plant ‰). In contrast, a low amount of DNA *F. graminearum* was observed in the second ear, reaching a level of 2.7 DNA fungi/ DNA plant ‰. In general, DNA concentration was more abundant in the plant compared to *F. culmorum* (Fig. 56, Table 33).

At ripening growth stage (GS 85) and concerning the section below the ear node, *F. graminearum* was detected in various amounts in nodes and internodes, reaching levels of 146 fungi/ DNA plant ‰ in the root, while DNA concentration of the first node and internode was lower (15.7 and 49 DNA fungi/ DNA plant ‰, respectively). Here, significant differences were observed between the corresponding organs (Fig. 56, page 128).

At this stage, the nodes were largely and significantly infected with *F. graminearum* compared to other corresponding internodes whereas the infection the second node reached (56.7 DNA fungi/ DNA plant ‰) which significantly differed from the nodes (N3, N4, N5, N6, N7, N8, N9) as well as the root and the second ear. Significant differences were observed between the other nodes and internodes (Table 33, appendix).

At the same stage, concerning the distribution of *Fusarium culmorum* in maize plant it was observed that DNA concentration was largely available in the root compared to the other corresponding organs except the main ear, reaching a level of 28.1 DNA fungi/ DNA plant ‰, whereas fungal DNA concentration was 15.1 and 32 DNA fungi/ DNA plant ‰ in the first node and internode, respectively. After that, this amount decreased at the fourth node and internode, reaching levels of 4.5 and 10.8 DNA fungi/ DNA plant ‰, respectively. Thereafter, DNA amounts roughly increased and were systemically distributed until the last node, whereas this concentration significantly increased in the main ear, reaching a level of 181 DNA fungi/ DNA plant ‰. Similar to *F. graminearum*, this amount was lower in the second ear with a level of 0.5 DNA fungi/ DNA plant ‰.

Generally, the organs were more severely contaminated with DNA of *F. graminearum* than *F. culmorum* at this stage with significant differences were observed in the nodes (N2, N3, N4, N5, N6, N7) and internodes (IN5, IN6, IN8) as well as the root (Table 33, appendix).

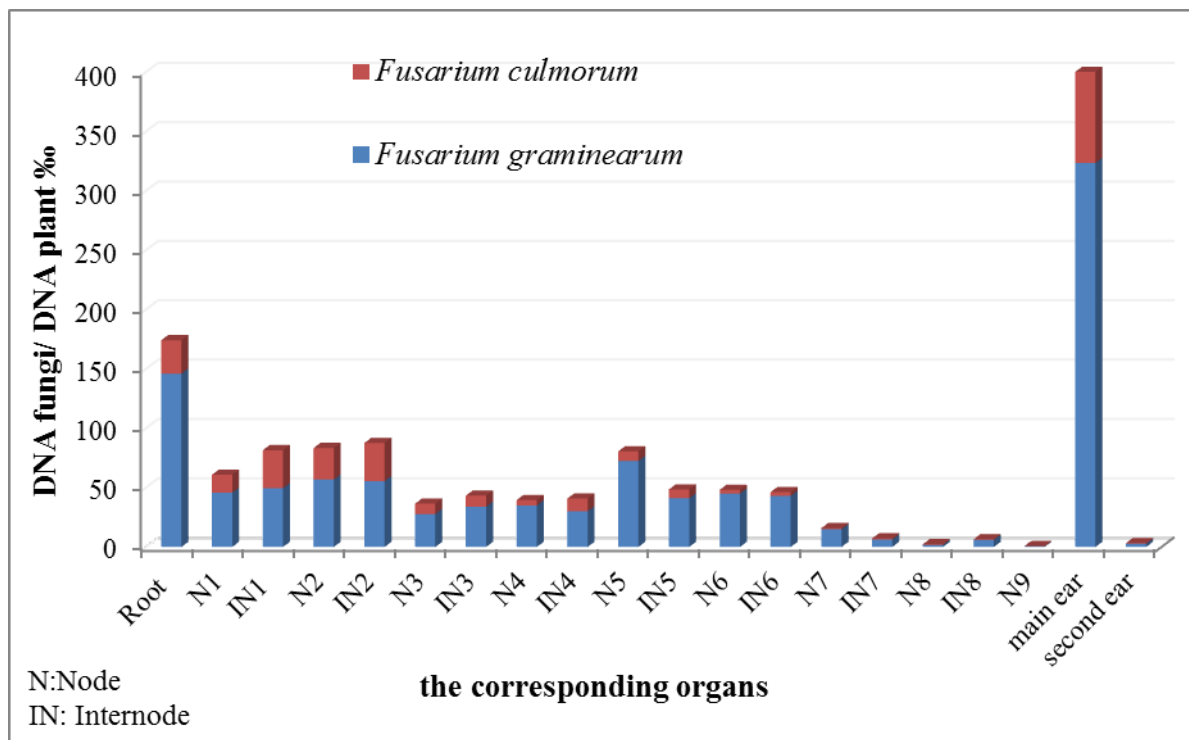


Figure 56. Quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize (GS 85).

4.6.5 Rainfall and temperature in the experimental field

It was observed that rain events varied during maize growth. Rainfall was largely recorded during May reaching levels of 16.2 mm; then, rainfall in June exceeds 16 mm but dry periods were recorded during this month. On the other hand, varying amounts were recorded during the other months which ranged from 0.1-19.9 mm. Generally, warm conditions were available during the plant growth with mean daily temperatures reaching 22.3 °C during August (Fig. 57).

The flowering stage occurred from 3 until 16 August with mean temperature of 20.2 °C. rain events were low and irregular varied, where dry days were recorded. The maximum mean precipitation reached 18.7 mm during this stage. The average maximum temperature during this stage was 22.2 °C and the average minimum temperature was 17.8 °C.

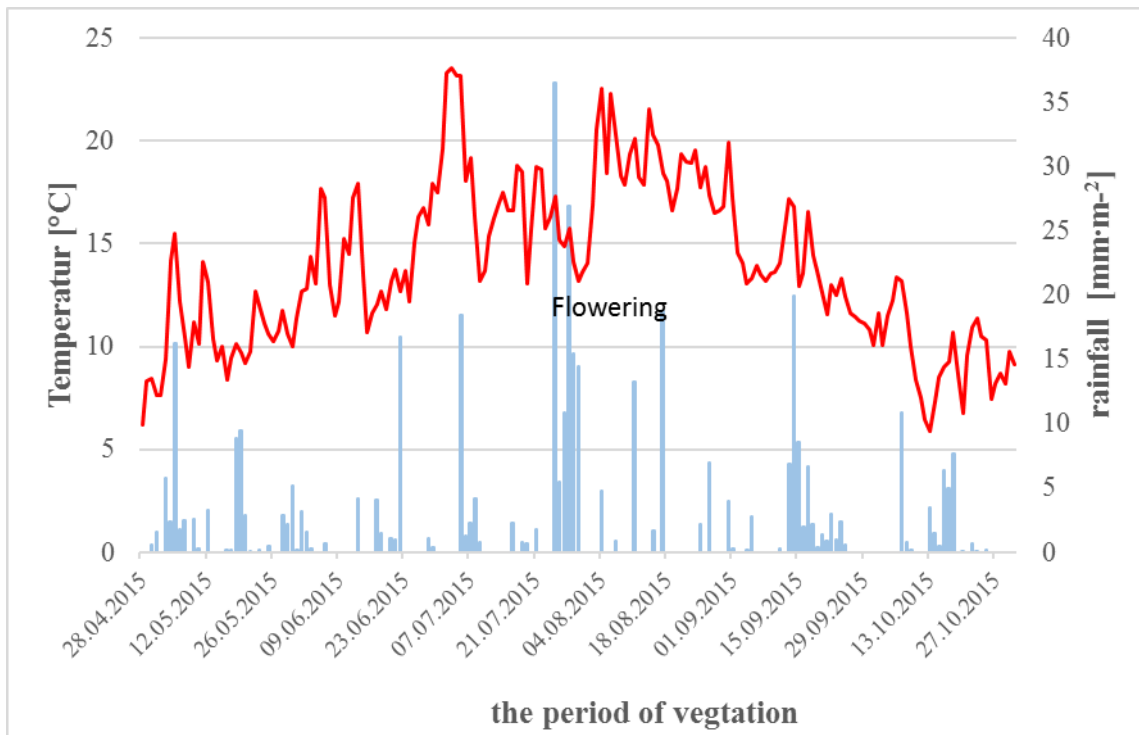


Figure 57. Rainfall and temperature in the experimental field (Hohenschulen) between April and October 2015.

Generally, the distribution of *Fusarium* species was considerably different throughout the development of maize plant. Additionally, Environmental conditions including rainfall and temperature have serious effects on the development of these fungi; for that reason, the management of these diseases during the earliest stages of disease progress is considered to be the most effective way of disease control.

5. Chapter IV
Discussion

The present work consists of six different experiments, for that reason, the discussion of the results were separately presented for each one.

5.1 Sensitivity of *Fusarium* species isolates to metconazole and epoxiconazole and comparison of CYP51 sequence of *Fusarium* species with CYP51 in *Septoria tritici*

This study was done to determine the role of point mutation in causing different sensitivity levels in *Fusarium* spp. isolates by comparing CYP51 sequence of mutant and wild type *Septoria tritici*. There is limited information in the literature about the sensitivity of several *Fusarium* species against DMI concerning the CYP51 gene. Major studies on *Fusarium* species focus only on *F. graminearum* or *F. asiaticum*.

This study aimed to determine the sensitivity of various isolates obtained from different geographical origins in Germany and other countries as well as to investigate possible mechanisms of reduced sensitivity to DMIs and its correlation with alterations in the target-encoding CYP51 gene. It also investigates whether the mutations of *Fusarium* species CYP51 confer different sensitivity levels by comparing with CYP51 sequence of *Septoria tritici* which resistance towards DMI fungicides was previously reported (Kiesner, 2012)

On the whole, the results demonstrated different sensitivities of *Fusarium* isolates towards metconazole and epoxiconazole fungicides (Table 11, 12, 13). Metconazole was the most effective in reducing the fungal growth on carrot agar medium. This activity was significantly stronger than epoxiconazole activity and differed considerably between the different isolates. The observation that metconazole activity is most effective is in agreement with the findings of other authors (Edwards et al., 2001; Simpson et al., 2001; Mesterházy et al., 2003). Additionally, Ivic et al. (2011) reported that metconazole was more efficient in reducing mycelia growth of *Fusarium* spp. on potato dextrose agar medium.

Epoxiconazole, on the other hand, was less effective. The small effect of epoxiconazole was also noted by Menniti et al. (2003).

In this study, it was suggested that lower concentrations of DMI fungicides only have slight effects on the fungal growth, which are similar to those reported by Audenaert et al. (2010). In the same study, it was suggested that lower doses of DMI fungicides did not have a significant effect on conidial germination; consequently, this small effect of fungicide has been reflected in the amount of fungal biomass.

Our results suggested that only one gene of CYP B 51 (FGSG 01003.3) was present in all *Fusarium* spp. isolates. This finding was similar to those reported by Klix. (2007). With regard to the sensitivity of *Fusarium* species to both DMI fungicides, our results demonstrated that the observed differences in sensitivity to the fungicides did not depend on differences in the CYP51 gene sequence. In other words, point mutations (missense) in CYP51 gene were not associated with fungicide sensitivity in these isolates because the isolates with different levels of sensitivity had the same amino acid sequence. This observation is in agreement with the findings of Yin et al. (2009), who concluded that there were no mutations associated with DMI resistance by analysing the deduced amino acid sequence of CYP51A and CYP51B, whereas three resistant isolates of *F. asiaticum* and *F.graminearum* isolates were resistant to tebuconazole and metconazole in China. Additionally, they concluded that DMI resistance was not associated with the expression of the CYP51 gene.

In the same regard, Liu et al. (2011) reported that no change in DMI sensitivity was observed in CYP51B deletion mutants. These authors also found that the sensitivity of CYP51C, CYP51A deletion mutants to some DMI fungicides raised compared to the parent strain; however, these mutations did not influence the mycelial growth indicating that none of these genes is an efficient factor for this growth stage.

Tateish et al. (2010) suggested that the substitutions of amino acid residues in FGSG 01003.3 were not correlated with the sensitivity to metconazole and one single amino acid substitution was observed in four isolates of *F. graminearum* tested. They reasoned that, because the isolates with MIC of 6.25 and 1.56 had the same amino acid sequence as isolates which had MIC of 3.13 and 0.39.

Leroux et al. (2007) and Kiesner (2012) reported that changes in sensitivity of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) to DMI fungicides were associated with mutations in the CYP51 gene, and they concluded that several important substitutions from CYP51 gene are involved in different levels of sensitivity to DMI fungicides. But in our results, no similar amino acid substitutions were observed, owing to the observation that the isolates with different levels of sensitivity to DMI had the same amino acid substitutions at defined positions 50, 136, 137, 188, 379, 381, 459, 460, 461, 510, and 513 of wild *Septoria tritici*, indicating that such changes did not influence the sensitivity level.

Several factors such as overexpression of the target CYP51 gene and overexpression of ATP-binding cassette (ABC) membrane transporters could be involved in the mechanism causing the difference in sensitivity (Becher and Wirsal, 2012). Further investigations on the influence of these factors will be interesting and helpful in this regard. Additionally, our results suggested that sensitivities of *Fusarium* species were distributed in a narrow range with metconazole efficacy being high. But FHB resistance against DMI were documented in many places all over the world. For that reason it will be worthwhile to incorporate antiresistance strategies in management of FHB with DMI fungicides. However, the monitoring of pathogen populations for fungicide sensitivity over the time is still an efficient way to determine whether the resistance is developing.

5.2 *In vitro* sensitivity of isolates of *Fusarium* species to carbendazim and investigation of the contribution of β -tubulin to its sensitivity

This experiment was conducted to determine sensitivity to carbendazim in *Fusarium* isolates collected from wheat and maize in Germany as well as to investigate the contribution of *tub2* to the sensitivity of *Fusarium* spp. to carbendazim. It is one of several members of a small family that are fundamental for different cellular activities (Zhao et al., 2014).

Sensitivities of isolates towards carbendazim were determined by calculating percent growth inhibition at different concentrations. Our results suggested that all *Fusarium graminearum* isolates had a low sensitivity against this fungicide (Table 16). The small effect of carbendazim was also noted by Chen and Zhou (2009). From the 70s of the 20th century, carbendazim was generally used in management of FHB and because of the widespread application of MBC fungicides, their effectiveness dramatically declined and was threatened by the emergence of resistant pathogen population in the field.

Our results demonstrated a point mutation in tested strains at the position 198. This mutation results in a single amino acid substitution, in which glutamic acid was converted to glutamine in all isolates. This observation is in agreement with the findings of Liu et al. (2010) who concluded that point mutations at codons 167, 198, 200 are associated with decreased levels of sensitivity against carbendazim. However, the resistance was high when double point mutations were at codons 200 and 198. Additionally, other studies suggested that the

carbendazim resistance was associated with certain substitutions in *tub2* of *Fusarium* population (Chen et al., 2009; Liu et al., 2010; Zhao et al., 2014).

In conclusion, continuous monitoring for resistant *Fusarium* spp. will be worthwhile to reveal the diversity of point mutations in resistant isolates.

5.3 Application timing and dose rate of triazoles alone or in mixture with pyraclostrobin for the control of *Fusarium* head blight and deoxynivalenol in wheat and maize

This experiment was aimed to evaluate the effect of protective and curative application and dose rate on the efficacy of metconazole, single applications of epoxiconazole and pyraclostrobin fungicides or in mixture (pyraclostrobin and epoxiconazole) in reducing *Fusarium* occurrences in maize, wheat and DON accumulation in grain. Additionally, to compare the effect of the mixture on *Fusarium* development and DON accumulation in wheat and maize grain.

There is limited information in the literature for the use of other strobilurins such as pyraclostrobin in FHB management (Spolti et al., 2013). In the two years of this study, we determined the protective and curative activity of two commonly used triazole fungicides (metconazole and epoxiconazole), pyraclostrobin alone and a mixture of epoxiconazole and pyraclostrobin against *Fusarium graminearum* in the greenhouse. We observed that there is significant difference between the protective and curative activities (Fig 18, 19, 20, 21). All treatments had a good effect in reducing the disease indicators FHB, FDK, DNA content and DON content when applied pre-or during inoculation, whereas the curative activity was significantly lower and differed strongly between the different fungicides (Fig 22, 23, 24, 25, 26, 27, 28)

The observation that protective treatment is most effective is in agreement with the findings of Hasan (1993) who suggested that fungicides are more effective at the early stages of *Fusarium* infection process as spore germination and growth of the germ tube. Also the two main critical factors in the use of fungicides to control FHB are the timing and the dose of application (Mesterházy et al, 2003) The same observation was also noted by Qingmei et al. (2005), who reported that the protective application of triazole fungicides reduced FHB index by 76.47-82.06% and DON content by 42.43-88.39% compared to the untreated wheat plants

when applied 2 or 3d before the inoculation. In the same regard, five-year research concluded that applying fungicides containing prothioconazole at the start of anthesis greatly reduced FHB index (Gilbert and Haber, 2013). In different study, it was suggested that epoxiconazole and pyraclostrobin combination had a high effectiveness against *Fusarium* spp. in maize grain when applied at early stages of maize growth, whereas DON was significantly reduced by 92% compared to the control. In the same study, the combination of metconazole and pyraclostrobin also decreased *Fusarium* spp. severity and inhibited mycotoxin production. (Urban, 2013)

In our study, metconazole was most effective in reducing the observed disease indicators in wheat and maize when applied as a curative treatment, indicating that this fungicide is very effective against *F. graminearum*. This observation is in agreement with the findings of other authors (Edwards et al., 2001; Simpson et al., 2001; Mesterházy et al., 2003). In the same regard, Matthies and Buchenauer (2000) reported that metconazole; prothioconazole and tebuconazole were found to be effective, resulting in reductions of FHB severity and mycotoxin contamination by 50- 80% and 5-90%, respectively. In other studies, tebuconazole and metconazole showed high efficiency against *F. culmorum* and other fungal pathogens in wheat plants (Simpson et al., 2001; Ioos et al., 2005).

In the same regard, the application of metconazole effectively reduced FHB, FDK, DNA and DON levels in grain, which indicates that this compound is very effective in controlling *F. culmorum* and *F. graminearum* (Pirgozliev et al., 2002). Another triazole fungicide, prothioconazole, was effectively able to reduce the mycelial growth of some *Fusarium* species (*F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichioides*, *F. tricinctum*) (Kotowicz et al., 2014). Amarasinghe et al. (2013) reported that metconazole with other triazole fungicides were effectively reduced FHB index and DON levels, whereas metconazole reduced both variables, reaching level of 81.4% and 75.8 %, respectively during the first year of the experimental study. Urban (2013) suggested that metconazole largely reduced DON in maize grain when applied alone or in a mixture with pyraclostrobin at the early stages of maize growth. Whereas DON reduction exceeded 60% compared to the control during the second year of the field study.

Epoxiconazole, on the other hand, was hardly effective in reducing FHB, FDK, DNA and DON levels when applied on 1d post-inoculation during the first year, while had a better

efficacy in reducing DNA and DON levels when applied at the same time point during the second year of the experimental work. On the other hand, epoxiconazole treatment showed no effect or little effect when applied on 3d post-inoculation. We even observed a significant increase when this fungicide was applied in sublethal doses post-inoculation on maize and wheat grain and also DON content seemed to increase for this dose. However, the full recommended dose of this treatment reduced both DON and DNA content in maize and wheat grain when applied on 1d post-inoculation. Although this treatment could not demonstrate significance difference for this effect on DON and DNA levels when applied on 3d post-inoculation. The small effect of epoxiconazole was also noted by Menniti et al. (2003), who, reported that a single application of epoxiconazole reduced the incidence and severity of FHB when infection pressure was low to medium while it was ineffective in reducing DON content. An increase in mycotoxin concentration after epoxiconazole treatment was also noted by other authors (Milus and Parsons). Application of epoxiconazole may therefore represent a risk, potentially resulting in high DON contamination in grain. On the same regard, Klix et al. (2007) reported that the inhibitory effect of epoxiconazole on mycelial growth decreased at a rate of 0.22% per year, while this efficacy was strongest in isolates isolated in 1987, among other isolates which were isolated between 1987-2004 in Germany. Klix et al. (2007) attributed the declining sensitivity to fungal adaptation to triazole fungicides.

Our results suggested that pyraclostrobin was less effective compared to epoxiconazole when applied on 1d post- inoculated, however this treatment slightly reduced the other disease variables when applied at this time point, while showed from no effect to little effect when applied on 3d post-inoculation. Whereas did not reduce DON concentration in maize grain under to the EU limit value when applied on 1d or 3d post inoculation.

In the first trial, our study also suggested that the mixture of pyraclostrobin and epoxiconazole significantly reduces FDK levels when applied in full dose post-inoculation. However, FHB index was only reduced when treatment took place 1d post-inoculation. We observed small effect of this fungicide on DON content for the curative treatment. On the other hand, this mixture treatment resulted in good reduction for most disease variables when applied on 1d post inoculation expect DON concentration in maize and wheat grain during the second year of the study, while roughly showed no effect or little effect on FHB, FDK, DNA and DON levels when applied on 3d post-inoculation during the second trial of the study. This is in agreement with the findings of Spolti et al. (2013), who used a similar mixture of triazole and

strobilurin, and suggested that the mixture of metconazole and pyraclostrobin had no significant effect on DON content but could be effective and led to high increase in yield when used in two sequential sprays. Integrated use of pyraclostrobin and epoxiconazole provided high control efficacy of FHB at two locations in China (Chen et al., 2012) only when used in two sequential sprays but this study did not analyse DON content. In the same regard, Dietrichs (2012) concluded that the strobilurin-triazole mixtures mainly lead to increase the toxin concentration in maize grain.

Most studies on the use of strobilurins for FHB control have focused on azoxystrobin and the results varied from no effect on yield or DON to an increase in DON accumulation over the non-treated control (Haidukowski et al., 2005; Gilbert and Haber, 2013). Simpson et al. (2001) observed that application of the strobilurin fungicide, azoxystrobin increased the DON content in infected grains. Also Pirgozliev et al. (2002) found a strong relationship between the amount of *Fusarium* present and the DON concentrations, and noted that azoxystrobin was significantly less effective against *Fusarium* than the triazole fungicide used for comparison, metconazole.

The small effect of strobilurins was interpreted by Menniti et al. (2003) that strobilurin do not control *F. graminearum*. Audenaert et al. (2011) also reported that strobilurins often result in increased DON levels and attributed this observation to the activity spectrum of strobilurins, which mainly target *Microdochium nivale* while being less effective against *F. graminearum*. It is thus possible that the niches that are no longer occupied by *M. nivale* are taken by *F. graminearum* and that this consequently leads to increased DON content. In the same regard, it was attributed in a more recent study that there were no significant difference neither Tri5 expression nor DON accumulation between the sets of azoxystrobin fungicide-treated and control wheat plants. Generally, Strobilurin fungicides are known to have lower fungitoxicity to FHB, they usually used in mixtures with triazoles to increase the spectrum of protection against multiple leaf diseases of wheat and consequently lead to higher yield compared to triazole alone. (Gilbert and Haber, 2013)

In this study, it was suggested that DON content increases after the sublethal fungicides application, this result is in agreement with was noted by Audenaert et al. (2010) that the sublethal triazole application resulted in a significant increase in DON production by *F. graminearum*. It was observed that sublethal triazole application led to increase H₂O₂

accumulation compared to the control. The stimulated DON production was preceded by a prompt induction of H₂O₂, suggesting that the proliferated DON accumulation was induced by an oxidative stress response in the fungus. Also, DON production by *F. graminearum* is the result of the interaction of fungal genomics and external triggers.

This present work attributed that disease variables including FHB, FDK, DNA, DON had a high correlation (from Fig 35 until Fig 46); only FHB index was slightly lower correlated to DNA concentration compared to FDK-DNA, also poor correlation was observed between FHB - DON for the dose 100%. This observation is in agreement with Palazzini et al. (2015) who concluded that *Fusarium*-damaged kernel has been considered as subjective parameter to correlate DON concentration of *F. graminearum* in grains. In the same study, a strong correlation was revealed among DNA fungal and DON concentration.

Paul et al. (2005) suggested that over 50% of the DON variation was explained by FDK values. In the same regard, Beyer et al. (2007) reported that 90% of DON level was explained by FDK level in artificial combined lots. Generally, FDK calculated after harvest and showed the highest association with DON while FHB index was quantified before many days of the harvest, thus, DON concentration could be increased in the grain (Palazzini et al. 2015). As result, the understanding of the association among disease variables may be useful way in developing of DON predictive models which can be used to monitor the potential inoculum and for managing this disease. Additionally, these measurements of qPCR could be used to predict the potential level of DON accumulation in the field or during storage. In the same regard, some researchers have suggested a strong association between FHB visual ratings and DNA levels in infected grain, while others reported that DON level is either only had a moderate relationship or not correlated with FHB index (Gilbert and Haber, 2013).

Generally, DNA, DON concentrations were considerably concentrated in maize grain compared to wheat grain. It was reported that maize is the crop which shows the highest contamination with mycotoxins of *Fusarium*. Additionally, the amounts of *Fusarium* metabolites in maize grain are about ten times than those present in wheat kernel when the intensity of infection is similar and the same *Fusarium* species are involved (Munkvold, 2003; Dorn et al., 2009).

It is worthwhile to refer that the growth stages of wheat plant were rather different when the inoculation applied at flowering stage, thus, the rate of resulted damaged *Fusarium* heads was rather slight compared to the first year of the study. As consequence, DON concentrations determined were low during the second year of the study.

The results of this study suggest that the protective treatments can be used to control FHB efficiently. Our findings, also confirm that epoxiconazole is less effectiveness, and the metconazole was the most effective fungicide. In addition that strobilurin alone or in mixture with triazole showed a low efficacy or increased DON content in grain. To determine an optimal treatment protocol for FHB control, additional studies under conditions more favorable for disease development and mycotoxin synthesis will be necessary. It also should be examined whether application of mixture fungicides can effectively contribute to better yield and grain quality. The selection of wheat cultivars with the highest level of resistance to FHB combined with application of fungicide should be tested to provide the best protection for the crop against FHB at the lowest possible fungicide use.

5.4 Aggressiveness of *Fusarium graminearum* isolates using several assays

This work investigated different aggressiveness assays (Petri-dish test, single floret inoculation, DNA quantification) and compared the results of Petri-dish test, which was suggested by PuraHong et al. (2012) as a simple and stable method, compared with the other assays. This is the first study done that compares the aggressiveness of *F. graminearum* isolates from different geographic origins using the Petri-dish test in addition to two assays, thus ensuring that their aggressiveness is stably and correctly quantified.

In our study, all *F. graminearum* isolates tested were pathogenic, causing disease across all different aggressiveness assays. This observation agrees with a previous report that pathogenicity is an important characteristic of *F. graminearum* (Von der ohe et al., 2010).

Our results showed a large range of aggressiveness between *F. graminearum* isolates (Table 29); this is in line with studies which reported that there is a large variation in aggressiveness among *F. graminearum* isolates sampled from various parts of the world (Bai and Shaner, 1996; Miedaner et al., 2001).

Here, in floret inoculation assay, the disease severity level of the *Fusarium graminearum* isolate reached 78% at the second assessment (14 days post- inoculation), while *Fusarium graminearum* isolate to5 was considered moderately aggressive, causing only 53% disease severity at the same date, whereas the *F. graminearum* Egy1 isolate showed the lowest aggressiveness with a disease severity level of 34.9% on heads of wheat plants at the second assessment. A similar observation about the difference in the development of the disease symptoms according to aggressiveness level was reported by Purahong et al. (2014). However, Miedaner et al. (2000) interpreted that qualitative and quantitative differences in the synthesis of enzymes and mycotoxins might be responsible for the great variability in aggressiveness observed between *F. graminearum* populations.

In our study, evaluation of *F. graminearum* aggressiveness was conducted with a limited number of isolates but from different geographic origins in order to cause high and different levels of infection that provide a different spectrum of aggressiveness.

The fact that we observe the same sequence in aggressiveness for the three isolates in all three assays confirms that the Petri-dish test can be used for aggressiveness screening of *F. graminearum* isolates on wheat. These results also agree with those reported by Purahong et al. (2012) that the Petri-dish test is reliable, stable and yields highly significant correlation with floret inoculation. Wu et al. (2005) attributed the high correlation between the Petri-dish test and the single floret assay to the fact that in both assays, all biotic and abiotic factors are strictly controlled. In addition, the situation is similar in both assays because the fungal inocula were put directly on the wheat seeds (without glumes) and they could directly penetrate and infect germinating seeds.

Our findings confirmed that DNA contamination of wheat grains is a consequence of the severity of FHB symptoms and agree with the results obtained by Klix et al. (2007).

The present work is the first attempt to analyze and relate both the capacity of *F. graminearum* isolates from different geographic origins to induce FHB symptoms and DNA production with the AUDPC standard (Petri-dish test). Therefore, the clarification of such relationship between the three aggressiveness assays remains an issue of great importance because it could greatly help the breeding of wheat genotypes tolerant or resistant to FHB.

Our results indicate that the Petri-dish test can clearly differentiate the aggressiveness of *F. graminearum* isolates, because the results of this aggressiveness assay were confirmed by the

two other assays used. Gilbert et al. (2001) mentioned that a good correlation between any two measurements may have screening of *F. graminearum* isolates on wheat. Our results also agree with those reported by Purahong et al. (2012) that the Petri-dish test is reliable, stable and yields highly significant correlation with floret inoculation. Wu et al. (2005) attributed the high correlation between the Petri-dish test and the single floret assay to the fact that in both assays all biotic and abiotic factors are strictly controlled. In addition, the situation is similar in both assays because the fungal inocula were put directly on the wheat seeds (without glumes) and they could directly penetrate and infect germinating seeds.

So the highly significant correlation of AUDPC_{standard} to disease severity in floret inoculation and DNA production confirms that Petri-dish test is a good test for screening the most aggressive *F. graminearum* isolates for FHB resistance breeding of wheat and could be used for similar studies with other cereal crops.

5.5 Distribution of *Fusarium* species in maize plant

This experiment was aimed to assess and compare the appearance and distribution of *Fusarium* species in different organs of forage maize. There is little information in literature about the *Fusarium* species in the entire plant used for silage (Eckard et al., 2011), which is the most important animal feed source all over the world. The different parts of maize plant can be attacked by *Fusarium* species including the remaining crop, maize kernels, the whole plant used as silage, as well as stalk pieces (Munkvold, 2003a). Our results confirmed that all maize plant parts were contaminated with DNA of *Fusarium* species, however in various amounts depending on the growth stage and the part of maize plant.

Recently, qPCR has been largely used for quantifying the fungal biomass of *Fusarium* spp. in various parts of maize plant in the field, which has allowed huge advances in our understanding of these fungi.

Our results demonstrated that DNA of *Fusarium* species was found in low or undetectable concentrations in the tested organs until the flowering stage, then DNA concentration began to increase during the development of fruit and ripening growth stages. This observation is in agreement with Parry et al. (1995) and Munkvold (2003b) who concluded that silks of maize kernels are the main infection pathway, which are susceptible during the first 6 days after silk emergence.

In our study, the flowering stage occurred from 28 July until 11 August with mean temperature reached 20 °C and mean precipitation was 35.8 mm. The average maximum temperature during this stage was 25.5 °C and the average minimum temperature was 15.3 °. Thus, these conditions are suitable for the development of the disease. It is known that the infection occurs after spores reached the silks by water splashes, wind dispersal or insect vectors. During growth stage of flowering, the precipitation ranged from 30-44 mm and the temperature was 18-25 which is considered favourable for the development of these fungal pathogens. In general, the optimum temperature for *F. graminearum* has been documented as 24-26°C or about 28°C; on the other hand, for *F. culmorum*, *F. poae* and *F. avenaceum*, the optimum temperature is documented as 20-25 °C. The infection of *Gibberella* ear rot is facilitated by high levels of moisture around silking, followed by reasonable temperatures and high rainfall during the maturation stage, thus, environmental conditions have serious effects on the development of these fungal species (Logrieco et al., 2002; Doohan et al., 2003; Munkvold, 2003b).

Other studies suggested that, the infection of *F. graminearum* needs temperature reaching level of 29°C > 48 h while the required temperature of the mycelial growth is (10-30°C) with optimum temperature of 25°C. Concerning the required temperature of *F. culmorum* infection reached (10-30°C) with optimum temperature of (20-25°C), while the required temperature of the infection is 26.5°C for 72 h. On the other hand; infection and mycelial growth temperatures of *F. poae* were similar to *F. culmorum*, while temperature was roughly lower for mycelial growth of *F. avenaceum*, with optimum temperature 20°C (Rossi et al., 2001; Brennan et al., 2003; Dufault et al., 2006).

Thus, environmental conditions including temperature and rainfall have been effected on the development of these fungal species. However, the predominant *Fusarium* species in the maize plant after flowering stage was *F. graminearum*, followed by *F. culmorum*, while *F. poae* was the less prevalent. This observation is in agreement with other studies which suggested *F. graminearum* as the dominant species in Schleswig-Holstein (Dietrichs, 2012; Birr, 2013). Dietrichs (2012) reported that *F. graminearum* and *F. crookwellense* were the most dominant species while Birr (2013) reported that *F. graminearum* was the dominant species, followed by *F. culmorum*, *F. avenaceum* and *F. poae*. *F. tricinctum*, *F. langsethiae*, and *F. equiseti* were detected with only small amounts.

The present work suggested that the quantity of individual fungal species varied among the samples and was distributed in various amounts depending on the growth stage. It was observed that DNA concentration gradually increased with the plant age. On the whole it was observed that the spindles and kernels had the highest concentrations of fungal DNA at the ripening stage of maize plant. Additionally, the leaf sheath and leaf blade were largely contaminated with *Fusarium* species, indicating that they can contribute to the contamination of harvest material with *Fusarium* mycotoxin later. These findings are in agreement with Oldenburg (2006) who suggested that mycotoxin accumulation was in the leaf sheath and leaf blade more than in nodes and internodes, whereas this value increases according to the growth stage.

On the same line, Foley (1962) attributed that the stalk infection also occurred through leaf sheaths and that symptomless kernel infection resulted in symptomless systemic infection of the stalk and eventually the kernels. In the same regard, Xuan (2014) suggested that the infection of maize leaves by *F. graminearum*, *F. proliferatum* and *F. verticillioides* resulted in cob and kernel infection and later may lead to reduce yield quality as well as mycotoxin contamination in maize plant, the author attributed that maize plants inoculated by *Fusarium* species at growth stage (15) resulted in higher infection rates compared with those inoculated at growth stage (35). Additionally, higher levels of colonization were observed with higher spore concentration and humidity. Additionally, Xuan (2014) considered that leaf sheaths and leaf blades lead to *Fusarium* biomass observed in the infected maize organs. Sutton (1982) reported that macroconidia and ascospores released from debris on the soil are disspread in the air, then infect the wheat spikes as well as leaf sheaths and ears of maize.

The observation about the presence of fungal DNA before flowering stage could be interpreted by mycotoxin absorbance from the soil. Logrieco et al. (2002) and (Shaner, 2003) also suggested that the *Fusarium* inoculum originates either from the soil or from *Fusarium*-infected crop residues of several crop species, such as wheat, corn, barley, soybean and rice on the soil surface which act as the primary sources of inoculum. Consequently, *Fusarium* species can survive on the soil and penetrate the plant. In the same regard, it was reported that the infection can begin from both conidia and mycelium which can be found either on the seed surface or inside the seeds (Foley, 1962; Munkvold et al., 1997a; Munkvold et al.,

1997b., Oren et al., 2003). Lawrence (1981) reported that the roots or mesocotyle could be infected by *Fusarium* through wounds or natural opening as well as direct penetration.

Overall, the distribution of *Fusarium* species was considerably different throughout the development of the maize plant. Additionally, the environmental conditions including rainfall and temperature affected this appearance and distribution. However, the predominant *Fusarium* species in the maize plant after the flowering stage was *F. graminearum*, followed by *F. culmorum*, while *F. poae* was the less prevalent.

In conclusion, *Fusarium* species lead to huge yield losses and additionally, they produce a large range of mycotoxins before and after harvest, ultimately causing animal health problems because of contaminated feed. So future efforts and advanced procedures are recommended toward understanding of the infection and contamination process in order to find genotype differences in the resistance of forage maize genotypes against *Fusarium* infection.

5.6 Systemic growth of *Fusarium graminearum* and *Fusarium culmorum* in maize plant

This work was aimed to assess and compare the appearance and distribution of *Fusarium* species in different organs of forage maize and, additionally, to determine the incidence of systemic infection of fungal species in the corresponding plant organs. Little information is available in literature about the distribution of *Fusarium* species in the entire plant used for silage in order to know whether certain plant organs are more favourable to these fungi. On the other hand, a lot of information about the incidence of *Fusarium* species infection and their toxins in maize grain under natural infection is available (Eckard et al., 2011; Xuan, 2014).

Different parts of the maize plant can be attacked by *Fusarium* species including the remaining crop, maize kernels, the whole plant used as silage, as well as stalk pieces. The assessment of *Fusarium* infection in maize plant parts is complicated because this infection could lead to the appearance of symptoms but also symptomless infections are reported. Recently, qPCR has been largely used for quantifying the fungal biomass of *Fusarium* spp. in various parts of maize plant in the field (Munkvold, 2003a; Oiah et al., 2006).

Our results confirmed that all maize plant parts were contaminated with DNA of *Fusarium* species, however, in various amounts depending on the growth stage and the part of maize plant.

Our results demonstrated that roots were largely contaminated with DNA of *Fusarium* species compared to the other organs. DNA values decreased from the root to the section below the main ear and are systemically distributed during the stalk representing the nodes and internodes until the ear node. This observation is in agreement with Munkvold and Carlton. (1997) who concluded that *Fusarium* can systemically grow in maize plant parts and described four steps for this movement, i) transmission from the seed to the seedling, ii) the movement of the fungus within the stalk, iii) the movement into the ear, and iv) the spread within the ear. On the same line, Logrieco et al. (2002) and Shaner (2003) also suggested that the *Fusarium* inoculum originates either from the soil or from *Fusarium*-infected crop residues of several crop species, such as wheat, corn, barley, soybean and rice on the soil surface which act as the primary sources of inoculum.

Additionally, our results suggest that the roots were largely contaminated with DNA; these results are consistent with observations of Oren et al. (2003) and Scauflaire et al. (2011) that roots are the main contact between the fungus and the host lateral roots and the mesocotyl, which is the tissue connecting the emerging root with the emerging shoot. In the same study it was reported that fungi were detected in these tissues as early as 72 h after planting seeds in infected soil. After penetration the development of fungi begins in intercellular spaces and then they directly grow through the cuticle and epidermis of these tissues. Foley (1962) suggested that hyphae grow out of seeds and during the roots, mesocotyls and stalks of the new plants. This study used the term systemic growth to describe the colonization of the plants because it was thought that the fungi spread from the roots to the remainder parts of the plant.

The systemic infection can begin from both conidia and mycelium which can be found either on the seed surface or inside the seeds (Foley. 1962; Munkvold et al., 1997a; Munkvold et al., 1997b., Oren et al., 2003). On the same line, Lawrence (1981) suggested that the roots or mesocotyle could be infected by *Fusarium* through wounds or natural opening as well as direct penetration.

Regard with the movement of *Fusarium* from seeds to stalk and kernels, Kedera et al. (1997) tracked specific strains using vegetative compatibility groups as a marker, thus, found an evidence that the fungus moves from the infected seeds to the kernels by recovering the isolate inoculated from approximately 10% of the kernels.

Similar results were reported in another studies (Leslie et al., 1990; Munkvold et al., 1997a; Munkvold et al., 1997b; Oren et al., 2003), these studies reported that infected seeds may lead to the infection of the kernel, whereas the inoculum surviving in crop residues and in the soil may cause to the systemic infection.

It was documented that the systemic infection of *F.verticillioides* from seeds to roots and stalks can cause rotting in all plant parts, whereas it is considered a cause of stalk root, but its importance appears to be low in kernel infection. Also the movement of the fungus from the seedling crown to the stalk tissue is considered the rate-limiting cause in fungal infection from infected seeds to the upper organs of the plant (Oren et al., 2003). In other words; the seed-transmitted strains do not progress to aboveground parts after pollination.

The present work suggests that the quantity of individual fungal species varied among the samples and was distributed in various amounts depending on the growth stage. It was observed that DNA concentration gradually increased with the plant age, with low infection rates during the growth stage of stem elongation (GS 33), whereas the root was contaminated with biggest amounts of DNA (28.4, 4.1 DNA fungi/ DNA plant ‰) of *F. graminearum* and *F. culmorum*, respectively. At the subsequent course of flowering (GS 65), *F. graminearum* and *F. culmorum* were detected in higher amounts in nodes and internodes, reaching levels of 19.9, 11.3 and 13.4, 11 DNA fungi/ DNA plant ‰, in the first node and internode, respectively. This observation about increasing concentration of fungal DNA is in agreement with Oren et al. (1997) who concluded that colonization and infection of *Fusarium* spp. largely occurred after the flowering stage and increased until the harvest depending on the environmental conditions.

Generally, in the section above the ear node, the fifth node was largely infected with DNA *F. graminearum* and *F. culmorum* compared to other corresponding expect root at the development of fruit reaching levels (64.9, 7.5 DNA fungi/ DNA plant ‰) respectively, and

(72.4, 8.1 DNA fungi/ DNA plant %) respectively at ripening stage. Then, DNA concentration decreased through the nodes and internodes and reached low amounts in the last nodes and internodes. These results are in agreement with Wiese (2015) who concluded the infection distributed from the lower to the middle nodes, which the ear node was largely contaminated with DNA *Fusarium* species, Wiese (2015) suggested that infection occurred because of soil-borne infections at early growth stages, as well as, infections of the rudimentary ears and leaf sheaths from which the pathogen spreads systemically throughout the plant parts. Regarding the systemic infection of *F. graminearum* and about the same previous result, Young and Miller. (1984) suggested that both deoxynivalenol and ergosterol were differently found inside the stalk and considerably appear in the sections above the ear-attachment point.

The highest amounts of *F. graminearum* and *F. culmorum* DNA were determined in the corresponding main ear after flowering stage and gradually increased until the ripening growth stage, reaching levels of 323.3 and 181 DNA fungi/ DNA plant %, respectively. These findings are in agreement with several other publications which suggested that the most effective way of infection was through the silks, followed by kernel infection with airborne conidia as well as through wounds caused by insects (Munkvold et al., 1997b; Munkvold, 2003a; Oren et al., 2003; Scaufaire et al., 2011). Foley (1962) attributed that the stalk infection also occurred through leaf sheaths and that symptomless kernel infection resulted in symptomless systemic infection of the stalk and eventually the kernels.

In our study it was observed that rain events varied during maize growth; rainfall was largely recorded during May reaching levels of 16.3 mm, and exceeds in June 16 mm but dry periods were recorded during this month. Additionally, rain events were more concentrated from 25 to 30 July which ranged from 5.5-36.5 mm. On the other hand, varying amounts of rainfall were recorded during the other months which ranged from 0.1-19.9 mm. Generally, warm conditions were available during the plant growth with maximum mean daily temperatures reaching 22.3 °C during August. The flowering stage occurred from 3 until 16 August with mean temperature of 20.2 °C. Rain events were low and irregular varied, whereas dry days were recorded. The maximum mean precipitation reached 18.7 mm during this stage. The average maximum temperature during this stage was 22.2 °C and the average minimum temperature was 17.8 °C

Researchers reported that the silks are susceptible during the first 6 days after silk emergence. The infection occurs after spores reached the silks by water splashes, wind dispersal or insect vectors. During the flowering stage, the precipitation ranged from 30-44 mm and the temperature was 18-25 °C which is considered suitable for the development of these fungal pathogens. In general, the optimum temperature for *F. graminearum* has been documented as 24-26°C or about 28°C; on the other hand, for *F. culmorum* the optimum temperature is documented as 20-25 °C. The infection of *Gibberella* ear rot is facilitated by high levels of moisture around silking, followed by reasonable temperatures and high rainfall during the maturation stage (Logrieco et al., 2002; Doohan et al., 2003; Munkvold, 2003a; Munkvold, 2003b; Kubicek et al., 2008; Görtz et al., 2010).

Generally, the infection of *F. graminearum* needs temperature reaching level of 29°C > 48 h while the required temperature of the mycelial growth is (10-30°C) with optimum temperature of 25°C. concerning the required temperature of *F. culmorum* infection ranged (10-30°C) with optimum temperature of (20-25°C), while the required temperature of the infection is 26.5°C for 72 h. (Rossi et al., 2001; Brennan et al., 2003; Dufault et al., 2006). Thus, environmental conditions have serious effects on the development of these fungal species in plant maize.

It was observed that *F.graminearum* was the dominant species; this observation is in consistent with other researchers who suggested *F.graminearum* as the dominant species in Schleswig-Holstein (Dietrichs, 2012; Birr, 2013). Dietrichs (2012) suggested that *F. graminearum* and *F. crookwellense* were the most dominant species. in the other study, *F. graminearum* was the dominant species, followed by *F. culmorum*, *F. avenaceum* and *F. poae*. *F. tricinatum*, *F. langsethiae*, and *F. equiseti* were detected with only small amounts.

Overall, the distribution of *Fusarium* species was largely different throughout development of maize plants. However, this distribution was systemically during of stem development growth stage and afterwards a high concentration of DNA fungal was determined in the ear node additionally to the main ears.

It is known that the genetic background of the host and the pathogen plays an important role in disease development. For that reason, future efforts and advanced procedures are recommended for a better understanding of the infection and contamination process in order to find genotype differences in the resistance of forage maize genotypes against *Fusarium* infection. Additionally, up to now, fungicides are not allowed for using to control *Fusarium* spp. In Europe, except seed treatment, while the other practices as cultural had only limited success because of affecting by the environmental conditions and cropping factors.

6. Summary

The protective (applied pre- and during inoculation) and curative (applied post-inoculation) activity of the fungicides metconazole, epoxiconazole, pyraclostrobin and a mixture of epoxiconazole and pyraclostrobin against *Fusarium graminearum*, the primary pathogen causing *Fusarium* head blight (FHB) in wheat and *Gibberella* ear rot in maize, was analyzed. All fungicides had protective activity, even when applied at 50% of the dose recommended by the manufacturer. All fungicides reduced the disease indicators FDK (*Fusarium*-damaged kernels), FHB index, DON (deoxynivalenol) content and fungal DNA, with metconazole being most effective, closely followed by the mixture, and then by epoxiconazole and pyraclostrobin, each being applied singly.

Thirty-nine *Fusarium* isolates showed various levels of sensitivity against the demethylation inhibitors (DMI) metconazole and epoxiconazole. An analysis of the deduced amino acid sequence of CYP51B gene showed that the differences in sensitivity against DMI were not due to the substitution of amino acids in CYP51B. Subsequently, CYP51 of *Fusarium* spp. was compared with CYP51 of wild-type and resistant isolates of *Septoria tritici*. The results showed no variations in DNA sequence of CYP51 at specific positions indicating that mutations in CYP51 do not play a role in sensitivity level against *Fusarium* spp.

Growth inhibitions of *F. graminearum* isolates caused by carbendazim were largely distributed in similar ranges at all concentrations. The DNA sequence of β 2-tubulin was analysed in four isolates. A point mutation at position 198 was identified in all tested isolates; this mutation resulted in a single amino acid substitution, where glutamic acid was converted to glutamine.

Three assays (Petri-dish test, single floret inoculation and DNA quantification) were applied in order to quantify the aggressiveness of *Fusarium graminearum* isolates on wheat. It could be shown that the highly and weakly aggressive isolates were stable in the mentioned assays, confirming that the Petri-dish test can be used for screening the most aggressive isolates of *F. graminearum*.

DNA of *Fusarium* species was found in low or undetectable concentrations in the tested maize organs until the flowering stage. After that, the abundance of individual species varied among the samples and according to the growth stage.

The systemic infection of *Fusarium* spp. during maize growth and development demonstrated that roots more severely contaminated with DNA of *Fusarium* species than the

other organs. DNA concentrations decreased from the root to the section below the main ear and were systemically distributed within the stalk representing the nodes and internodes until the ear node. The highest amounts DNA were determined in the main ear after flowering stage and they further gradually increased until the ripening stage.

7. Zusammenfassung

Die protektive (vor und während der Inokulation angewandte) und kurative (angewandt nach der Inokulation) Wirkung der Fungizide Metconazol, Epoxiconazol, Pyraclostrobin und einer Wirkstoffkombination aus Epoxiconazol und Pyraclostrobin gegen *Fusarium graminearum*, den Haupterreger von Ährenfusariosen (*Fusarium* head blight, FHB) in Weizen und Gibberella Ährenfäule in Mais, wurde analysiert. Alle Fungizide besaßen protektive Wirksamkeit, selbst bei 50% der vom Hersteller empfohlenen Dosis. Alle Fungizide reduzierten darüber hinaus die Krankheitsindikatoren FDK (durch *Fusarium* geschädigte Körner), FHB-Index, DON (Deoxynivalenol)-Gehalt und Pilz-DNA, wobei Metconazol am wirksamsten war, dicht gefolgt von der Wirkstoffkombination und dann von Epoxiconazol und Pyraclostrobin, die jeweils einzeln angewandt wurden.

Neununddreißig *Fusarium*-Isolate zeigten unterschiedliche Anfälligkeit gegenüber den Demethylierungshemmern (DMI) Metconazol und Epoxiconazol. Eine Analyse der vom CYP51B-Gen abgeleiteten Aminosäuresequenzen zeigte, dass die Unterschiede in der Anfälligkeit gegenüber DMI auf die Substitution von Aminosäuren im CYP51B-Gen zurückzuführen waren. Anschließend wurde CYP51 von *Fusarium* spp. mit CYP51 des Wildtyps und resistenten Isolaten von *Septoria tritici* verglichen. Die Ergebnisse zeigten keine Unterschiede in der DNA-Sequenz von CYP51 an spezifischen Positionen, was darauf hindeutet, dass Mutationen in CYP51 keine Rolle für die Anfälligkeit gegen *Fusarium* spp. spielen.

Durch Carbendazim verursachte Wachstumshemmungen von *F. graminearum*-Isolaten waren bei allen Konzentrationen weitgehend in ähnlichen Bereichen verteilt. Die DNA-Sequenz von β 2-Tubulin wurde in vier Isolate analysiert. Eine Punktmutation an Position 198 wurde in allen getesteten Isolaten identifiziert; diese Mutation resultierte in einer einzigen Aminosäure-Substitution, wobei Glutaminsäure durch Glutamin ausgetauscht wurde.

Drei verschiedene Tests (Petrischalen-Test, Einzelblüten-Inokulation und DNA-Quantifizierung) wurden angewandt, um die Aggressivität von *Fusarium graminearum*-Isolaten an Weizen zu quantifizieren. Es konnte gezeigt werden, dass die hoch und schwach aggressiven Isolate in den drei Tests einheitlich reagierten, was bestätigt, dass der Petrischalen-Test für das Screening der aggressivsten Isolate von *F. graminearum* verwendet werden kann.

DNA von *Fusarium*-Arten wurde in geringen oder nicht nachweisbaren Konzentrationen in den untersuchten Maisorganen bis zum Blütenstadium gefunden. Danach variiert die Zusammensetzung der Arten je nach Probe und Wachstumsstadium.

Die systemische Infektion von *Fusarium* spp. während des Wachstums und der Entwicklung von Mais zeigte, dass Wurzeln stärker mit DNA von *Fusarium*-Arten kontaminiert waren als die anderen Pflanzenorgane. DNA-Konzentrationen verringerten sich von der Wurzel bis zum Abschnitt unter dem Hauptkolben und waren systemisch im Stängel verteilt, das heißt in den Nodien und Internodien bis zum Kolben. Die höchsten DNA-Konzentrationen wurden im Hauptkolben nach dem Stadium der Blüte nachgewiesen und sie erhöhten sich allmählich bis zur Reife.

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Table 33. Significant differences of quantification of *Fusarium graminearum* and *Fusarium culmorum* in maize plant (GS 85) N: node, IN: internode, *F. gra*: *F.graminearum*, *F.cul*: *F. culmorum*. Only p values less than 0.1 are shown (p< 0.1).

<i>Fuarium</i> spp.	fraction	Estimated mean difference	P value
<i>F. cul</i>	IN6 - IN5	-4.000e+00	<0.01 ***
<i>F. cul</i>	IN8 - IN5	-6.500e+00	<0.01 ***
<i>F. cul</i>	N2 - IN5	1.900e+01	<0.01 ***
<i>F. cul</i>	N4 - IN5	-3.000e+00	0.0196 *
<i>F. cul</i>	N6 - IN5	-4.000e+00	<0.01 ***
<i>F. cul</i>	N7 - IN5	-6.200e+00	<0.01 ***
<i>F. cul</i>	N8 - IN5	-6.500e+00	<0.01 ***
<i>F. cul</i>	N9 - IN5	-6.800e+00	<0.01 ***
<i>F. cul</i>	second_ear - IN5	-6.500e+00	0.0375*
<i>F. cul</i>	IN8 - IN6	-2.500e+00	0.0742 .
<i>F. cul</i>	N2 - IN6	2.300e+01	<0.01 ***
<i>F. cul</i>	N3 - IN6	4.333e+00	<0.01 ***
<i>F. cul</i>	N5 - IN6	5.000e+00	<0.01 ***
<i>F. cul</i>	N9 - IN6	-2.800e+00	<0.01 **
<i>F. cul</i>	N2 - IN7	2.513e+01	<0.01 ***
<i>F. cul</i>	N2 - IN8	2.550e+01	<0.01 ***
<i>F. cul</i>	N3 - IN8	6.833e+00	<0.01 ***
<i>F. cul</i>	N4 - IN8	3.500e+00	<0.01 ***
<i>F. cul</i>	N5 - IN8	7.500e+00	<0.01 ***
<i>F. cul</i>	N6 - IN8	2.500e+00	0.0753 .
<i>F. cul</i>	N3 - N2	-1.867e+01	<0.01 ***
<i>F. cul</i>	N4 - N2	-2.200e+01	<0.01 ***
<i>F. cul</i>	N5 - N2	-1.800e+01	<0.01 ***
<i>F. cul</i>	N6 - N2	-2.300e+01	<0.01 ***
<i>F. cul</i>	N7 - N2	-2.520e+01	<0.01 ***
<i>F. cul</i>	N8 - N2	-2.550e+01	<0.01 ***
<i>F. cul</i>	N9 - N2	-2.580e+01	<0.01 ***
<i>F. cul</i>	second_ear - N2	-2.550e+01	<0.01 ***
<i>F. cul</i>	N4 - N3	-3.333e+00	<0.01 **
<i>F. cul</i>	N6 - N3	-4.333e+00	<0.01 ***
<i>F. cul</i>	N7 - N3	-6.533e+00	<0.01 ***
<i>F. cul</i>	N8 - N3	-6.833e+00	<0.01 ***
<i>F. cul</i>	N9 - N3	-7.133e+00	<0.01 ***
<i>F. cul</i>	second_ear - N3	-6.833e+00	0.0188 *
<i>F. cul</i>	N5 - N4	4.000e+00	<0.01 **
<i>F. cul</i>	N7 - N4	-3.200e+00	<0.01 **
<i>F. cul</i>	N9 - N4	-3.800e+00	<0.01 ***
<i>F. cul</i>	N6 - N5	-5.000e+00	<0.01 ***
<i>F. cul</i>	N7 - N5	-7.200e+00	<0.01 ***
<i>F. cul</i>	N8 - N5	-7.500e+00	<0.01 ***
<i>F. cul</i>	N9 - N5	-7.800e+00	<0.01 ***
<i>F. cul</i>	second_ear - N5	-7.500e+00	<0.01 **
<i>F. cul</i>	N9 - N6	-2.800e+00	<0.01 **
<i>F. gra</i>	IN7 - IN2	-4.930e+01	0.0145 *
<i>F. gra</i>	IN8 - IN2	-5.057e+01	<0.01 **

<i>F. gra</i>	N8 - IN2	-5.370e+01	<0.01 **
<i>F. gra</i>	N9 - IN2	-5.523e+01	<0.01 **
<i>F. gra</i>	root - IN2	9.043e+01	<0.01 **
<i>F. gra</i>	second_ear - IN2	-5.283e+01	<0.01 **
<i>F. gra</i>	N5 - IN3	3.907e+01	<0.01 **
<i>F. gra</i>	root - IN3	1.121e+02	<0.01 ***
<i>F. gra</i>	N5 - IN4	4.300e+01	<0.01 ***
<i>F. gra</i>	N8 - IN4	-2.813e+01	0.0387 *
<i>F. gra</i>	N9 - IN4	-2.967e+01	0.0179*
<i>F. gra</i>	root - IN4	1.160e+02	<0.01 ***
<i>F. gra</i>	IN7 - IN5	-3.500e+01	<0.01 ***
<i>F. gra</i>	IN8 - IN5	-3.627e+01	<0.01 ***
<i>F. gra</i>	N2 - IN5	1.507e+01	<0.01 ***
<i>F. gra</i>	N3 - IN5	-1.327e+01	<0.01 ***
<i>F. gra</i>	N4 - IN5	-6.267e+00	<0.01 ***
<i>F. gra</i>	N5 - IN5	3.173e+01	<0.01 ***
<i>F. gra</i>	N6 - IN5	3.733e+00	<0.01 ***
<i>F. gra</i>	N7 - IN5	-2.627e+01	<0.01 ***
<i>F. gra</i>	N8 - IN5	-3.940e+01	<0.01 ***
<i>F. gra</i>	N9 - IN5	-4.093e+01	<0.01 ***
<i>F. gra</i>	root - IN5	1.047e+02	<0.01 ***
<i>F. gra</i>	second_ear - IN5	-3.853e+01	<0.01 ***
<i>F. gra</i>	IN7 - IN6	-3.673e+01	<0.01 ***
<i>F. gra</i>	IN8 - IN6	-3.800e+01	<0.01 ***
<i>F. gra</i>	N2 - IN6	1.333e+01	<0.01 ***
<i>F. gra</i>	N3 - IN6	-1.500e+01	<0.01 ***
<i>F. gra</i>	N4 - IN6	-8.000e+00	<0.01 ***
<i>F. gra</i>	N5 - IN6	3.000e+01	<0.01 ***
<i>F. gra</i>	N7 - IN6	-2.800e+01	<0.01 ***
<i>F. gra</i>	N8 - IN6	-4.113e+01	<0.01 ***
<i>F. gra</i>	N9 - IN6	-4.267e+01	<0.01 ***
<i>F. gra</i>	root - IN6	1.030e+02	<0.01 ***
<i>F. gra</i>	second_ear - IN6	-4.027e+01	<0.01 ***
<i>F. gra</i>	N1 - IN7	3.973e+01	<0.01 ***
<i>F. gra</i>	N2 - IN7	5.007e+01	<0.01 ***
<i>F. gra</i>	N3 - IN7	2.173e+01	<0.01 ***
<i>F. gra</i>	N4 - IN7	2.873e+01	<0.01 ***
<i>F. gra</i>	N5 - IN7	6.673e+01	<0.01 ***
<i>F. gra</i>	N6 - IN7	3.873e+01	<0.01 ***
<i>F. gra</i>	N7 - IN7	8.733e+00	0.0941 .
<i>F. gra</i>	root - IN7	1.397e+02	<0.01 ***
<i>F. gra</i>	N1 - IN8	4.100e+01	<0.01 ***
<i>F. gra</i>	N2 - IN8	5.133e+01	<0.01 ***
<i>F. gra</i>	N7 - IN7	8.733e+00	0.0941 .
<i>F. gra</i>	root - IN7	1.397e+02	<0.01 ***
<i>F. gra</i>	N3 - IN8	2.300e+01	<0.01 ***
<i>F. gra</i>	N4 - IN8	3.000e+01	<0.01 ***
<i>F. gra</i>	N6 - IN8	4.000e+01	<0.01 ***
<i>F. gra</i>	N5 - IN8	6.800e+01	<0.01 ***

<i>F. gra</i>	N7 - IN8	1.000e+01	<0.01 ***
<i>F. gra</i>	N9 - IN8	-4.667e+00	<0.01 ***
<i>F. gra</i>	root - IN8	1.410e+02	<0.01 ***
<i>F. gra</i>	N8 - N1	-4.413e+01	<0.01 ***
<i>F. gra</i>	N9 - N1	-4.567e+01	<0.01 ***
<i>F. gra</i>	root - N1	1.000e+02	<0.01 ***
<i>F. gra</i>	second_ear - N1	-4.327e+01	<0.01 ***
<i>F. gra</i>	N3 - N2	-2.833e+01	<0.01 ***
<i>F. gra</i>	N4 - N2	-2.133e+01	<0.01 ***
<i>F. gra</i>	N7 - N2	-4.133e+01	<0.01 ***
<i>F. gra</i>	N8 - N2	-5.447e+01	<0.01 ***
<i>F. gra</i>	N9 - N2	-5.600e+01	<0.01 ***
<i>F. gra</i>	root - N2	8.967e+01	<0.01 ***
<i>F. gra</i>	second_ear - N2	-5.360e+01	<0.01 ***
<i>F. gra</i>	N4 - N3	7.000e+00	<0.01 ***
<i>F. gra</i>	N5 - N3	4.500e+01	<0.01 ***
<i>F. gra</i>	N6 - N3	1.700e+01	<0.01 ***
<i>F. gra</i>	N7 - N3	-1.300e+01	<0.01 ***
<i>F. gra</i>	N8 - N3	-2.613e+01	<0.01 ***
<i>F. gra</i>	N9 - N3	-2.767e+01	<0.01 ***
<i>F. gra</i>	root - N3	1.180e+02	<0.01 ***
<i>F. gra</i>	second_ear - N3	-2.527e+01	<0.01 ***
<i>F. gra</i>	N5 - N4	3.800e+01	<0.01 ***
<i>F. gra</i>	N6 - N4	1.000e+01	<0.01 ***
<i>F. gra</i>	N7 - N4	-2.000e+01	<0.01 ***
<i>F. gra</i>	N8 - N4	-3.327e+01	<0.01 ***
<i>F. gra</i>	N9 - N4	-3.467e+01	<0.01 ***
<i>F. gra</i>	root - N4	1.110e+02	<0.01 ***
<i>F. gra</i>	second_ear - N4	-3.227e+01	<0.01 ***
<i>F. gra</i>	N6 - N5	-2.800e+01	<0.01 ***
<i>F. gra</i>	N7 - N5	-5.800e+01	<0.01 ***
<i>F. gra</i>	N8 - N5	-7.113e+01	<0.01 ***
<i>F. gra</i>	N9 - N5	-7.267e+01	<0.01 ***
<i>F. gra</i>	root - N5	7.300e+01	0.0118 *
<i>F. gra</i>	second_ear - N5	-7.027e+01	<0.01 ***
<i>F. gra</i>	N7 - N6	-3.000e+01	<0.01 ***
<i>F. gra</i>	N8 - N6	-4.313e+01	<0.01 ***
<i>F. gra</i>	N9 - N6	-4.467e+01	<0.01 ***
<i>F. gra</i>	root - N6	1.010e+02	<0.01 ***
<i>F. gra</i>	second_ear - N6	-4.227e+01	<0.01 ***
<i>F. gra</i>	N8 - N7	-1.313e+01	<0.01 ***
<i>F. gra</i>	N9 - N7	-1.467e+01	<0.01 ***
<i>F. gra</i>	root - N7	1.310e+02	<0.01 ***
<i>F. gra</i>	second_ear - N7	-1.227e+01	<0.01 ***
<i>F. gra</i>	root - N8	1.441e+02	<0.01 ***
<i>F. gra</i>	root - N9	1.457e+02	<0.01 ***
<i>F. gra</i>	second_ear - root	-1.433e+02	<0.01 ***
<i>F. gra</i> - <i>F. cul</i>	IN5	34.2667	< 1e-05 ***
<i>F. gra</i> - <i>F. cul</i>	IN6	40.0000	< 1e-05 ***

<i>F. gra</i> – <i>F. cul</i>	IN8	4.5000	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	N2	30.3333	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	N3	20.6667	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	N4	31.0000	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	N5	65.0000	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	N6	42.0000	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	N7	14.2000	< 1e-05 ***
<i>F. gra</i> – <i>F. cul</i>	root	117.8667	0.000201 ***

Significant codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1. All other hiding means are not significantly different.

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2001-2002 Diplom im Fachbereich „Pflanzenschutz“ mit „sehr gut“
2002-2004 Beschäftigung als Agraringenieurin am Zentrum für Landwirtschaftliche Forschung Lattakia, Mitwirkung an Labor- und Feldversuchen
2004-2007 Magister im Fachgebiet „Fungizide“, Stipendiatin des Hochschulministeriums
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