

**Effect of mycotoxin production on interactions between *Fusarium* species
during maize infection and on the production of volatile metabolites**

Dissertation

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Contents

Chapter 1: General introduction	1
Figures	7
References	9
Chapter 2: Role of trichothecenes and fumonisins in interaction between <i>Fusarium graminearum</i> and <i>F. verticillioides</i> in maize ears	
Abstract	17
Introduction	18
Materials and Methods	21
Results	24
Discussion	27
Tables	32
Figures	34
References	41
Chapter 3: Volatiles emitted from maize ears simultaneously infected with two <i>Fusarium</i> species mirror the most competitive fungal pathogen	
Abstract	48
Introduction	49
Materials and Methods	51
Results	54
Discussion	57
Tables	62
Figures	64
References	68

Chapter 4: Role of trichothecenes and fumonisins in interaction between <i>Fusarium graminearum</i> and <i>F. verticillioides in vitro</i>	
Abstract	73
Introduction	73
Materials and Methods	74
Results and Discussion	75
Tables	77
Figures	78
References	79
Chapter 5: General discussion	82
References	90
Summary	99
Acknowledgements	101
Publications & Workshops	102
<i>Curriculum vitae</i>	104

Chapter 1: General Introduction

Cultivated maize (*Zea mays* L. spp. *mays*) was domesticated from wild grass teosinte (*Zea mays* ssp. *parviglumis*) about 7000 thousand years ago in Central America (Doebley, 1990; Matsuoka et al., 2002), (**Figure 1**). Today, the harvested maize areas cover more than 180 million ha. Maize currently accounts about 37 % of world cereal production, with more than one billion tons, followed by rice, wheat, barley, sorghum and millet (FAOSTAT, 2014). Hence, maize provides a staple food for humans and fodder for animals, besides its industrial uses (O’Sullivan and Edwards, 2003). The three major important products come from processed maize are sweeteners, ethanol and starch (**Figure 2**). Corn fields all over the globe are threatened by microbial fungal diseases including those caused by genus of *Fusarium*, which in specific instances is responsible for a severe yield loss up to 48% of maize ears and kernels (Vigier et al., 2001).

The genus *Fusarium* contains soilborne, hemibiotrophic, plant pathogenic fungi with many species that cause serious plant diseases around the world (Agrios, 2005). Some *Fusarium* species reproduce sexually by producing ascospores in perithecia to overwinter and mycelia or chlamydospores which survive in infected plant debris. In the spring, ascospores are released and carried by the wind to corn stalks or ears and cause infection. Fungal non-sexual conidia spores in infected plant parts serve as the secondary inoculum for renewed infection during the season of growing crop (Munkvold, 2003).

Fusarium species are responsible for the ear rot and scab in corn and small grains (Agrios, 2005). Due to the fact that *Fusarium* species grow saprophytically and can also survive within maize seedlings and kernels in asymptomatic habit (Headrick and Pataky, 1991), it hinders either seed treatment or systemic fungicide applications at the late phase of plant growth (Bacon et al., 2001). Hence, an unconventional control strategy is required. Early disease diagnosis and the knowledge that comes from *Fusarium* epidemiology research and maize resistance research can eventually lead to efficient control/resistance strategies against fusarioses in maize crop (Munkvold, 2003; Mesterhazy et al., 2012).

Fusarium graminearum Schwabe [teleomorph = *Gibberella zea* (Schwein) Petch] is the causal agent of *Gibberella* ear rot in maize crop. It needs succulent silk tissues (Reid et

al., 1992) resulting in pink or reddish mold in kernels. *F. graminearum* development requires warm temperatures and wetness (Miller, 1994; Munkvold, 2003). *Fusarium verticilloides* (Saccardo) Nirenberg [synonym=*F.moniliforme* J. Sheldon, teleomorph = *Gibberella moniliformis* Wineland] is the causal agent of *Fusarium* ear rot in maize. *F. verticillioides* favours higher temperatures and dry conditions, resulting in a whitish colored mold (Headrick and Pataky, 1991; Vigier et al., 1997).

F. verticillioides infects maize plants as a symptomless endophyte (Bacon et al., 1992; Bacon and Hinton, 1996). *F. verticillioides* is known as a weak ear rot pathogen that remains systemically in maize plants and develop less visible symptoms on maize (Munkvold et al., 1997 a,b), (**Figure 3**) until external abiotic or biotic stress intervenes (Yates and Jaworski, 2000; Bacon et al., 2008; Kurtz et al., 2010). Yates et al., (2005) demonstrated that mature maize plants grown under three different growing seasons from seeds inoculated with *F.verticillioides*, had equal or even greater vegetative growth and yield production than those from non-inoculated seeds.

The infection routes through which *F. graminearum* and *F. verticillioides* enter maize ears differ. On one side *F. graminearum* grows intensively internally and externally along the silks, penetrating the ovaries or colonizing the interkernels spaces, and subsequently reaching the rachis (Miller et al., 2007). On the other side, *F. verticillioides* does not follow ear silks to infect maize (Zummo and Scott, 1990; Duncan and Howard, 2010). Examination of matured asymptomatic corn kernels infected with *F. verticillioides* with scanning microscopy revealed the presence of the fungal mycelium only intercellularly in the kernel pedicles (Bacon et al., 1992). Furthermore, following the progression of *F. verticillioides* strain expressing a fluorescent protein, Duncan and Howard (2010) demonstrated that the fungus was able to enter maize kernels through the styler canal, a natural opening in the kernel's surface located below the site of silk attachment.

Mycotoxins produced by *Fusarium* spp. commonly contaminate and severely impair the quality of the grain. Mycotoxins are low molecular weight fungal metabolites that are toxic to human and livestock. The mycotoxins produced by *F. graminearum* are mainly trichothecenes type B [(nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives of nivalenol and deoxynivalenol] and zearalenone (ZEN). Trichothecenes-producing strains of *F. graminearum* can be found as specific chemotypes synthesizing either nivalenol or deoxynivalenol and its acetylated derivatives (Desjardins, 2008;

McCormick et al., 2011). *F. verticillioides* mainly produces fumonisins (fumonisin B series: FB1, FB2 and FB3), fusarin C and fusaric acid (Han et al., 2014).

Pathogenicity and aggressiveness of *Fusarium* spp. are quite variable within a species. there are considerable differences in *Fusarium* aggressiveness and toxin production as well depending not only on the environmental conditions, but also on the inocula (i.e. fungal strain) (Garcia et al., 2009, Marin et al., 2008; Mesterhazy et al., 2012). Several studies showed that the severity of infection is highly correlated to toxin accumulation (Pascale et al., 1997; Perkowski et al., 1997; Reid and Sinha, 1998; Schaafsma et al., 2006; Balconi et al., 2014). This correlation between severity of infection and toxin accumulation does not necessarily implicate a role of the toxin in infection, the effect may be because of the large biomass of the pathogen.

Indeed the trichothecenes produced by *F.graminearum* were shown to be involved in plant pathogenesis (Proctor et al., 2002, Maier et al., 2006) and act as virulence factors on wheat spikes, but not on barley (Proctor et al., 1995; Desjardins et al., 1996; Jansen et al., 2005; Maier et al., 2006). Although the positive correlation between disease severity and trichothecenes accumulation in maize ears (Reid et al., 1996; Bolduan et al., 2009; Becker et al., 2014), the role of trichotecenes in maize ears is controversial since some studies demonstrated that trichothecene production is necessary for the virulence of *F. graminearum* on maize (Proctor et al., 1995; Harris et al., 1999). Using *F. graminearum* DON and NIV chemotypes disrupted in their ability to synthesize DON or NIV, Maier et al. (2006) concluded that DON did not act as a virulence factor in maize however NIV did so to a certain extent.

Similarly to trichotecenes, the role of fumonisins in disease development has been studied with various outcomes. On one side, fumonisin accumulation in maize seedlings seems to correlate well with disease symptoms on leaves (Glenn et al., 2008), while only a moderate correlation was observed on maize ears (Kleinschmid et al., 2005). On the other side, fumonisins were detected in fully asymptomatic maize kernels (Bullerman and Tsai, 1994), demonstrating that they do not always induce symptoms. Additionally, it was concluded that they are neither required for ear rot symptoms to appear (Desjardins and Plattner, 2000; Desjardins et al., 2002). Overall this demonstrated that mycotoxins might influence the virulence of their producer, however this highly depends on the host plant (Jansen et al., 2005; Maier et al., 2006).

Maize/*Fusarium* pathosystem is complex. The natural infection is initiated by a mixture of the local *Fusarium* spp., where several *Fusarium* spp. are able to infect maize plants,

but usually one species predominates (Logrieco et al., 2002; Doohan et al., 2003). *F. graminearum* and *F. verticilloides*, are considered as the major causal agents of *Gibberella* ear rot and *Fusarium* ear rot on maize, respectively. The co-occurrence of several *Fusarium* species colonizing same host plant prompts *Fusarium* interspecific interaction, which subsequently influence on the dominant disease and toxin production in maize crop fields (Mesterhazy et al., 2012).

Secondary fungal metabolites are ecologically significant and confer the fitness to the producing organism, but there is only very little knowledge about the biological role of secondary metabolites including mycotoxins in a complex and variable environment (Fox and Howlett, 2008), except for the common concept that these metabolites can guarantee better growth and protection to their producers inside their ecological niche (Fox and Howlett, 2008). As mentioned earlier mycotoxins might act as virulence factors during pathogenesis in plants, they may also be involved in microbial antagonism (Karlovsky, 2008). This antagonistic function of mycotoxins in microbial interactions *in vitro* was demonstrated early (Cuero et al., 1988; Ramakrishna et al., 1996).

The interaction between *F. graminearum* and *F. verticilliodes* that are infecting and colonizing maize ears together have been observed and discussed since *F. verticilliodes* was able to take advantage over *F. graminearum* in terms of fungal growth and mycotoxin accumulation in infected kernels of the host plant (Reid et al., 1999; Picot et al., 2012), while the role of mycotoxins in this interaction is still questionable. Thus, an empirical greenhouse study using the experimental maize variety of Gaspé Flint (*Zea mays* var. Gaspé Flint, **Figure 4**) was launched in **Chapter 2** to elucidate the role of mycotoxins in *Fusarium* interspecific interactions *in planta*. Additionally, the interaction between *F. graminearum* and *F. verticilliodes* *in vitro* on synthetic media was also tested and recorded in **Chapter 4**.

Volatile organic compounds (VOCs) are secondary metabolites with low molecular weight, lipophilic nature and high vapor pressure produced by the living organism (Dudareva et al., 2006). Due to the fact that the plant is exposed to divergent stress factors (biotic/abiotic), the blend of produced volatiles varies accordingly (Mumm and Dicke, 2010). The primary functions of released volatiles are i) preparing plants for defense against pathogens and herbivores (Fujita et al., 2006; Piesik et al., 2013) via producing plant hormones such as ethylene, nitric oxide, methyl jasmonate and methyl salicylate, ii) attracting insect pollinators and seed transmitters (Pichersky and

Gershenzon, 2002). Plants produce over a thousand of different VOC (Knudsen et al., 2006; Baldwin, 2010; Schenkel et al., 2015) from all different organs above and below the ground. These volatile compounds are mainly represented by terpenoids, phenylpropanoids/benzenoids, fatty acid and amino acid derivatives (Dudareva et al., 2004). They are considered as communication tool to transmit information between plants, additionally they are considered as indicators for the physiological status of the plant under certain environmental conditions (Baldwin, 2010; Wenke et al., 2010; Clavijo-McCormick et al., 2012).

VOCs have been implemented as a characteristic tool to understand the complex interaction within the living organisms (plant-plant, plant-insect and plant-microbe). In mean time, they represent powerful indicators to evaluate the plant health and food quality (Dudareva et al., 2006). Infected plants are known to emit plethora of volatiles (Jansen et al., 2011), which play a signaling role for defense response activation, or a direct inhibitors against the pathogen. Obviously, monitoring of such indicator volatiles emitted by diseased plants as early as possible during plant lifecycle is considered as significant indication for an action to be taken. Application of plant VOCs in plant disease diagnosis has been proposed (Turner and Magan, 2004; Tholl et al., 2006). The detection of emitted VOCs from infected plants has become a promising tool in plant disease diagnosis, especially as a non-invasive indirect method for large scale disease diagnosis (Sankaran et al., 2010; Aksenov et al., 2013).

Generally speaking, emitted compounds by stressed plants include; short-chained alcohols, aldehydes lipoxigenase, shikimate and specific mono- and sesquiterpenes (Beauchamp et al., 2005; Blande et al., 2007), which are the constituents of volatile biomarkers in plant health evaluation. In addition, several studies identified different blends of volatiles from different plant-microbe pathosystems (Jansen et al., 2009; Cruz et al., 2012; Hantao et al., 2013).

In cereal plants, the foliar infection with *Fusarium* spp. on barley and wheat plants induced eleven VOCs compared to non-infected plants (Piesik et al., 2013). Leaf and root infection of maize plants with *Fusarium* spp. induced several VOCs in high quantities upon infection (Piesik et al., 2011). Moreover, Girotti et al., (2012), introduced trichodiene volatile as a marker for *Fusarium* head blight disease on wheat cultivars at early stages of the infection. In addition, Becker et al., (2014), detected twenty two volatiles emitted from maize ears infected with single *Fusarium* spp.

The effect of simultaneous infections with two fungal species of *F. graminearum* and *F. verticillioides* on maize ears, in correlation with the change in emitted plant volatiles from infected ears was investigated in **Chapter 3**. The study adopted two varieties of maize plants and strains of *F. graminearum* and *F. verticillioides* that were different in their virulence to their host varieties.

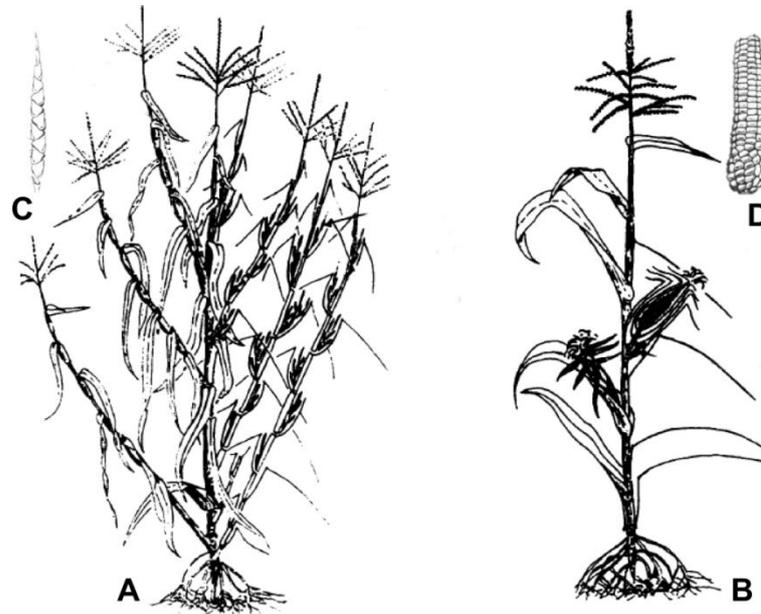
Figures

Figure 1: Teosinte and maize phenotypes. (A) Teosinte plant, (B) Maize plant, (C) Teosinte ear, (D) Maize ear. (Source: modified from O’Sullivan and Edwards, 2003; after Doebley et al., 1990).

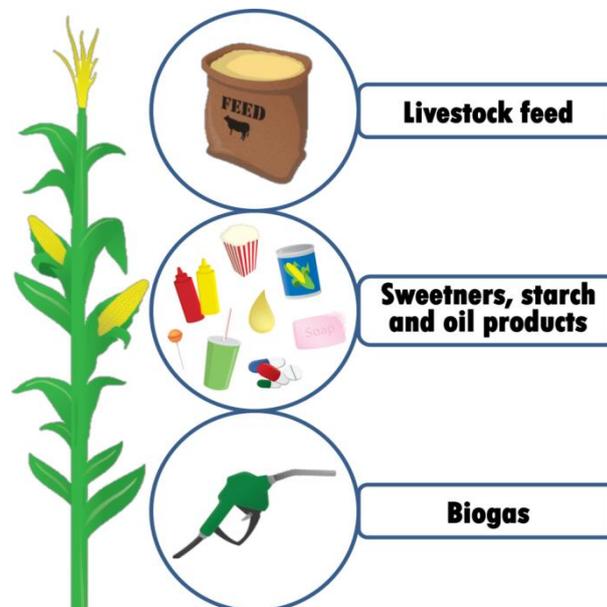


Figure 2: Different uses of raw and processed maize (Source: modified from Iowa State University- Center for Crops Utilization Research ©2009).

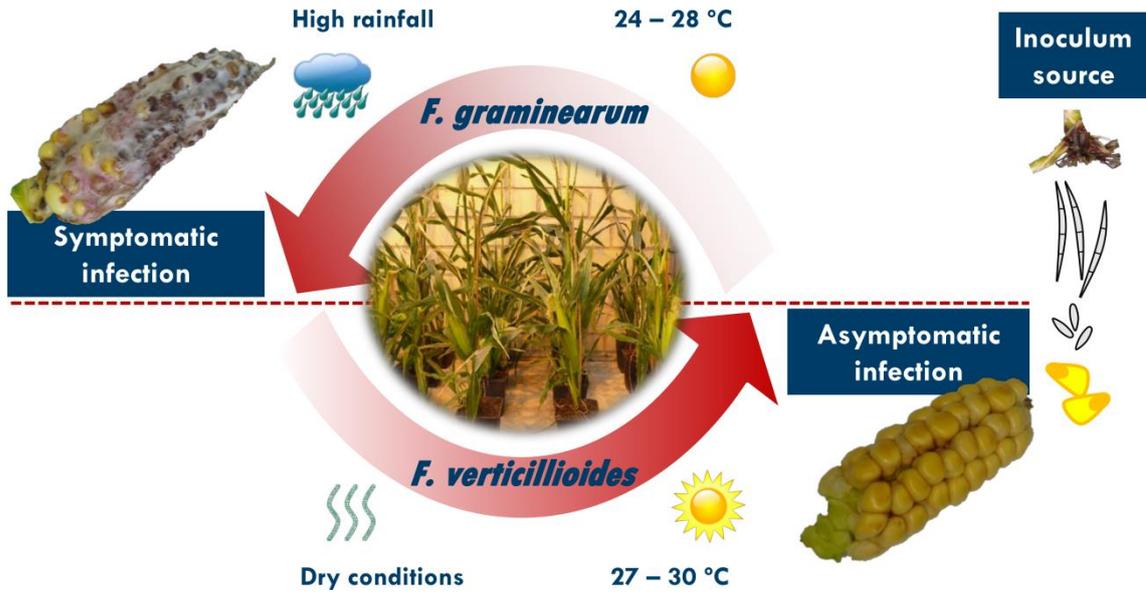


Figure 3: Schematic diagram represents maize ear rot disease caused by *F. graminearum* and *F. verticillioides* with favored climatic conditions (Source: by Mohammed Sherif).

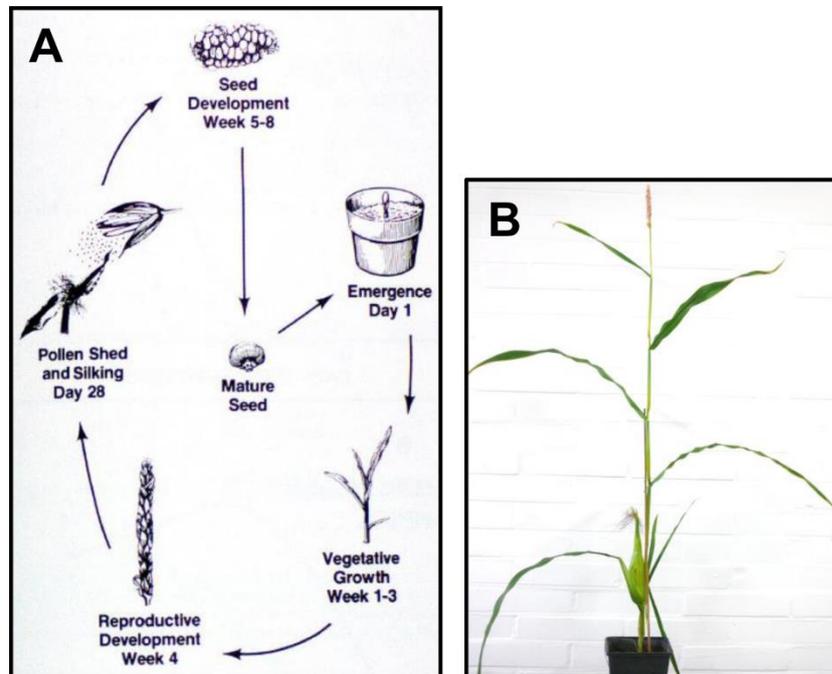


Figure 4: Maize (*Zea mays* L. var. Gaspe Flint). (A) Life cycle of Gaspe variety (Source: Hourcade et al., 1986), (B) Whole plant of Gaspe Flint (Source: by Mohammed Sherif)

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Chapter 2: Role of trichothecenes and fumonisins in interaction between *Fusarium graminearum* and *F. verticillioides* in maize ears

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Abstract

Concomitant infection of maize in the field with several *Fusarium* species is common. It is established that mixed infection of maize ears with *Fusarium graminearum* and *Fusarium verticillioides* facilitates the growth of *F. verticillioides* while suppressing *F. graminearum*. Suppression of host defense by trichothecenes of *F. graminearum* and inhibition of *F. graminearum* by fumonisins of *F. verticillioides* were hypothesized as the causes of these effects. We used *Fusarium* strains with deactivated synthesis of deoxynivalenol, nivalenol and fumonisins to clarify the role of these mycotoxins in mixed infection of maize. *F. verticillioides* growth *in planta* was stimulated by co-infection with *F. graminearum* regardless of the ability of *F. graminearum* to produce deoxynivalenol or nivalenol. The growth of a deoxynivalenol-producing *F. graminearum* strain and its nonproducing mutant was suppressed by co-inoculation with *F. verticillioides* regardless of the ability of *F. verticillioides* to produce fumonisins. Disease symptoms caused by a deoxynivalenol-producing strain of *F. graminearum* and its mutant but not by a nivalenol-producing strain and its mutant were reduced in mixed inoculation with *F. verticillioides* regardless of the ability of *F. verticillioides* to produce fumonisins. These and other results corroborated the role of

nivalenol as a virulence factor of *F. graminearum* in maize ears and showed that neither trichothecenes nor fumonisins are involved in the suppression of *F. graminearum* and stimulation of *F. verticillioides* growth during mixed infection of maize ears.

Keywords: maize, *Fusarium graminearum*, *Fusarium verticillioides*, interaction, ear rot, fumonisins, trichothecenes

Introduction

Maize (*Zea mays* L.) was domesticated from wild teosinte plants several thousand years ago (Doebley, 1990; Matsuoka et al., 2002). Since then, maize has become a major commodity crop, with more than one billion tons harvested per year (FAOSTAT, 2014). Maize plants are threatened by numerous fungal pathogens which reduce crop yield and contaminate grains with mycotoxins that are harmful to humans and livestock. Pathogenic fungi of the genus *Fusarium* are major pathogens of maize and other grain crops.

The most important *Fusarium* species infecting maize ears are *F. graminearum*, a dominant species in moderate to cold growing areas (Central Europe, Canada) and *F. verticillioides*, a dominant species in warm growing areas (Southern Europe, Africa, Midwestern United States). Maize ear infestation with *F. graminearum* Schwabe, the causal agent of *Gibberella* ear rot, can be recognized through the pinkish or reddish fungal mycelium on kernels. It typically begins at the tip of the cob (Oldenburg and Ellner, 2015) and requires warm temperatures (24-26°C) and high humidity (Munkvold, 2003). Infection of maize with *F. verticilloides* (Saccardo) Nirenberg, the causal agent of *Fusarium* ear rot, is characterized by scattered colonization of kernels along the cob and whitish mycelium in late stages; it is favored by temperatures close to 30°C and dry conditions (Vigier et al., 1997; Reid et al., 1999). *F. verticillioides* is regarded as less aggressive in maize ears than *F. graminearum*. Asymptomatic infection of maize ears with *F. verticillioides* and endophytic growth of the fungus in maize plants were often observed (Bacon and Hinton, 1996; Munkvold et al., 1997a, b). *F. graminearum* can colonize host plants endophytically, too (Larran et al., 2007; Pinaria et al., 2010; Quazi et al., 2010) but little is known about endophytic growth of the fungus in maize plants. Maize ears are commonly infected by several *Fusarium* species simultaneously

(Doohan et al., 2003). Thus, successful disease management of *Fusarium* infection in the field requires understanding interactions among fungi colonizing the same niche.

F. graminearum and *F. verticillioides* infect maize ears through distinct routes. Infestation with both pathogens occurs at flowering stage when maize ears develop silks. *F. graminearum* grows in and on silks, penetrates the ovaries, colonizes inter-kernel space and eventually reaches the rachis (Miller et al., 2007). Since Koehler (1942), numerous plant pathologists observed that the most important infection pathway for *F. verticillioides* is also through silks, causing infection in up to 84% of kernels (Munkvold et al., 1997b; Vigier et al., 1997; Munkvold, 2003). While many researchers have been using silk inoculation routinely, Duncan and Howard (2010) were unable to achieve kernel infection by spraying silks with conidia of *F. verticillioides*. They therefore injected conidia between the husks with a hypodermic needle. With the help of *F. verticillioides* strain expressing a fluorescent protein they demonstrated that the fungus entered maize kernels through the styler canal, which is a natural opening on the kernel's surface located below the site of silk attachment (Duncan and Howard, 2010). *F. verticillioides* can also infect maize asymptotically (Bacon and Hinton, 1996; Munkvold et al., 1997b; Yates et al., 2005; Bacon et al., 2008; Dastjerdi and Karlovsky, 2015). Scanning electron microscopy of matured asymptomatic maize kernels infected with *F. verticillioides* revealed the presence of fungal mycelium in intercellular space of kernel pedicles (Bacon et al., 1992).

F. graminearum and *F. verticillioides* produce several mycotoxins. Major mycotoxins of *F. graminearum* are type B trichothecenes (nivalenol; deoxynivalenol and their acetylated derivatives) and zearalenone. *F. verticillioides* produces mainly fumonisins B1, B2 and B3 (FB1, FB2, FB3), fusarin C and fusaric acid (Han et al., 2014). Mixed contamination of maize grains with several mycotoxins is common (Adejumo et al. 2007b; Hossain et al., 2015). *F. graminearum* strains can be divided into chemotypes synthesizing mainly deoxynivalenol, nivalenol, or acetylated derivatives of deoxynivalenol (Desjardins, 2008). Trichothecenes produced by *F. graminearum* act as virulence factors on wheat spikes but not on barley spikes (Proctor et al., 1995; Jansen et al., 2005; Maier et al., 2006). Several studies found that trichothecenes facilitated disease development in maize ears (Proctor et al., 1995; Harris et al., 1999). Using *F. graminearum* mutants unable to synthesize deoxynivalenol and nivalenol, Maier et al. (2006) showed that deoxynivalenol did not act as a virulence factor in maize but the ability to produce nivalenol increased the aggressiveness of the pathogen. The role of

fumonisin in disease development has been controversial. Fumonisin was detected in fully asymptomatic maize kernels (Bullerman and Tsai, 1994), showing that they do not induce disease symptoms. Strong induction of fumonisin synthesis in maize kernels indicated that fumonisins might be involved in the colonization of plant tissue (Han et al., 2014) but low levels of fumonisins in maize kernels colonized with fumonisin producer *F. verticillioides* (Adejumo et al. 2007b) did not support this hypothesis. Most experiments with *F. verticillioides* strains producing different amounts of fumonisins as well as with mutants of disrupted fumonisin synthesis indicated that fumonisins were not required for the infection of maize ears (Jardine and Leslie, 1999; Desjardins and Plattner, 2000; Desjardins et al., 2002) and maize seedlings (Dastjerdi and Karlovsky, 2015). Results of other studies supported the role of fumonisins as virulence factors in maize seedlings (Williams et al., 2007; Glenn et al., 2008).

Apart from their function as virulence factors, some mycotoxins likely mediate microbial antagonism (Karlovsky, 2008). A rigorous proof of the involvement of a mycotoxin in microbial antagonism was only provided in a few cases; fusaric acid (Notz et al., 2002), zearalenone (Utermark and Karlovsky, 2007) and gliotoxin (Coleman et al., 2011) are well-studied examples. Indirect evidence for the role of mycotoxins in fungal interactions was provided by studies of mycotoxin production in mixed cultures and by the effects of mycotoxins on fungi *in vitro* (Yates et al., 1999; Velluti et al., 2000; Barberis et al., 2014; Hua et al., 2014; Chatterjee et al., 2016). When several pathogens infect the same plant, the effects of their secondary metabolites on the plant and on each other are likely to modulate the outcome of the infection in ways that cannot be explained alone by competition. For instance, metabolites secreted by one pathogen acting as virulence factors may facilitate colonization of the tissue by another pathogen; on the other hand, metabolites that induce defense responses of the host might slow down infection by unrelated pathogens. Several studies addressed mixed infection of maize by *F. graminearum* and *F. verticillioides*. Reid et al. (1999) found that *F. verticillioides* outcompeted *F. graminearum* in mixed inoculation of maize. Picot et al. (2012) reported that the growth of *F. graminearum* was suppressed (or unaffected) and *F. verticillioides* stimulated (or unaffected) in mixed infection, corroborating observations from field trials that artificial inoculation with *F. graminearum* stimulated the growth of spontaneously infecting *F. verticillioides* (Picot et al., 2012). The inhibition and facilitation of growth of *F. graminearum* and *F. verticillioides*, respectively, in mixed infection was observed with different maize cultivars but the

mechanism remains unknown. Because trichothecenes act as virulence factors (Maier et al., 2006) and fumonisins were reported to inhibit fungal growth (Keyser et al., 1999), in this study we investigated the role of trichothecenes and fumonisins produced by *F. graminearum* and *F. verticillioides* in mixed infection of maize ears using mutants of both species with disrupted mycotoxin biosynthesis pathways.

Materials and Methods

Fungal strains

Three mycotoxin-producing strains of two *Fusarium* species and their mutants deficient in the production of distinctive mycotoxins were used (**Table 1**). *F. graminearum* deoxynivalenol chemotype (DON+), its deoxynivalenol-deficient mutant (DON-) with disrupted *Tri5* gene, *F. graminearum* nivalenol chemotype (NIV+) and its nivalenol-deficient mutant (NIV-) with disrupted *Tri5* gene (Maier et al., 2006) were kindly provided by W. Schäfer, University of Hamburg, Germany. *F. verticillioides* fumonisin producer (FUM+) and its fumonisin-deficient mutant (FUM-) with disrupted *FUM1* gene (Proctor et al., 1999) were kindly provided by R. Proctor, USDA/ARS, Peoria, USA. Sporulation was achieved in liquid mung bean medium (Bai and Shaner, 1996) as modified by Becker et al. (2014). The number of spores in fresh cultures was counted with a Thoma chamber and spore concentration was adjusted for further artificial ear inoculations. Spore suspensions in sterile tap water amended with 15% glycerol were stored at -70°C until use.

Plant material and growth conditions

In our study we used Gaspé Flint (provided by Roberto Tuberosa, University of Bologna, Italy), which is a very early maturing, yellow-seeded dwarf maize landrace of the Northern Flint race originally collected in Quebec, Canada (accession no. CN 33817, Plant Gene Resources of Canada, http://pgrc3.agr.gc.ca/acc/search-recherche_e.html#add). Gaspé Flint completes its life cycle in approximately 65 days, reaches a height of 1 m and produces ears of about 10 cm length with up to 8 rows of kernels (Desjardins et al. 2008, Hourcade et al., 1986). Before seeding, kernels were sterilized with sodium hypochlorite 4% for 15 min and rinsed with distilled sterilized water 3 times for 10 min each. To check for possible seed contaminations, seeds were

pre-germinated in the dark on sterilized wetted filter papers at 28 °C for 5 days (Warham et al., 1996). Healthy seedlings were placed individually into plastic pots (13 x13 x 11 cm), containing autoclaved mixture of topsoil and sand (v/v = 2:1) and transferred to a glass house (25 °C, 14h photoperiod). Plants were irrigated with tap water as required and supplemented weekly with the mineral fertilizer Hakaphos® (15% N, 0.01% B, 0.02% Cu, 0.075% Fe, 0.05% Mn, 0.001% Mo, 0.015 %Zn, 10% P₂O₅, 15% K₂O, 2% MgO).

Inoculation

Maize ears were inoculated 5 days after silking. 0.50 mL sterilized water (control plants) or aqueous spore suspension (1×10^5 spores/mL) were injected into the upper part of the ear through silk channel using a 1 mL hypodermic syringe. All strains (wild types and mutants) were inoculated separately (single inoculations). *F. graminearum* and *F. verticillioides* strains were also inoculated concurrently (0.50 mL of 1×10^5 spores/mL, 1:1 ratio) and sequentially: the first species 5 days after silking (0.50 mL, 1×10^5 spores/mL), the second species 10 days after silking (0.50 mL, 1×10^5 spores/mL). **Table 2** lists all combinations of fungal strains used for the inoculation. Five ears on five independent growing plants were inoculated with each strain or strains combination in each of two experiments, performed in early and late summer 2013 according to a completely randomized design.

Ear harvesting and symptom evaluation

Ears were harvested 18 days post inoculations, which corresponds to the early dough growth stage (Hanway and Ritchie, 1986), and immediately processed. Disease symptoms on dehusked maize ears involving discoloration, rotting, and/or fungal mycelium, were indexed using a scale modified from Reid et al. (1999). Ears were visually divided into two symmetrical faces and each face was crossed by a vertical and a horizontal line, resulting in four sections per face and eight sections per ear. Sections showing signs of infection were counted, resulting in disease index values from zero (healthy) to eight (fully infected) (**Figure 1**).

After disease symptom scoring, the ears were detached from plants, all kernels were separated from rachides using a knife and the rachides were discarded. The kernels were freeze-dried and ground with a beadbeater (Retsch-Germany). Homogenized flour was used for the determination of mycotoxin content and fungal DNA.

DNA extraction and quantification of fungal biomass

DNA was extracted from aliquots of 100 mg freeze-dried maize kernels following a protocol of Brandfass and Karlovsky (2008). Extracted samples were diluted to approximately 10-15 ng μL^{-1} of total DNA based on comparison of the intensity of DNA bands after agarose electrophoresis with DNA standards, the dilutions were recorded and DNA was subjected to qPCR using primers Fg16NF/Fg16NR for *F. graminearum* (Nicholson et al., 1998) and VER1/VER2 for *F. verticillioides* (Mulè et al., 2004) essentially as previously described (Brandfass and Karlovsky, 2008; Nutz et al., 2011). In brief, the reaction mixture contained PCR buffer with 2.5 mM MgCl_2 , 200 μM dNTP, 0.3 μM of each primer, SYBR Green I diluted according to manufacturer's instruction, bovine serum albumin 1.0 mg/mL and 10-15 ng total DNA. The thermocycler program for *F. verticillioides* consisted of 2 min at 95°C, followed by 34 cycles of 40 s at 94°C, 30 s at 62°C, and 40 s at 72°C, with a final extension for 4 min at 72°C. The thermocycler program for *F. graminearum* consisted of 2 min at 95°C, followed by 34 cycles of 30 s at 94 °C, 30 s at 64°C, and 30 s at 72°C, with a final extension for 5 min at 72°C. Standard curves were generated using DNA of *F. graminearum* and *F. verticillioides* in three-fold serial dilutions between 1 pg/ μL and 3.3 ng/ μL .

Mycotoxin extraction and quantification

Homogenized lyophilized maize flour (500 mg) was transferred to 15 mL centrifuge tubes and extracted with 5 mL acetonitrile/water (84:16, v/v) on a rotary shaker at 170 rpm overnight. After centrifugation at 4,800 g for 10 min, 1.0 mL of the supernatant was transferred to a 2 mL reaction tube and dried at 35°C in a vacuum concentrator. The residue was dissolved in 500 μL methanol-water (1:1, v/v); the solubilization was facilitated by sonication for 10 sec and repeated vortexing. A volume of 800 μL cyclohexane was added to dissolve residues for defatting. The samples were vortexed and centrifuged at 14,000 g for 10 min. Analytes recovered in the methanol/water phase were separated by HPLC on a reverse-phase column at 40°C with a methanol-water gradient, followed by electrospray ionization essentially as described (Ratzinger et al., 2009). deoxynivalenol and nivalenol were detected by HPLC coupled with a triple quadrupole mass spectrometer (Adejumo et al., 2007a). Fumonisin were quantified by HPLC coupled with an ion-trap tandem mass spectrometer (Adejumo et al., 2007b).

Statistical analysis

The experiment was repeated twice with five replicates per treatment in each experiment. The data from both experimental repetitions were pooled. DNA and mycotoxin concentrations were log transformed before statistical analysis. Statistical differences between mixed and single inoculations was computed for relative values (Figures 3,4 and 5) in Sigmaplot 11.0, using either ANOVA/Holm-Sidak or Kruskal-Wallis ANOVA on ranks/Dunn's tests depending on the homogeneity of the variance in the data. Statistical differences for the data presented in Figures S1, S2 and S3 (absolute values for disease severity, fungal DNA and mycotoxin content) was computed in PAST 3.04 (Hammer et al., 2001) using multiple comparisons (Mann-Whitney with Bonferroni corrections). Statistical tests applied are specified in figures legends. Mycotoxin content below LOQ was replaced by half the LOQ value (US Environmental Protection Agency, 2000).

Results

Disease severity, fungal biomass and mycotoxin accumulation after inoculation with single species

Maize ears were inoculated separately with mycotoxin producing strains of *F. verticillioides* and *F. graminearum* and their mycotoxin nonproducing mutants five days after silking. Disease severity was evaluated 18 days after inoculation. Control treatments (mock inoculation with water) did not show any disease symptoms. Inoculation with *F. graminearum* DON+ and DON- strains caused most severe symptoms, followed by *F. graminearum* NIV+ and NIV- strains; *F. verticillioides* FUM+ and FUM- strains exhibited the lowest aggressiveness (**Figure 2A**). Mycotoxin-producing and non-producing strains of the deoxynivalenol chemotype of *F. graminearum* and of *F. verticillioides* did not differ in aggressiveness. Inoculation with nivalenol-deficient mutant of *F. graminearum* led to less severe symptoms than inoculation with isogenic nivalenol-producing strain ($p = 0.048$, **Figure 2A**). Fungal biomass was determined as the amount of fungal DNA in kernels using species-specific qPCR. *F. graminearum* (both DON+ and DON- strains) accumulated the highest biomass *in planta*; *F. verticillioides* (both FUM+ and FUM- strains) produced the lowest biomass while *F. graminearum* (NIV+ and NIV-) reached intermediate values.

Fungal biomass of wildtype strains and their mycotoxin nonproducing mutants did not differ (**Figure 2B**). The accumulation of fumonisin B1, deoxynivalenol, and nivalenol by *F. verticillioides* (FUM+), *F. graminearum* (DON+), and *F. graminearum* (NIV+), respectively, was quantified by HPLC-MS/MS. Mycotoxin levels (**Figure 2C**) followed the same trend as the disease severity and fungal growth: Deoxynivalenol was found in the highest amounts (mean 490 mg/kg), followed by nivalenol (mean 2.30 mg/kg) and fumonisin B1 (0.15 mg/kg).

Disease development in mixed inoculations

For concurrent infection, maize ears were inoculated with *F. verticillioides* and *F. graminearum* 5 days after silking. Sequential inoculations were performed with the first species 5 days after silking and the second species 10 days after silking. Disease severity was evaluated 18 days post-inoculation in the concurrent infections and 18 days after the second inoculation in sequential inoculation. Disease severity in mixed inoculation was compared to disease severity after inoculation with single species and expressed as relative values (**Figure 3**); the absolute values are shown in **S1**. Mixed inoculation with *F. verticillioides* (FUM+ or FUM-) and *F. graminearum* (DON+ or DON-), both concurrent and sequential, resulted in most instances to increased disease severity as compared to inoculation with *F. verticillioides* (FUM+ or FUM-) alone and decreased severity as compared to inoculations with *F. graminearum* (DON+ or DON-) alone (**Figure 3**). Concurrent and sequential inoculation with *F. graminearum* (NIV+ or NIV-) and *F. verticillioides* (FUM+ or FUM-) lead to unchanged or increased disease severity as compared to inoculation with *F. verticillioides* (FUM+ or FUM-) alone. Specifically concurrent and sequential inoculations comprising *F. graminearum* (NIV+) did not influence disease symptoms compared to single inoculations with (NIV+). In the contrary, concurrent and sequential inoculations comprising *F. graminearum* (NIV-) increased disease severity in three (FUM-/NIV-, FUM+/NIV- and NIV-/FUM+) out of five cases as compared to the single inoculation with *F. graminearum* (NIV-) alone (**Figure 3**).

Fungal biomass in mixed inoculation

In the same way as for disease severity, fungal biomass in kernels from plants infected concurrently or sequentially with strains of both species was compared to the biomass reached by each strain in single-species inoculations and expressed as relative values

(absolute values are shown in **S2**). Concurrent and sequential inoculations with *F. verticillioides* (FUM+ or FUM-) and *F. graminearum* (DON+ or DON-) resulted in increased or unchanged biomass of *F. verticillioides* (FUM+ or FUM-) and decreased biomass of *F. graminearum* (DON+ or DON-) (**Figure 4**). These effects were less prominent in concurrent and sequential inoculations with *F. verticillioides* (FUM+ or FUM-) and *F. graminearum* (NIV+ or NIV-), even though in some instances an increase in the biomass of *F. verticillioides* and a decrease of the biomass of *F. graminearum* occurred (**Figure 4**). In most cases, fungal biomass pattern was similar to the pattern of disease severity. Unlike for disease severity (**Figure 3**), the biomass of *F. graminearum* (NIV+ or NIV-) decreased in concurrent (FUM+/NIV+) and sequential (FUM-/NIV+ , FUM+/NIV-, and FUM-/NIV-) inoculations compared to single inoculations with *F. graminearum* (NIV+ or NIV-). Although *F. graminearum* (DON+, DON-, NIV+ or NIV-) was inoculated before *F. verticillioides* (FUM+) in sequential inoculation, the biomass of *F. graminearum* was comparable or lower (DON+/FUM+) than when *F. graminearum* had been inoculated alone. The growth of *F. verticillioides* (FUM+ or FUM-) was stimulated by concurrent or subsequent co-inoculation with *F. graminearum* in 10 strain combinations out of 20 (**Figure 4**).

Mycotoxin accumulation in kernels after mixed inoculation with *F. graminearum* and *F. verticillioides*

We compared mycotoxin levels in kernels inoculated with single species and co-inoculated with both species in different combinations of mycotoxin producing strains and non-producing mutants (**Figure 5** for relative values; **S3**, for absolute values), with raw mycotoxins data in **Table 3**. The average concentration of fumonisin B1 in one concurrent mixed inoculations (FUM+/DON-) and all three out of four (FUM+/DON+, FUM+/DON-, FUM+/NIV-) sequential inoculations with *F. verticillioides* followed by *F. graminearum* was significantly increased (**Figure 5**). In sequential mixed inoculations with *F. graminearum* followed by *F. verticillioides*, the average concentration of fumonisin B1 was unchanged compared to the average concentration of fumonisin B1 in single *F. verticillioides* inoculation (**Figure 5**). The accumulation of trichothecene deoxynivalenol was reduced in three (FUM-/DON+, FUM+/DON+ and FUM-/DON+)out of five inoculations (concurrent and sequential) whereas nivalenol was unaffected and was below the limit of quantification in three (FUM+/NIV+,

FUM+/NIV+ and FUM-/NIV+) out of five inoculations (concurrent and sequential) (**Figure 5, Table 3**).

Discussion

We investigated the role of nivalenol, deoxynivalenol and fumonisin fumonisin B1 in mixed infection of maize ears with *F. graminearum* and *F. verticillioides*. The strains used differed in their aggressiveness and in the fungal biomass accumulated in infected tissue. *F. graminearum* deoxynivalenol chemotype and its mutant were the most aggressive strains. They were followed by *F. graminearum* nivalenol chemotype and its mutant, which achieved moderate infection. Both *F. verticillioides* strains caused the mildest disease symptoms and achieved the lowest biomass accumulation. This species ranking is in line with previous results (Reid et al., 1999; Reid et al., 2002; Miedaner et al., 2010). A lower aggressiveness of a nivalenol chemotype of *F. graminearum* as compared to deoxynivalenol chemotype was reported previously on winter rye (Miedaner et al., 2000).

The only mycotoxin potentially acting as a virulence factor in our experiments was nivalenol. The non-producing mutant of nivalenol chemotype of *F. graminearum* caused fewer symptoms than the nivalenol-producing strain (**Figure 2A**), though the biomass of the strains in infected kernels was unaffected (**Figure 2B**). This result corroborated earlier finding that nivalenol was a virulence factor of *F. graminearum* in maize ears (Maier et al., 2006). The observation that nivalenol producer caused more extensive disease symptoms than the non-producing mutant while both strains accumulated the same biomass indicates that nivalenol might have directly contributed to disease symptoms rather than facilitating colonization. Unlike in the results of Maier et al. (2006), a marked difference in aggressiveness between nivalenol and deoxynivalenol chemotypes of *F. graminearum* was observed in terms of disease symptoms, fungal biomass and mycotoxin accumulation. Because the strains used in both experiments were the same, differences among maize varieties and different time spans allowed for disease development may have played a role. Gaspe Flint, used in our experiments, appeared more susceptible to *F. graminearum* strain FG 2311 (deoxynivalenol chemotype) than the inbred line A188 used by Maier et al. (2006). Different susceptibility to deoxynivalenol was not responsible for this difference

because deoxynivalenol non-producing mutant was as aggressive as the deoxynivalenol producer in both studies. The relative aggressiveness of *F. graminearum* (nivalenol chemotype) and *F. verticilloides* on maize hybrid Ronaldinio and Gaspé Flint was similar: both cultivars were more susceptible to *F. graminearum* than to *F. verticilloides* (Becker et al., 2014). Gaspé Flint was slightly more susceptible to *F. graminearum* than hybrid maize (100% versus 85% disease severity) but less susceptible to *F. verticilloides* (20% versus 60% disease severity) (Becker et al., 2014). It is a trivial fact that maize genotype affects disease susceptibility to the infection with a mixture of *Fusarium* species. The effect of mixed inoculation of *F. graminearum* and *F. verticilloides* on pathogen growth *in planta* was however found to be the same in all maize cultivars tested so far. It is unlikely that in different cultivars different mechanisms are responsible for the same interaction pattern.

The main goal of our study was to clarify the role of mycotoxins on the interaction between *F. graminearum* and *F. verticilloides* in maize ears. Reid et al. (1999) and Picot et al. (2012) established that mixed inoculation facilitated *F. verticilloides* growth and suppressed the growth of *F. graminearum*. In spite of high variability inherent to field trials and pervasive spontaneous infection with *F. verticilloides*, which in some experiments exceeded *F. verticilloides* levels in artificially inoculated plants, both effects were significant in most experiments in both studies. Because trichothecenes act as virulence factors of *F. graminearum*, suppression of defense response of the host by trichothecenes could explain the facilitation of growth of *F. verticilloides* by co-infecting *F. graminearum*. Fumonisins inhibit fungal growth (Keyser et al., 1999); local concentrations of fumonisins in plant tissue may considerably exceed average concentrations determined in grain extracts and inhibit the growth of competing *F. graminearum*. Hence, we used *Fusarium* mutants impaired in the synthesis of fumonisins, deoxynivalenol and nivalenol to clarify the role of mycotoxins in these effects.

In line with previous finding, *F. verticilloides* suppressed disease symptoms caused by *F. graminearum* DON⁺ and its mutant DON⁻ as well as their growth *in planta* both when *F. graminearum* was inoculated earlier than and concurrently with *F. verticilloides*. Production of deoxynivalenol did not influence these effects, indicating that suppression of plant defense response by trichothecenes does not account for the stimulation of *F. verticilloides* growth in mixed inoculation with *F. graminearum* deoxynivalenol chemotype. Co-inoculation of *F. verticilloides* with

nivalenol chemotype of *F. graminearum* was studied in this work for the first time. While stimulation of *F. verticillioides* growth *in planta* by *F. graminearum* NIV+ and its mutant NIV- was similar to the stimulation by the deoxynivalenol chemotype, suppression of *F. graminearum* growth in mixed inoculation was less pronounced for the NIV+ and its mutant NIV- than for DON+ and its mutant DON- (**Figure 4**). This difference was reflected by the effect on disease severity. No significant disease suppression by *F. verticillioides* was observed for the nivalenol producing strain. For its trichothecene-nonproducing mutant, co-inoculation with *F. verticillioides* actually increased disease symptoms; this happened in all five combinations of strains and infection modes (sequential and concurrent), in three of them the increase was statistically significant (**Figure 3**). The relevance of these results should however not be overestimated since disease rating was based on a visual semi-quantitative scale. The production of nivalenol did not seem to modulate the effect of *F. verticillioides* on *F. graminearum* growth because similar results were obtained for NIV+ and NIV-. The effect of *F. verticillioides* on disease severity appeared to differ for the NIV+ and NIV- but we caution against generalizing these findings. Taking all results into account, the cause of differences between the effect of interaction with *F. verticillioides* on *F. graminearum* strains do not seem to depend on which trichothecene is produced by *F. graminearum* but on other characteristics of the strains which are so far unknown. However, sequential inoculation with *F. verticillioides* prior to *F. graminearum* which has been tested in this study revealed strong effect of stimulation and inhibition on *F. verticillioides* and *F. graminearum* respectively in terms of fungal growth and mycotoxin accumulation (**Figure 4, 5**). Overall, *F. verticillioides* growth was stimulated in plants co-infected with *F. graminearum* as statistically observed in 10 out of 20 strain combinations for which we obtained quantitative data (**Figure. 4**).

Our results convincingly excluded the role of fumonisins, either direct or indirect via modulation of plant defense in suppression of *F. graminearum* *in planta* by co-infection with *F. verticillioides*. It remains puzzling why fumonisin synthesis is induced *in planta* (Han et al., 2014) when fumonisins do not act as virulence factors.

Seasonal fluctuation in *Fusarium* species epidemics has been well-known among plant growers. Climatic conditions have been suggested to explain these fluctuations (Schaafsma et al. 1997; Reid et al. 1999). Interaction among co-infecting species affects disease progression; because the effect of weather conditions on infection efficiency differs among *Fusarium* species, interspecies interactions add another source of

variance to the dependence of disease progression on the climate. For instance, high temperature does not favor *F. graminearum* infection (Reid et al., 1999) but if *F. verticillioides* is present, its negative effect on disease symptoms caused by certain strains of *F. graminearum* (**Figure 3**) might obscure the effect of temperature. In addition to *F. graminearum* and *F. verticillioides*, a number of further *Fusarium* species and other fungi (Doohan et al., 2003) infect maize ears in the field and are likely to affect each other's ability to colonize the ears and cause disease. These interactions should be taken into account in studies of the effect of abiotic factors on fungal diseases of maize.

Author Contributions

PK, MS and RS designed the work. MS performed fungal inoculations, analyzed disease severity, and quantified fungal DNA. MS prepared the figures with inputs from all co-authors. MS and KP analyzed mycotoxins and interpreted the data. MS and RS did the statistics. MS, RS and PK drafted the manuscript, which was critically revised by all co-authors.

Conflict of Interest

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

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Supplementary Material

S1, Disease severity on maize ears

S2, Fungal DNA quantification in maize ears

S3, Mycotoxin content in maize ears

Tables

Table 1. *F. graminearum* and *F. verticillioides* strains

Strain	Code	Abbreviation	Source
<i>F. graminearum</i> DON	FG 2311 WT	DON+	
<i>F. graminearum</i> DON-deficient ^a	FG 2311#2899	DON-	Wilhelm Schäfer, Hamburg University, Hamburg, Germany
<i>F. graminearum</i> NIV	FG 06 WT	NIV+	
<i>F. graminearum</i> NIV-deficient ^b	FG 06#7	NIV-	
<i>F. verticillioides</i> WT	M-3125	FUM+	Robert Proctor, National Center for Agricultural Utilization Research/ U. S. Department of Agriculture Peoria, Illinois, USA
<i>F. verticillioides</i> FUM-deficient ^c	GfA2364	FUM-	

^a Mutant strain of FG 2311 with disrupted *Tri5* gene (Maier et al., 2006)

^b Mutant strain of FG 06 with disrupted *Tri5* gene (Maier et al., 2006)

^c Mutant strain of M-3125 with disrupted *FUM1* gene (Proctor et al., 1999)

Table 2. Inoculation experiments

Inoculation type	Fungal chemotype(s) ^a
Single	DON+, DON-, NIV+, NIV-, FUM+, FUM-
Concurrent	FUM+/DON+, FUM+/DON-, FUM+/NIV+, FUM+/NIV-, FUM-/DON+, FUM-/DON-, FUM-/NIV+, FUM-/NIV-
Sequential ^b	FUM+/DON+, FUM+/DON-, FUM+/NIV+, FUM+/NIV-, FUM-/DON+, FUM-/DON-, FUM-/NIV+, FUM-/NIV-, DON+/FUM+, DON-/FUM+, NIV+/FUM+, NIV-/FUM+
Control	Mock inoculation with water

^a FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364, DON+: *F. graminearum* FG2311, DON-: *F. graminearum* FG2311#2899, NIV+: *F. graminearum* FG06, NIV-: *F. graminearum* FG06#7

^b The strain on the left was inoculated first, followed by the strain on the right after 5 d

Table 3. Trichothecenes and fumonisins content ($\mu\text{g}/\text{kg}$) in infected maize kernels

Treatment	Single inoculation			Concurrent inoculation										
	FUM+	DON+	NIV+	FUM+/DON+		FUM+/DON-	FUM+/NIV+			FUM+/NIV-	FUM-/DON+	FUM-/NIV+		
Mycotoxin	Fumonisin B1	Deoxynivalenol	Nivalenol	Fumonisin B1	Deoxynivalenol	Fumonisin B1	Fumonisin B1	Nivalenol	Fumonisin B1	Deoxynivalenol	Nivalenol			
1	<LOQ	16.000	7.300	2.600	12.000	6.200	810	<LOQ	58	300.000	<LOQ			
2	<LOQ	260.000	<LOQ	<LOQ	<LOQ	3.000	1.200	<LOQ	<LOQ	240.000	<LOQ			
3	<LOQ	130.000	<LOQ	<LOQ	<LOQ	11.000	<LOQ	<LOQ	<LOQ	2.800	<LOQ			
4	<LOQ	320.000	<LOQ	<LOQ	<LOQ	170	<LOQ	<LOQ	<LOQ	1.900	<LOQ			
5	<LOQ	750.000	nd	<LOQ	<LOQ	4.200	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ			
6	nd	660.000	8.500	480	190.000	370	370	<LOQ	910	53.000	4.400			
7	340	920.000	1.500	510	210.000	800	770	<LOQ	790	12.000	2.300			
8	310	830.000	1.200	380	370.000	780	650	<LOQ	1.700	18.000	4.100			
9	310	500.000	1.000	380	240.000	880	670	nd	1.200	21.000	3.800			
10	300	nd	1.100	440	210.000	580	430	nd	980	21.000	3.400			
Treatment	Sequential inoculation													
	FUM+/DON+		FUM+/DON-	FUM+/NIV+		FUM+/NIV-	FUM-/DON+	FUM-/NIV+	DON+/FUM+		DON-/FUM+	NIV+/FUM+		NIV-/FUM+
Mycotoxin	Fumonisin B1	Deoxynivalenol	Fumonisin B1	Fumonisin B1	Nivalenol	Fumonisin B1	Deoxynivalenol	Nivalenol	Fumonisin B1	Deoxynivalenol	Fumonisin B1	Fumonisin B1	Nivalenol	Fumonisin B1
1	180	<LOQ	3.400	6.300	<LOQ	900	nd	<LOQ	52	<LOQ	920	750	<LOQ	110
2	15.000	<LOQ	73	<LOQ	<LOQ	3.000	<LOQ	<LOQ	36	<LOQ	380	73.000	<LOQ	950
3	28	12.000	4.300	<LOQ	<LOQ	6.100	nd	<LOQ	600	21.000	650	36.000	<LOQ	<LOQ
4	350	<LOQ	nd	<LOQ	<LOQ	nd	27.000	<LOQ	420	31.000	<LOQ	11.000	1.200	<LOQ
5	57.000	<LOQ	8.100	<LOQ	<LOQ	nd	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	nd	<LOQ
6	4.000	<LOQ	1.600	1.100	<LOQ	490	14.000	<LOQ	<LOQ	100.000	<LOQ	<LOQ	<LOQ	<LOQ
7	4.600	<LOQ	1.300	1.000	<LOQ	1.600	22.000	<LOQ	<LOQ	140.000	<LOQ	<LOQ	<LOQ	<LOQ
8	6.300	<LOQ	1.600	630	<LOQ	860	22.000	<LOQ	<LOQ	160.000	<LOQ	<LOQ	<LOQ	<LOQ
9	3.800	<LOQ	1.900	600	<LOQ	880	19.000	<LOQ	<LOQ	260.000	<LOQ	<LOQ	<LOQ	<LOQ
10	7.300	nd	570	840	<LOQ	1.300	54.000	nd	<LOQ	120.000	<LOQ	<LOQ	<LOQ	<LOQ

FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364, DON+: *F. graminearum* FG2311, DON-: *F. graminearum* FG2311#2899, NIV+: *F. graminearum* FG06, NIV-: *F. graminearum* FG06#7. LOQ: limit of quantification; for fumonisin B1= 50 $\mu\text{g}/\text{kg}$; for deoxynivalenol and nivalenol= 100 $\mu\text{g}/\text{kg}$. nd: not detected.

Figures

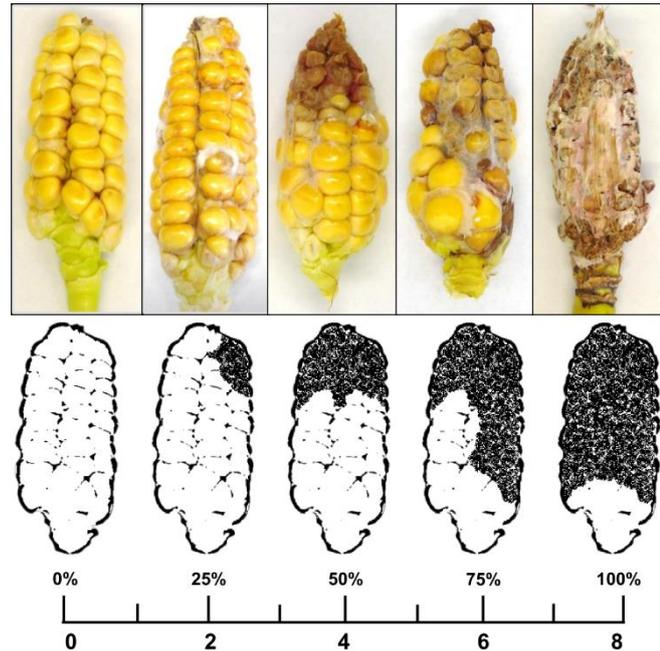


Figure 1. Ear rot disease scale. Symptom on both (front and back) faces are rated from zero = no symptom (0%) to eight = fully deteriorated (100%).

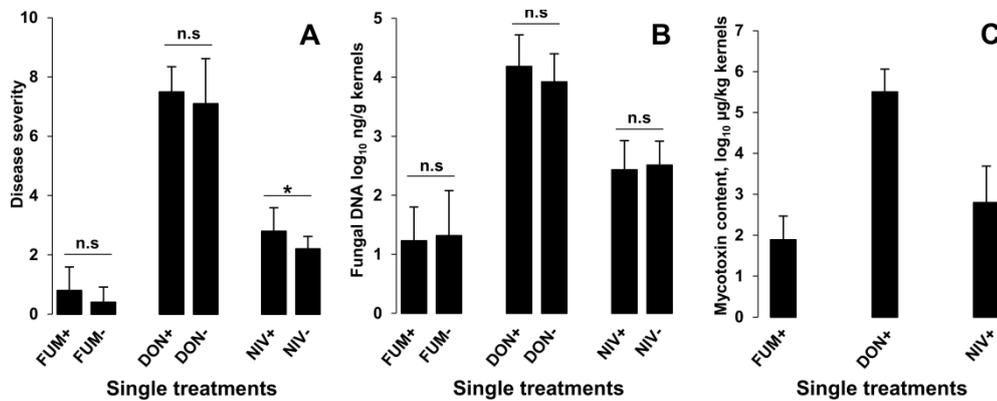


Figure 2. Single-strain inoculation of maize ears. A) Disease severity, B) fungal DNA, C) mycotoxin concentration. FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenol chemotype), DON-: *F. graminearum* FG2311#2899 (mutant of FG2311 with disrupted deoxynivalenolsynthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. Asterisk indicates a significant difference ($P = 0.048$), t test. n.s = non-significant.

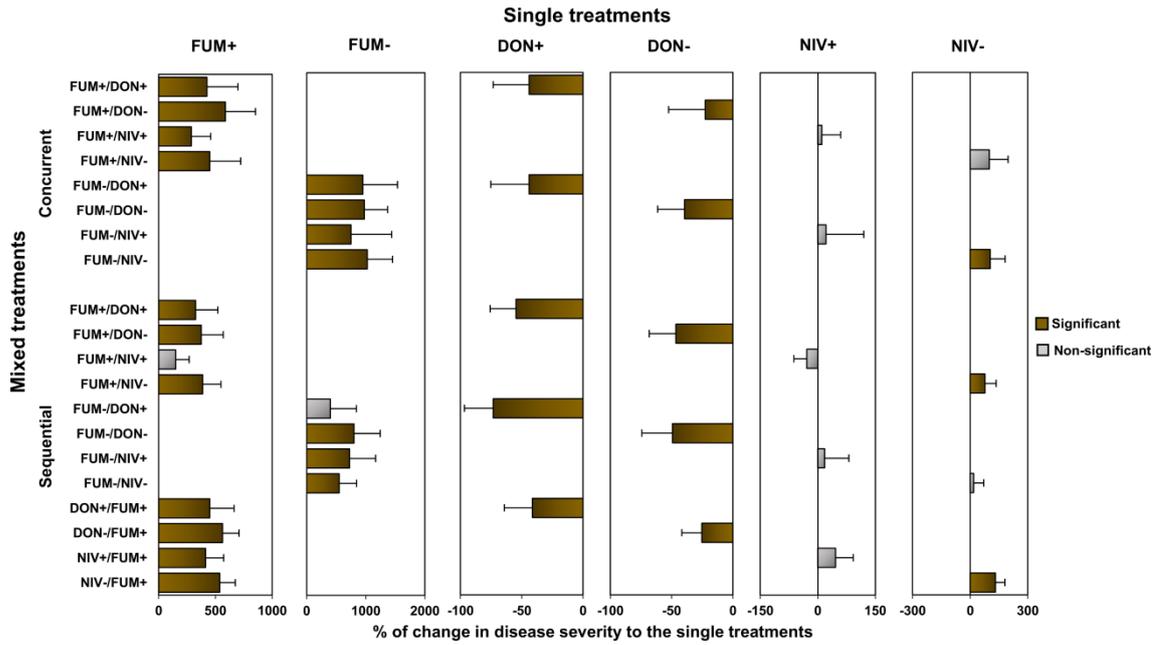


Figure 3. Comparison of disease severity of maize ears co-infected with *F. graminearum* and *F. verticillioides* (mixed strains listed on the left side) to disease severity inflicted by single-strain infection (strains listed above the panels). FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenolchemotype), DON-: *F. graminearum* FG2311# 2899 (mutant of FG2311 with disrupted deoxynivalenolsynthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. Brown columns indicates a significant difference ($p < 0.05$, Kruskal-Wallis ANOVA on ranks Dunn's test or ANOVA Holm-Sidak depending on the homogeneity of variance in the data) between mixed and single inoculations. Concurrent: simultaneous inoculation of both *Fusarium* species at the same time point. Sequential: interval of 5 days between the first/second inoculations of *Fusarium* species appears in order.

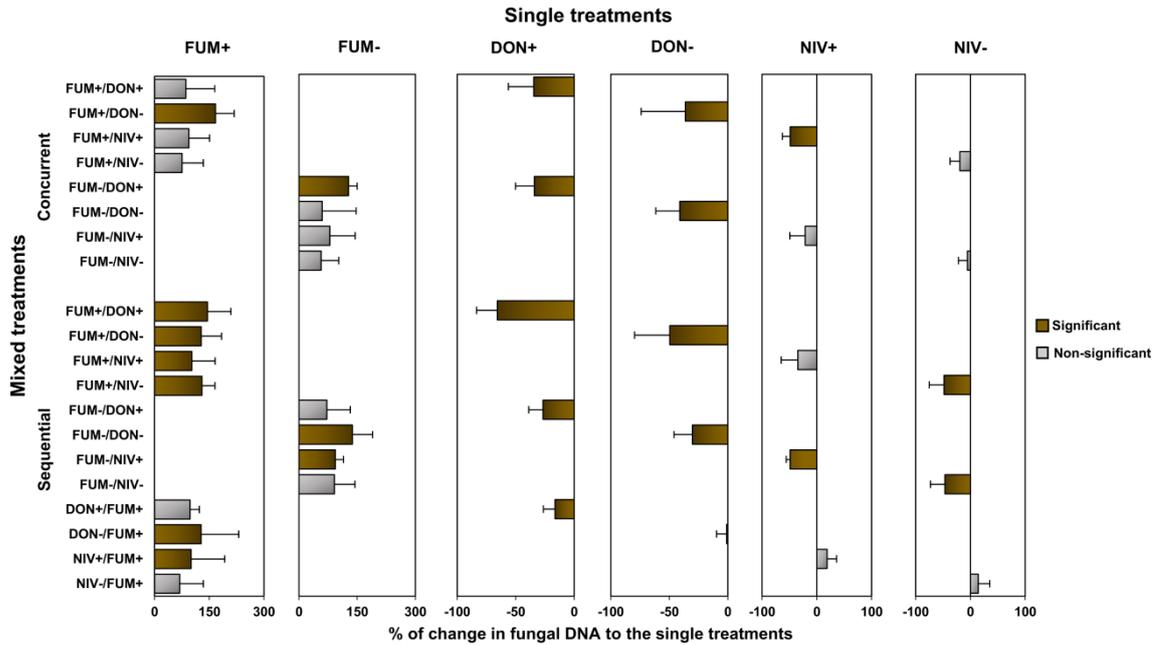


Figure 4. Comparison of fungal DNA in maize ears co-infected with *F. graminearum* and *F. verticillioides* (mixed strains listed on the left side) to fungal DNA inflicted by single-strain infection (strains listed above the panels). FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenolchemotype), DON-: *F. graminearum* FG2311# 2899 (mutant of FG2311 with disrupted deoxynivalenolsynthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. Brown columns indicates a significant difference ($p < 0.05$, Kruskal-Wallis ANOVA on ranks Dunn's test or ANOVA Holm-Sidak depending on the homogeneity of variance in the data) between mixed and single inoculations. Concurrent: simultaneous inoculation of both *Fusarium* species at the same time point. Sequential: interval of 5 days between the first/second inoculations of *Fusarium* species appears in order.

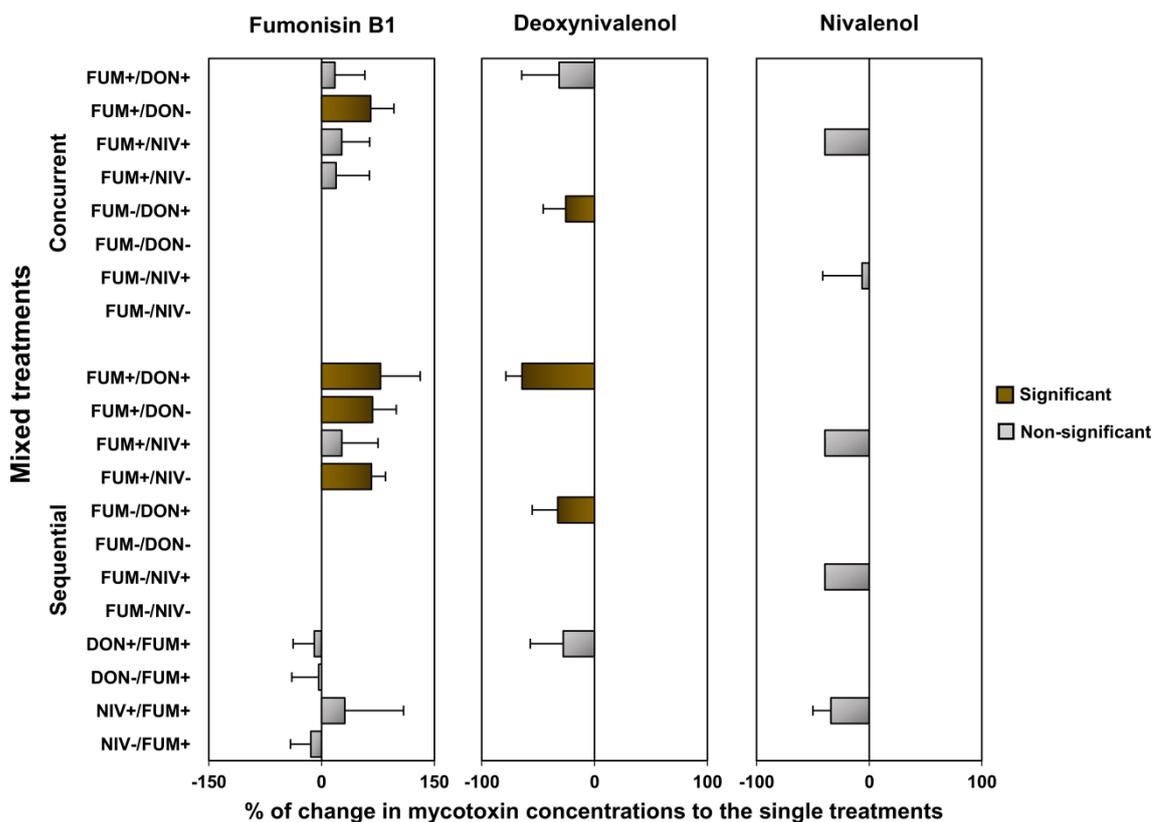
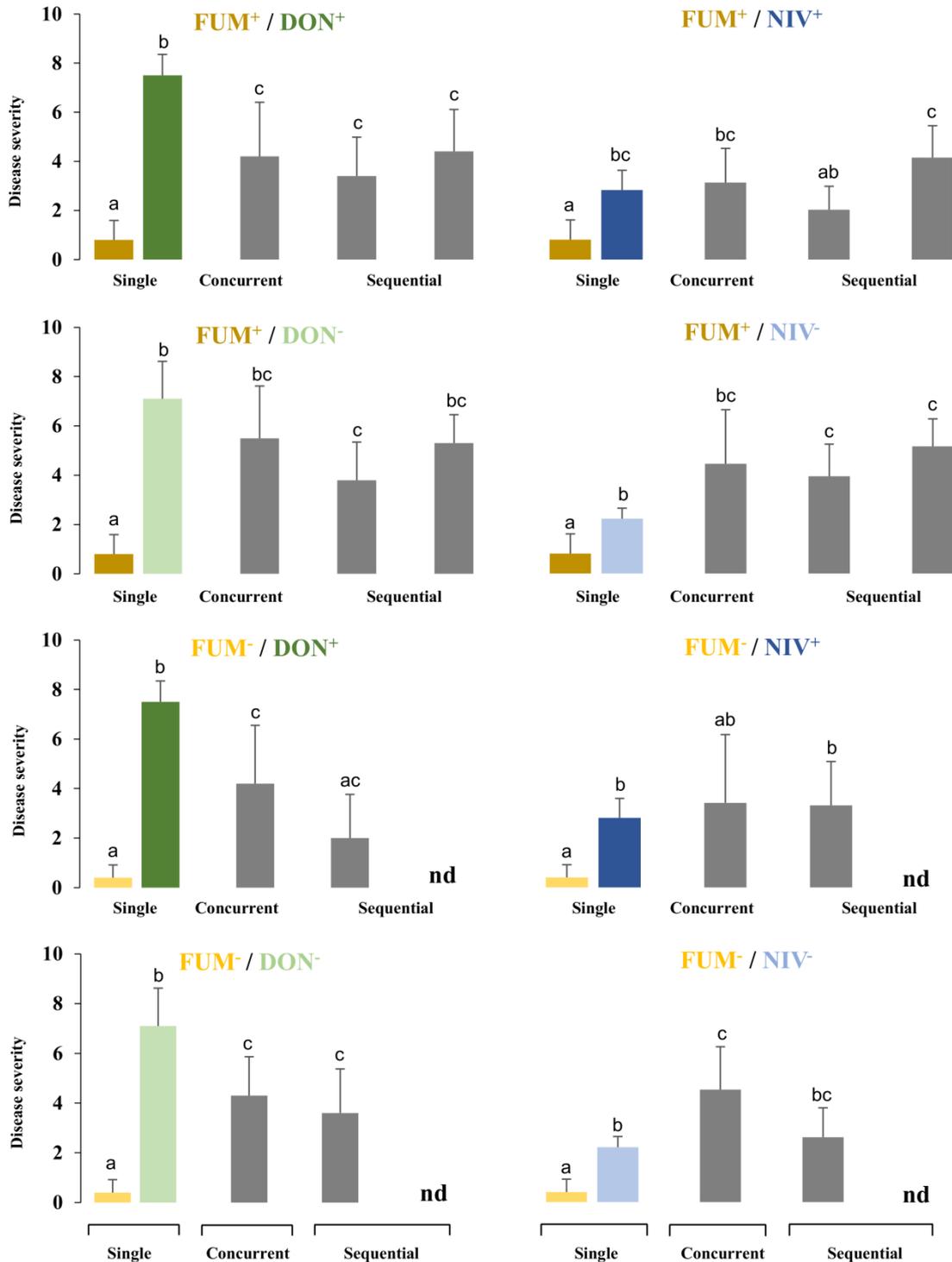
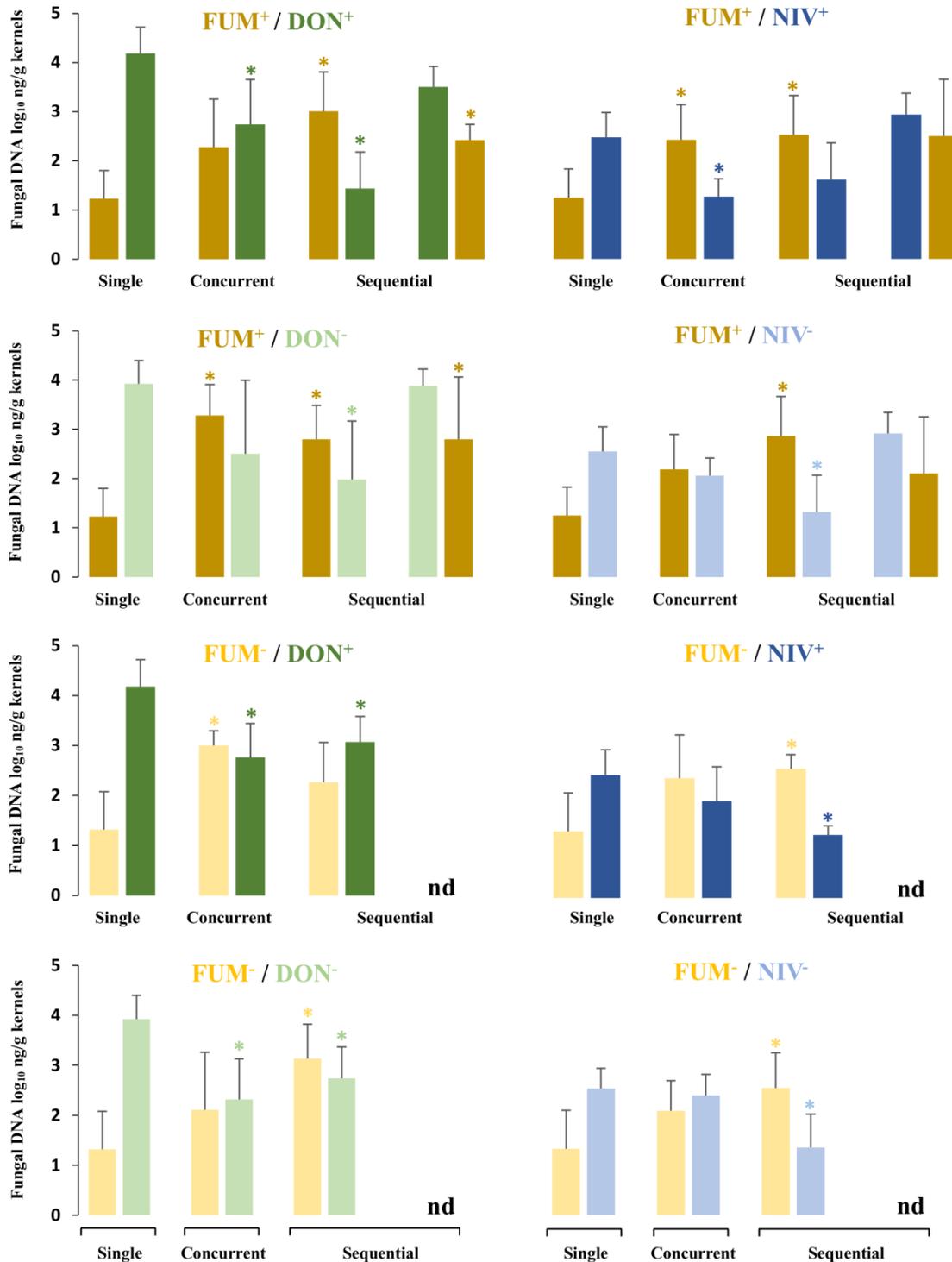


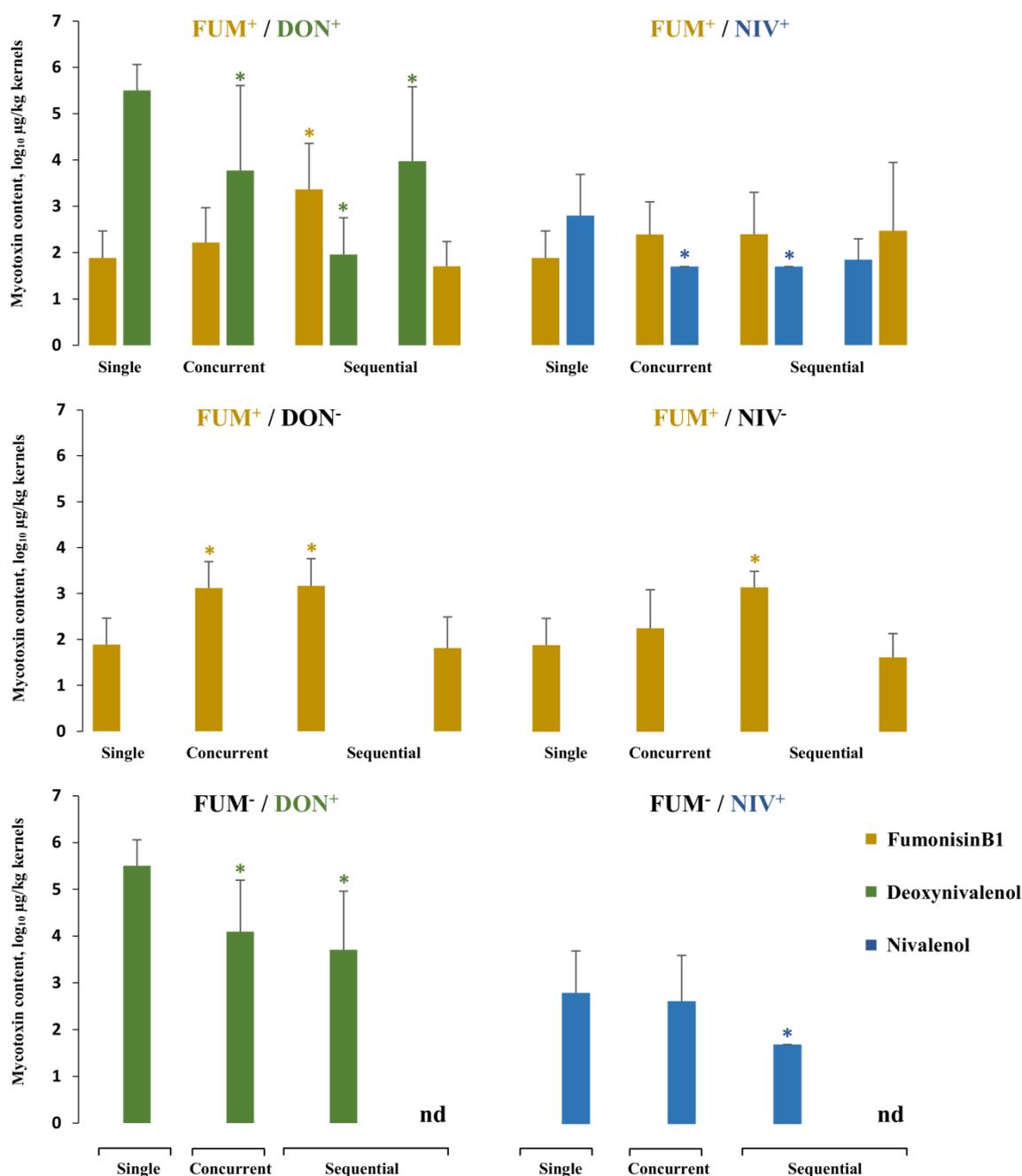
Figure 5. Comparison of mycotoxin content in maize ears co-infected with *F. graminearum* and *F. verticillioides* (mixed strains listed on the left side) to mycotoxin content inflicted by single-strain infection. FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenol chemotype), DON-: *F. graminearum* FG2311# 2899 (mutant of FG2311 with disrupted deoxynivalenol synthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. Brown columns indicates a significant difference ($p < 0.05$, Kruskal-Wallis ANOVA on ranks Dunn's test) between mixed and single inoculations. Concurrent: simultaneous inoculation of both *Fusarium* species at the same time point. Sequential: interval of 5 days between the first/second inoculations of *Fusarium* species appears in order.



S1: Disease severity on maize ears var. Gaspé Flint. y-axis represents disease index (0=healthy, 8= fully deteriorated). x-axis represents the treatments. Single = individual species inoculation, concurrent = simultaneous two species inoculation “one combination”, sequential = two species inoculation with 5 days intervals between primary and secondary inoculum”two combinations”, *F.verticillioides* then *F.graminearum* and vice versa in order. FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenol chemotype), DON-: *F. graminearum* FG2311# 2899 (mutant of FG2311 with disrupted deoxynivalenol synthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. nd = not detected. Different letters indicates significant differences (p < 0.05). Statistical analyses were conducted with PAST statistics’ software 3.04 (Hammer et al., 2001), Mann-Whitney pairwise test with Bonferroni corrected p values.



S2: Fungal DNA quantification by q-PCR in maize ears. Single = individual species inoculation, concurrent = simultaneous two species inoculation “one combination”, sequential = two species inoculation with 5 days intervals between primary and secondary inoculum “two combinations”, *F. verticillioides* then *F. graminearum* and vice versa in order. FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenol chemotype), DON-: *F. graminearum* FG2311# 2899 (mutant of FG2311 with disrupted deoxynivalenol synthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. nd = not detected. Asterisk indicates significant differences ($p < 0.05$) to the single inoculation treatments. Statistical analyses were conducted with PAST statistics⁷ software 3.04 (Hammer et al., 2001), Mann-Whitney pairwise test with Bonferroni corrected p values.



S3: Mycotoxins content in maize ears. Single = individual species inoculation, concurrent = simultaneous two species inoculation “one combination”, sequential = two species inoculation with 5 days intervals between primary and secondary inoculum”two combinations”, *F.verticillioides* then *F.graminearum* and vice versa in order. FUM+: *F. verticillioides* M-3125, FUM-: *Fusarium verticillioides* GfA2364 (mutant of M-3125 with disrupted fumonisin synthesis) DON+: *F. graminearum* FG2311 (deoxynivalenol chemotype), DON-: *F. graminearum* FG2311# 2899 (mutant of FG2311 with disrupted deoxynivalenol synthesis), NIV+: *F. graminearum* FG06 (nivalenol chemotype), NIV-: *F. graminearum* FG06#7 (mutant of FG06 with disrupted nivalenol synthesis). Values are means, error bars show standard deviations. nd = not detected. Asterisk indicates significant differences ($p \leq 0.05$) to the single inoculation treatments. Statistical analyses were conducted with PAST statistics’ software 3.04 (Hammer et al., 2001), Mann-Whitney pairwise test with Bonferroni corrected p values.

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Chapter 3: Volatiles emitted from maize ears simultaneously infected with two *Fusarium* species mirror the most competitive fungal pathogen

[Journal published article]

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Abstract

Along with barley and rice, maize provides staple food for more than half of the world population. Maize ears are regularly infected with fungal pathogens of the *Fusarium* genus, which, besides reducing yield, also taint grains with toxic metabolites. In an earlier work, we have shown that maize ears infection with single *Fusarium* strains was detectable through volatile sensing. In nature, infection most commonly occurs with more than a single fungal strain; hence we tested how the interactions of two strains would modulate volatile emission from infected ears. For this purpose, ears of a hybrid and a dwarf maize variety were simultaneously infected with different strains of *F. graminearum* and *F. verticillioides* and, the resulting volatile profiles were compared to

the ones of ears infected with single strains. Disease severity, fungal biomass and the concentration of an oxylipin 9-hydroxy octadecadienoic acid, a signaling molecule involved in plant defense, were monitored and correlated to volatile profiles.

Our results demonstrate that in simultaneous infections of hybrid and dwarf maize, the most competitive fungal strains had the largest influence on the volatile profile of infected ears. In both concurrent and single inoculations, volatile profiles reflected disease severity. Additionally, the data further indicate that dwarf maize and hybrid maize might emit common (i.e. sesquiterpenoids) and specific markers upon fungal infection. Overall this suggests that volatile profiles might be a good proxy for disease severity regardless of the fungal competition taking place in maize ears. With the appropriate sensitivity and reliability, volatile sensing thus appears as a promising tool for detecting fungal infection of maize ears under field conditions.

Keywords: maize, *Zea mays*, volatile organic compounds, sesquiterpenoids, *Fusarium* spp., fungal pathogens, oxylipins

Introduction

Maize fields cover about 180 million hectares worldwide and provide, along with wheat and rice, staple food for more than half of the world population (FAO, 1995). Maize cultivation suffers from numerous pathogens, which infect plant roots, stems, leaves and ears in the field. Some of the most devastating pathogens of maize belong to the *Fusarium* genus which is responsible for 10-30 % yield loss in major crops throughout the globe (Agrios, 2005). Maize ear infection is typically caused by a mixture of *Fusarium* species (Kedera et al., 1994, Doohan et al., 2003), the most common of which are *F. graminearum* Schwabe and *F. verticillioides* (Sacc.) Nirenberg (Vigier et al., 2001; Logrieco et al., 2002). Apart from causing yield losses, *Fusarium* species infecting maize produce mycotoxins potentially endangering the health of consumers and farm animals.

Controlling and detecting early infection of maize by *Fusarium* spp. is challenging. Disease symptoms may become visible at late stages of infection because the pathogen infect kernels through the rachis (Oldenburg and Ellner, 2015), or the infection may even proceed without visible symptoms (Bacon and Hinton, 1996). Serological and

molecular diagnostic techniques require sample destruction and are therefore not suitable for real-time monitoring (Nezhad, 2014). Volatile sensing has emerged as a promising alternative to detect disease in crops (Sankaran et al., 2010; Aksenov et al., 2013). The rationale for volatile sensing is that the volatile blend emitted by plants depends on their physiological status, which is affected by the presence of a pathogen. Comparison of volatile profiles of infected and non-infected plants might allow the identification of volatile biomarkers that can be used to monitor fungal infection in real time using non-invasive techniques.

Most volatiles emitted by plants and microbes are secondary metabolites with low molecular weight of a lipophilic nature and a high vapour pressure (Dudareva et al., 2006, Lemfack et al., 2014). To date nearly 2,000 volatiles have been described in plants (Knudsen et al., 2006; Dunkel et al., 2009, Schenkel et al., 2015), while a little more than 1,000 volatile compounds have been documented from bacteria and fungi (Lemfack et al., 2014; Schenkel et al., 2015). Most of these volatiles are terpenoids, phenylpropanoids/benzenoids, fatty acid and amino acid derivatives (Dudareva et al., 2004). Volatile metabolites mediate ecological interactions among plants, microbes and other organisms and may thus affect defense against pathogens and herbivores (Piesik et al., 2013, Peñuelas et al., 2014; Kanchiswamy et al., 2015; Schenkel et al., 2015). Technically, volatiles can also be considered as indicators for the physiological status of the plant (Baldwin, 2010; Wenke et al., 2010; Clavijo-McCormick et al., 2012). For example Jansen et al. (2009) showed that tomato plants infected with the fungal pathogen *Botrytis cinerea* released higher quantities of mono- and sesquiterpenes than their healthy counterparts. The alcohols 1-penten-3-ol and (3Z)-hexen-1-ol are induced in chickpea infected with the fungal pathogen *Ascochyta rabiei* (Cruz et al., 2012). We have similarly demonstrated that the emission of twenty two volatiles was regulated in maize ears infected with single strains of *Fusarium* spp. fungal pathogens (Becker et al., 2013, 2014). The most common biomarkers of *Fusarium* spp. infection were the sesquiterpenoids β -macrocarpene and β -bisabolene, however some other markers (octan-3-ol and β -farnesene) were strain specific (Becker et al., 2013, 2014).

Here, we extend our investigation to the volatile profiles of maize ears simultaneously and separately infected with *F. graminearum* and *F. verticillioides*, using strains that differ in their aggressiveness towards maize. We wanted to know if simultaneous infection would lead to a volatile profile which differed from single infections. For this purpose we concurrently inoculated maize ears with strains of *F. graminearum* and *F.*

verticillioides and compared their volatile profiles to the one of ears infested with single fungal strains. We also monitored fungal biomass and disease severity and overall interpret shifts in volatile profiles in light of competitive fungal interactions.

Material and Methods

Fungal species

Seven strains belong to *F. graminearum* and *F. verticillioides* were used (**Table 1**). Sporulation was achieved on Mung bean medium (Bai and Shaner, 1996; Becker et al., 2014). Spore density was determined using a Thoma chamber (0.0025 mm²) and adjusted to the desired concentrations in sterile water. Spore viability was checked on potato dextrose agar (PDA).

Plant material and cultivation

Two maize (*Zea mays* L.) varieties were employed here, the hybrid field variety Ronaldinio (KWS Saat AG, Einbeck, Germany) and the dwarf maize variety Gaspe Flint (collected in Quebec, Canada). Maize kernels were surface sterilized with 4% aqueous solution of sodium hypochlorite for 15 min and rinsed 3 times with sterile water. Kernels were planted into autoclaved soil (topsoil/sand; 2:1 v/v) filled in plastic pots. Seedlings were grown in a greenhouse (26 ± 4°C, 14h photoperiod) until full development of the maize ears and fertilized as required using mineral fertilizer Hakaphos[®] (COMPO Expert GmbH, Münster, Germany).

Fungal inoculation of maize ears

Hybrid and dwarf maize plants were infected at the main flowering stage either with a single strain or simultaneously with two *Fusarium* strains as a 50:50 mixture (**Table 2**). This time point corresponds to approximately 4 and 7 days after silking for the dwarf maize and hybrid maize, respectively. The concentration of inoculated spores was adjusted to approximately 10⁵ or 10⁶ spores/mL according to spores' viability and a volume of 0.5 mL (dwarf maize) or 1.0 mL (hybrid maize) inoculum were injected into the silk channel (**Table 2**). Mock inoculation with sterile water was used as a control. All treatments for fungal biomass quantification and volatile profiling were replicated on four plants (hybrid maize) and five plants (dwarf maize). Oxylipins were quantified

from hybrid maize using five ears (replicates) from control/uninfected plants, four to seven replicates for single inoculations with strains FG1, FG2, FV1, FV2; and seven replicates for each of the mixed inoculations FG1+FV1 or FG2+FV2.

Assessing disease severity and sampling of ears

Disease symptoms on infected maize ears were indexed 24 and 18 days post fungal inoculation in hybrid and dwarf maize varieties, respectively. The dehusked maize ears showing infection symptoms (i.e. fungal mycelium and/or rotting) were graded on an index scale from zero to eight as described earlier (Sherif et al., unpublished). Ear kernels were cut off and immediately collected for volatile profiling, fungal DNA quantification and oxylipin analysis as described in Becker et al. (2014).

Fungal biomass quantification

DNA was extracted from aliquots of 100 mg maize flour following a protocol of Brandfass and Karlovsky (2008). Fungal DNA was quantified in the samples (10-15 ng μL^{-1}) by qPCR using species specific primers for *F. graminearum* (Nicholson et al., 1998) and *F. verticillioides* (Mulè et al., 2004). DNA from control (uninfected) ears were also subjected to qPCR using the aforementioned primers to ascertain that there were not contaminated control plants.

Oxylipin analysis

Aliquots of freeze-dried maize material, corresponding to 2.0 g fresh weight, were extracted according to the protocol of Goebel et al. (2003) and methylated with trimethylsilyldiazomethane (2 M in hexane, Sigma-Aldrich, Taufkirchen, Germany). As an internal standard, (6Z,9Z,11E,13S)-13-hydroxy-6,9,11-octadecatrienoic acid was added. Hydroxyl fatty acids were purified on reverse phase-HPLC equipped with ET250/2 Nucleosil 120-5 C18 column (Macherey-Nagel, Dueren, Germany) as described in Goebel et al. (2003). Eluate fraction was collected between 8 and 13.5 min, evaporated to dryness and re-dissolved in 2 μL acetonitrile. After addition of 2 μL N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, Taufkirchen, Germany), analysis was carried out with an Agilent 6890 gas chromatograph equipped with a capillary DB-23 column (Agilent, Waldbronn, Germany, nominal diameter: 0.25 mm, length: 30 m, nominal film thickness: 0.25 μm) and coupled with an Agilent 5973 MS. Standard

curves were constructed by plotting ion intensities vs. molar amounts of known hydroxyl fatty acids.

Full volatile profiling

The samples of 2.0 g kernels were enclosed in 20 mL solid-phase microextraction (SPME) vials sealed air tight with a screw cap containing a silicon/polytetrafluoroethylene septum. Samples were extracted for 10min at 40°C using a 1.0 cm SPME (PDMS/DVB fiber) and, for the hybrid maize samples, volatile were profiled as described in Becker et al. (2014). For the dwarf maize variety, the temperature programming of the GC oven was modified to achieve a better separation of volatiles compared to Becker et al. (2014). Specifically the following parameters were used: 40 °C for 3 min, increasing at 1.5 °C min⁻¹ to 80 °C, followed by 80 °C min⁻¹ to 250 °C (7.21 min isothermic).

GC/MS output data was processed using two different approaches. TagFinder version 4.1 (Luedemann et al., 2008) was used for the dwarf maize data set with the following parameters; Timescale: 2, Low Mass: 40, High Mass: 400. Peakfinder tool; SmoothWidth Apex Finder: 1, Low Intensity Threshold: 20000 (non-Smooth Apex), Smooth Width +/- Apex Scan: 1 (non-Merge Peaks). Peak alignment; Time ScanWidth 4.0; Gliding Median Group Count 1; Min Fragment Intensity 50. Volatile profiles of hybrid maize were processed as described in Becker et al. (2014).

Volatiles were identified using Kovats retention indices, the NIST 2008 Mass Spectral library (version 2.0f), the ADAMS MS library (Adams, 2005), and authentic standards when available, specifically for: pentane, dimethyl sulfide, 3-methyl-butanal, 2-methyl-butanal, 3-hexene-1-ol, heptan-2-ol, octan-3-ol, 1-octen-3-ol, octan-3-one, α -selinene, β -selinene, β -bisabolene, β -macrocarpene.

Statistical analysis

Statistical analysis was performed using the statistics software PAST version 3.04 (Hammer et al. 2001) for the principal component analysis and cluster trees. Disease severity and fungal DNA (log transformed values) were compared among treatments with Tukey's pairwise test (PAST version 3.04). In addition, the nonparametric Kruskal-Wallis test performed in R, version 3.0.3 (R Development Core Team, 2008) was used for both maize varieties to identify volatile markers that significantly differed

among treatments (i.e. control plants, plants infected with one fungus, plants infected with two fungi).

Results

Two maize varieties, including a hybrid variety with wide commercial usage and a dwarf maize variety with short life cycle were selected for our experiments. Hybrid and dwarf maize were (i) infected with single strains of *F. graminearum* and *F. verticillioides*, (ii) simultaneously infected with different strains belonging to the two aforementioned species and (iii) uninfected (“mock-inoculated” with water). All strains used in this work are listed in **Table 1**, and specific combinations of maize varieties and strains are listed in **Table 2**. In short, hybrid maize was infected with either *F. graminearum* (FG) strain FG1, *F. verticillioides* (FV) strain FV1 and mixed strains FG1+FV1 or with FG2, FV2 and mixed strains FG2+FV2. By contrast dwarf maize was infected with strains FG3, FV3 and mixed strains FG3+FV3 or with strain FG4 and mixed strains FG4+FV3. Volatiles were profiled in all cases by SPME-GC/MS, submitted to statistics to identify infection biomarkers and highlight trends in the data.

Volatile profiles in mixed inoculations is governed by the most competitive fungal strain

Our first aim was to understand how competitive interactions between two *Fusarium* species affected the volatile profiles of hybrid and dwarf maize. For this purpose, principal component analysis (PCA) was performed on the volatile biomarkers of hybrid and dwarf maize, considering always four groups of samples made of (1) uninfected ears, (2) infected with *F. graminearum*, (3) infected with *F. verticillioides* and (4) infected with both species. Two different strain combinations were used for hybrid maize and two others for dwarf maize, resulting in four PCAs as shown in **Figure 1**. Depending on the cases, PCA could explain from 67 % - 82 % data variability in terms of volatile profiles (equivalent to the sum of the scatter plot scores for both axis/principal components PC1 and PC2).

In the case of hybrid maize, volatile profiles of single inoculations differed the most from uninfected ears for strains FG1 and FG2 (**Figure 1**), despite the fact that the latter strains accumulated comparable biomass to strains FV1 and FV2, respectively. In

simultaneous infections, the volatile profile of mixed inoculation FG1+FV1 was somehow comparable to the one of the single inoculation FV1, possibly reflecting the drop in biomass of FG1 combined with FV1 compared to single inoculation FG1. A comparable trend was observed for the concentration of the oxylipin 9-hydroxy octadecadienoic acid (9-HOD). Indeed its concentration in the simultaneous inoculation with FG1+FV1 was similar to the one of the single inoculation with FV1. The volatile profile of mixed inoculation FG2+FV2 differed from the one of single inoculations with the same strains, while fungal biomass and the concentration of 9-HOD remained unaffected (**Figure 1**).

In the case of dwarf maize, volatile profiles of single inoculations differed the most from uninfected ears for strains FV3 and FG4 (**Figure 1**) and, the highest biomass was reached by FG3 and FG4. In simultaneous infections, the volatile profile of mixed inoculation FG3+FV3 was half-way between the one of maize ears inoculated with single strains, and biomass accumulation was stimulated for FV3 and inhibited for FG3 compared to single inoculation with the same strain. The profile of mixed inoculation FG4+FV3 partially overlapped with the one of maize ears inoculated with single strains, which however displayed important data variability. In terms of biomass, FV3 was unaffected, however FG4 was inhibited compared to single inoculations.

Overall the data indicates that volatile profiles in mixed inoculations are governed by the most competitive fungal strain, and this does not correlate with their ability to produce a specific mycotoxin.

In both concurrent and single inoculations, volatile profiles reflect disease severity

In order to investigate a possible correlation between volatile profiles and disease severity, we applied cluster analysis to the volatile biomarkers of hybrid and dwarf maize and displayed the resulting analysis along with disease severity for each treatment and replicate. Results are shown in (**Figure 2**) for hybrid maize and dwarf maize. Considering clusters with boot-strap values larger than 60 %, two major clusters are visible for both maize varieties. For hybrid maize, one cluster includes all samples infected with FV1, either alone or with FG1 (FG1+FV1) (**Figure 2**, cluster I), while the other cluster includes all replicates infected with FG1 (**Figure 2**, cluster II). For dwarf maize, one cluster includes all replicates infected with FV3 alone, four of five replicates simultaneously infected with FG4+FV3 and one replicate infected with FG4 (**Figure 2**, cluster III), the other cluster includes four of five replicates infected with FG4 and one

replicate simultaneously infected with FG4+FV3 (**Figure 2**, cluster IV). Disease index depicted on the right side of the diagrams highlight that the clustering is dependent on disease severity for both hybrid and dwarf maize (**Figure 2**).

We further investigated the correlation between disease severity and the oxylipin 9-HOD (**Figure 1**). Disease severity and the concentrations of 9-HOD quantified in hybrid maize were significantly correlated ($p < 0.05$, two-tailed t test computed in PAST version 3.04 (Hammer et al. 2001)). Considering the overall data, $R^2 = 0.49$ ($p < 0.05$). Considering inoculations with single strains and uninfected controls, $R^2 = 0.67$ ($p < 0.05$). Considering mixed inoculations and uninfected controls, $R^2 = 0.23$ ($p < 0.05$).

In summary our results exemplify that the overall volatile profile of maize ears reflects disease severity regardless of the presence of one or more *Fusarium* species, and highlight that the correlation between disease severity and oxylipin concentrations (9-HOD) is higher in single inoculations compared to mixed inoculations.

Dwarf maize and hybrid maize share common and specific volatile infection markers

Volatile compound identification was achieved using Kovats retention indices, mass spectral libraries, and authentic standards when available. VOC markers which concentration significantly differed between healthy and infected plants included an alkane, a sulfur compound, alcohols, ketones and terpenoids and some unidentified compounds. From both maize varieties, twenty three volatile markers could be identified or tentatively identified, twelve from dwarf and fifteen from hybrid maize, and both varieties shared six common markers including; (+)-longifolene, β -farnesene, β -macrocarpene, trichodiene and two unidentified SQT (**Figure 3 and 4**). The pie chart in **Figure 3** illustrating the number of volatiles common and specific to both maize varieties includes unidentified volatiles in addition to the identified and tentatively identified ones listed in **Figure 4**. Differences in the volatile markers of both maize varieties could be ascribed to aldehydes, one alkane and a sulfur compound present in dwarf maize only whereas numerous sesquiterpenoids could solely be detected from hybrid maize (**Figure 4**).

Volatile profiles presented a quite important quantitative variability within replicates of the same treatment (independent ears infected with the same fungus) whereas a qualitative variability in volatile composition was observed upon infection of different *Fusarium* species. This can be seen in the heatmaps of **Figure 5** that have been colour

coded to represent the concentration of infection biomarkers in dwarf and hybrid maize. As an example of qualitative variability, in both maize varieties, the volatile trichodiene was only detected from *F. graminearum* but never from *F. verticillioides*. In dwarf maize, hexan-1-ol was induced by FV3 (FV3 alone, FG3+FV3, FG4+FV3) compared to single inoculations with FG3 and FG4 (**Figure 5**).

The heatmaps of **Figure 5** further illustrate to which extent co-inoculations with two strains modulate emission of volatiles compared to single strains inoculations. For example in dwarf maize, inoculation with *F. verticillioides* FV3 did hardly not induce sesquiterpenoids (i.e. (+)-longifolene, β -farnesene, α -muurolene, β -macrocarpene) whereas inoculation with *F. graminearum* FG4 did to a large extent. Simultaneous inoculation of dwarf maize ears with the two latter strains (FG4+FV3) lead to an intermediate situation where sesquiterpenoids were strongly induced in one ear/replicate only (similarly to FG4) but they were hardly not induced in the remaining four ears/replicates (similarly to FV3).

Overall the data highlight that hybrid and dwarf maize share common volatile markers mostly composed of sesquiterpenoids while they might differ in terms of volatiles belonging to other chemical classes.

Discussion

Previously we demonstrated that maize ears infected with single *Fusarium* strains (i.e. *F. graminearum*, *F. subglutinans*, *F. verticillioides*) emitted specific volatiles, or disease biomarkers, which revealed the presence of the fungus even at a very early infection stages (Becker et al., 2014). If some volatiles (i.e. β -macrocarpene) seemed induced by all *Fusarium* species, others were species or strain specific (i.e. octan-3-ol was only induced by *F. verticillioides* and β -farnesene by *F. verticillioides* and *F. subglutinans*). Practically, these volatile biomarkers could potentially serve to identify infected ears and even to specifically identify the infecting *Fusarium* species. However, because in the field infection generally occurs with more than one *Fusarium* strain/species, it is essential to understand how competitive interactions influence the volatile blend of infected maize ears. Specifically our aim here was to understand how interaction between the two widely occurring species *F. graminearum* and *F. verticillioides* modulated volatiles emitted by maize ears.

Our data demonstrate that the volatile profile of maize ears infected with two *Fusarium* strains was dependant on the most competitive strain (**Figure 1**). Interspecific fungal interactions are driven by either interference- or exploitation-type competition (Peay et al., 2008). Interference competition in fungi involves direct interactions such as overgrowth or chemical competition whereas exploitation competition involves indirect negative effects resulting for example from the use of a common resource (Wicklow, 1981; Chatterjee et al., 2016). What strategy *Fusarium* strains use to compete among each other is unclear, and it might well be a mixture of chemical and exploitation competition. Mycotoxins such as tricothecenes and fumonisins have for long been candidates for chemical competition, however, we have recently demonstrated that they were not involved in such competition on maize ears (Sherif et al., unpublished).

Our data further demonstrate that volatile profiles of maize ears reflect disease severity regardless of the presence of one or more fungal pathogens. This conclusion was reached based on cluster analysis of the volatile profiles of infected maize ears (**Figure 2**), however, more powerful statistical models might be able to distinguish among ears infected with one or two pathogens. For example Thorn et al. (2011) analysed the volatile profiles of eleven bacterial strains belonging to six species and, using a combination of similarity matrices, cluster analysis and multidimensional scaling could successfully distinguish among strains belonging to the same species. From a practical perspective, however, cluster analysis on the maize volatile profile presented here provides useful information that can be used as a proxy to estimate disease severity and hence to potentially treat or sort infected kernels.

Detecting volatiles in real time under field conditions nevertheless remains a challenge essentially due to sensitivity issues. Sesquiterpenes are indeed released by plants in the range of ten to thousands of $\text{ng g}^{-1}_{\text{DW}} \text{h}^{-1}$ (Duhl et al., 2008) which is far below the detection limit of most portable instruments, and this emission highly fluctuates as a function of the plant's circadian clock, but also further biotic and abiotic factors (Duhl et al., 2008; Loreto and Schnitzler 2010). The latest generation of proton transfer-mass spectrometers (PTR-MS) might be sensitive enough for real time detection of these volatile biomarkers, even though their cumbersome size and high price remain a hindrance for the agro-business sector. One cheaper alternative might be provided by laser based photoacoustic systems as described in a recent review (Harren and Cristescu, 2013).

Overall using volatile sensing in the field to detect and possibly treat infected maize

ears will require highly sensitive and affordable detection methods that operate reliably under variable weather conditions.

Our data also indicates that hybrid and dwarf maize share common volatile markers mostly composed of sesquiterpenoids while they differ in terms of other chemical classes of volatiles. These differences should be interpreted cautiously since we did not use the same fungal strains to infect hybrid and dwarf maize. Part of the differences observed among the two maize varieties might be attributed to the ability of either different *Fusarium* species/strains or of different maize cultivars to emit different volatiles. Variability in volatile profiles was indeed demonstrated for maize cultivars (Oluwafemi et al., 2012) and also for *Fusarium* species (Eifler et al., 2011). Nevertheless, the fact that a core volatile profile of sesquiterpenoids (β -macrocarpene, (+)-longifolene, β -farnesene, and trichodiene) was detected from both maize varieties is consistent with their ecological function. Indeed these volatiles can serve as building blocks for zealexins, metabolites involved in plant defence against fungal pathogens and insect pests (Huffaker et al., 2011). Interestingly in dwarf maize β -macrocarpene was not induced to detectable levels by single inoculations with *F. graminearum* FG3 and *F. verticillioides* FV3 nor by co-inoculation with the same strains (**Figure 5**). This suggests that similarly to what has been observed in maize root and stems and leaves (Köllner et al., 2008; Huffaker et al., 2011), β -macrocarpene might have been fully transformed into non-volatile zealexins.

A marked difference among *F. graminearum* strains was also detected for trichodiene, the volatile precursor of trichothecene toxins such as *nivalenol* and *deoxynivalenol* (Desjardin, 2006). In our study trichodiene was detectable from *F. graminearum* strains FG1, FG2 and FG4 but not from FG3 (**Figure 5**). These differences are supported by earlier quantifications of trichothecenes by the same strains. Indeed infection with FG3 results in the lowest trichothecene accumulation compared to the other strains [FG1 and FG2: > 50 mg/kg; FG3 <3.0 mg/kg; FG4: > 400 mg/kg, Becker et al. (2014) and Sherif et al. (unpublished)]. This observation suggests that not only the presence/absence of specific infection markers but also their concentrations should be taken into account to estimate the infection level of maize ears.

Maize has developed an array of defense metabolites (phytoalexins) in response to fungal infections and attacks by herbivores. Zealexins, acidic sesquiterpenoid phytoalexins, accumulate to very high levels at infection sites of fungi and stem herbivores (Huffaker et al. 2011). Oxylipins, which result from the peroxidation of fatty

acids by lipoxygenases (LOXs), are similarly involved in defense against pests and pathogens (Christensen et al., 2015) and the oxylipin 9-HOD (**Figure 1**) has been suggested as a biomarker for aflatoxin-resistance in maize lines (Wilson et al., 2001). The peroxidation of α -linolenic acid by 13-LOX yields 12-oxo-phytodienoic acid (12-OPDA) and downstream jasmonates, which includes the plant defense hormone jasmonic acid. By contrast, the peroxidation of α -linolenic and linoleic acid by 9-LOX lead to 10-oxo-11-phytodienoic acid (10-OPDA) and 10-oxo-11-phytoenoic acid (10-OPEA), which are involved in direct plant defense. Indeed, unlike jasmonates, 10-OPDA and 10-OPEA directly act as phytoalexins and display a significant phytotoxicity which highlights their involvement in localized cell death (Christensen et al, 2015).

In line with the latter studies, we observed earlier that several oxylipins and zealexins were induced upon infection of maize ears with single *Fusarium* strains and that disease severity correlated to oxylipins induction levels (Becker et al, 2014). The data presented with mixed inoculations in the current paper similarly indicates that disease severity correlates with the oxylipin 9-HOD in single and mixed inoculations (**Figure 1**). Linear correlation was however almost three times higher in single inoculations ($R^2=0.67$, $p<0.05$) compared to mixed inoculations ($R^2=0.23$) suggesting that fungal competition might somehow compromise plant response. Interestingly compromised plant response in terms of repressed transcriptional factors (9- and 13-LOX) and reduced concentrations of zealexins were documented in maize ears and stalks infected by *F. verticillioides* under elevated CO₂ concentration. Overall increased CO₂ lead to increased susceptibility and repressed levels of zealexins (Vaughan et al., 2014). This highlights that more than one biotic or abiotic factor (CO₂, competition) might compromise plant defense and begs for further studies to disentangle this complex interactions network.

Overall the data presented in this manuscript suggest that volatile profiles might be a good proxy for disease severity regardless of the fungal competition taking place in maize ears. With the appropriate sensitivity and reliability, volatile sensing thus appears as a promising tool for detecting fungal infection of maize ears under field conditions.

Author Contributions

PK, MS, EMB and RS designed the work. MS, EMB and RS analyzed volatile profiles,

disease severity and fungal biomass and interpreted the work with PK. IF, CH analyzed the oxylipins and interpreted the data together with EMB, PK, RS. MS and RS drafted the manuscript, which was critically revised by all co-authors.

Conflict of Interest

EMB, RS and PK declare having applied for a patent in 2013 describing the use of the volatile markers for identifying *Fusarium* infection in maize (patent application WO2013135889 A1). The other author(s) declare that they have no competing interests.

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Tables

Table 1: Fungal strains of *F. graminearum* and *F. verticillioides* used in maize ear infections

Fungal strain	Name	Abbreviation	Source
<i>F. graminearum</i>	Fg71 ^a	FG1	T. Miedaner, University of Hohenheim, Germany
<i>F. graminearum</i>	Fg210.1 wt ^b	FG2	Phytopathological strain collection, Division of Plant Pathology and Crop Protection, Georg-August-University Göttingen, Germany
<i>F. graminearum</i>	FG 06 ^a	FG3	Wilhelm Schäfer, Hamburg University, Hamburg, Germany
<i>F. graminearum</i>	FG 2311 ^b	FG4	
<i>F. verticillioides</i>	Fv Ita 1 ^c	FV1	A. Prodi, University of Bologna, Italy
<i>F. verticillioides</i>	FM8114 ^c	FV2	Fusarium Research Centre, Pennsylvania State University, USA
<i>F. verticillioides</i>	M-3125 ^c	FV3	Robert Proctor, National Center for Agricultural Utilization Research/U. S. Department of Agriculture Peoria, Illinois, USA

^a Nivalenol-producing fungal strain, (NIV chemotype).

^b Deoxynivalenol-producing fungal strain, (DON chemotype).

^c Fumonisin-producing fungal strain.

Table 2: Infection of maize plants with *Fusarium* species

Treatment	Fungal strain	Spore concentration	Volume	Host plant
Single	FG1	10^5 mL^{-1}	1.00 mL	Hybrid maize
	FG2	10^5 mL^{-1}	1.00 mL	Hybrid maize
	FG3	10^5 mL^{-1}	0.50 mL	Dwarf maize
	FG4	10^5 mL^{-1}	0.50 mL	Dwarf maize
	FV1	10^6 mL^{-1}	1.00 mL	Hybrid maize
	FV2	10^6 mL^{-1}	1.00 mL	Hybrid maize
	FV3	10^5 mL^{-1}	0.50 mL	Dwarf maize
Mix	FG1+FV1	10^5 mL^{-1} (50:50)	1.00 mL	Hybrid maize
	FG2+FV2	10^5 mL^{-1} (50:50)	1.00 mL	Hybrid maize
	FG3+FV3	10^5 mL^{-1} (50:50)	0.50 mL	Dwarf maize
	FG4+FV3	10^5 mL^{-1} (50:50)	0.50 mL	Dwarf maize

Figures

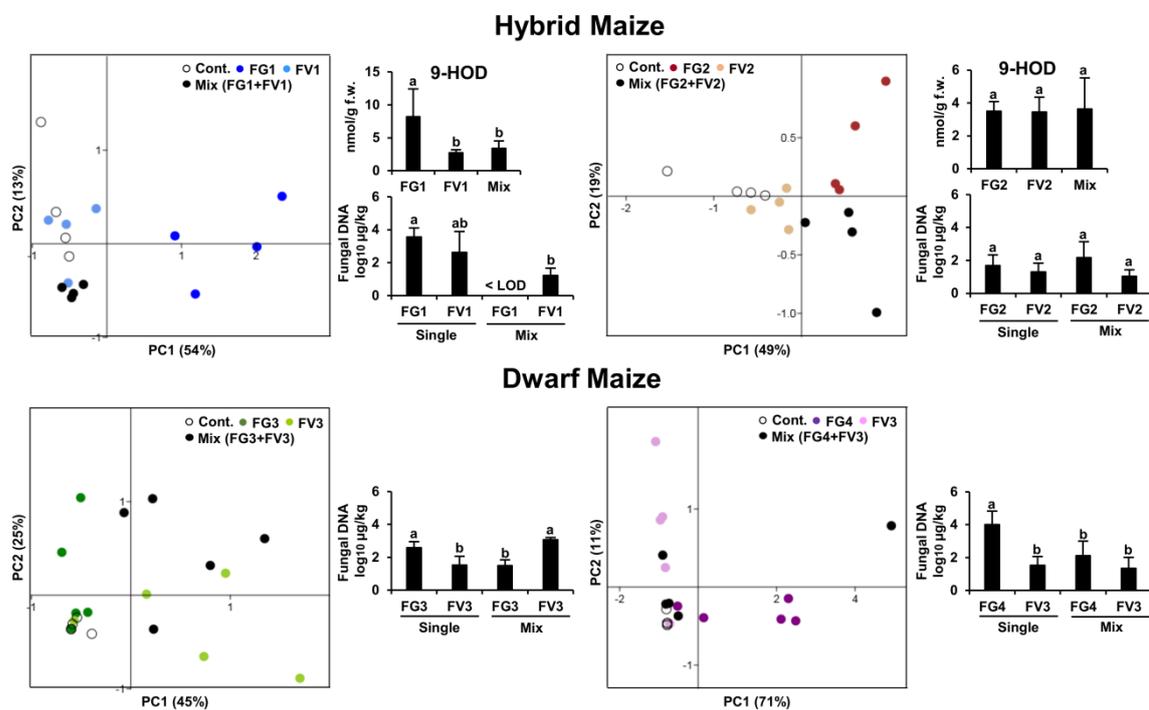


Figure 1. Principle Component Analysis (PCA) of the volatile infection biomarkers in infected and uninfected maize ears for hybrid and dwarf maize. Each dot represents one replicate from each treatment. 9-HOD: (10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid. F.W. = fresh weight. Different letters indicate statistical differences ($p < 0.05$) Tukey's pairwise test.

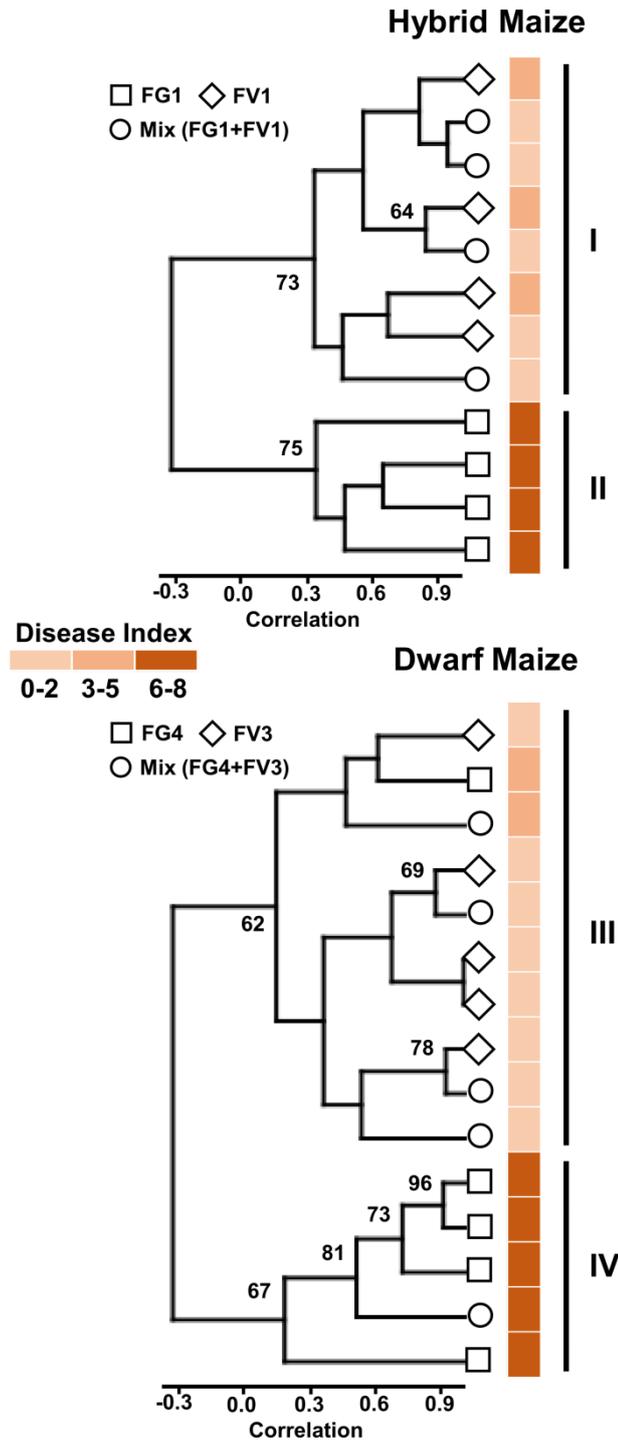


Figure 2. Cluster tree based on volatile infection biomarkers of maize ears infected with one or two *Fusarium* species. Bootstrap values > 60% are indicated (N=10,000 bootstraps) on the tree along with disease index (colour coded) for each individual samples (maize ears).

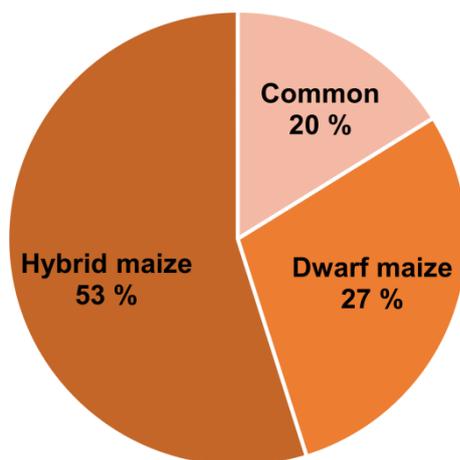


Figure 3. Pie chart representing the percentage of common and specific infection biomarkers to hybrid and dwarf maize. The numbers take into account identified, tentatively identified and unidentified volatiles.

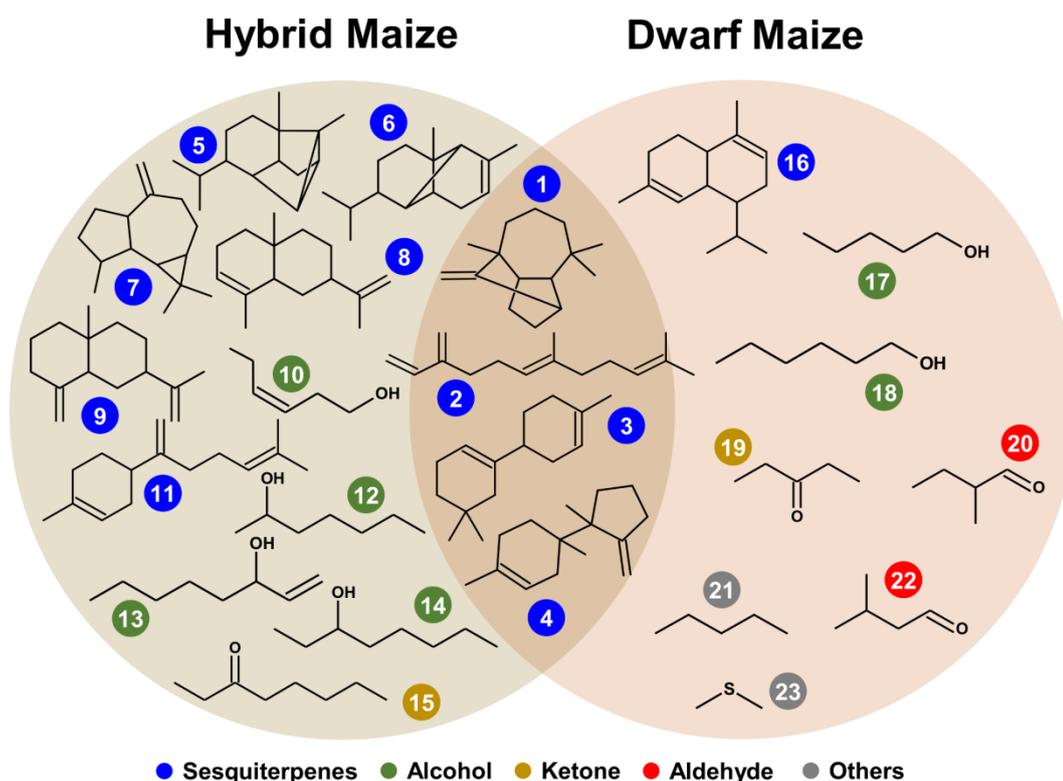


Figure 4. Structures of common and specific infection biomarkers in hybrid and dwarf maize. Colour coder represent chemical classes: (1) (+)-longifolene, (2) β -farnesene, (3) β -macrocarpene, (4) trichodiene, (5) (+)-cycloisositivene, (6) α -ylangene, (7) (+)-aromadendrene, (8) α -selinene, (9) β -selinene, (10) 3-hexen-1-ol, (11) β -bisabolene, (12) heptan-2-ol, (13) 1-octen-3-ol, (14) octan-3-ol, (15) octan-3-one, (16) α -muurolene, (17) pentan-1-ol, (18) hexan-1-ol, (19) pentan-3-one, (20) 2-methylbutanal, (21) pentane, (22) 3-methylbutanal, (23) dimethyl sulfide.

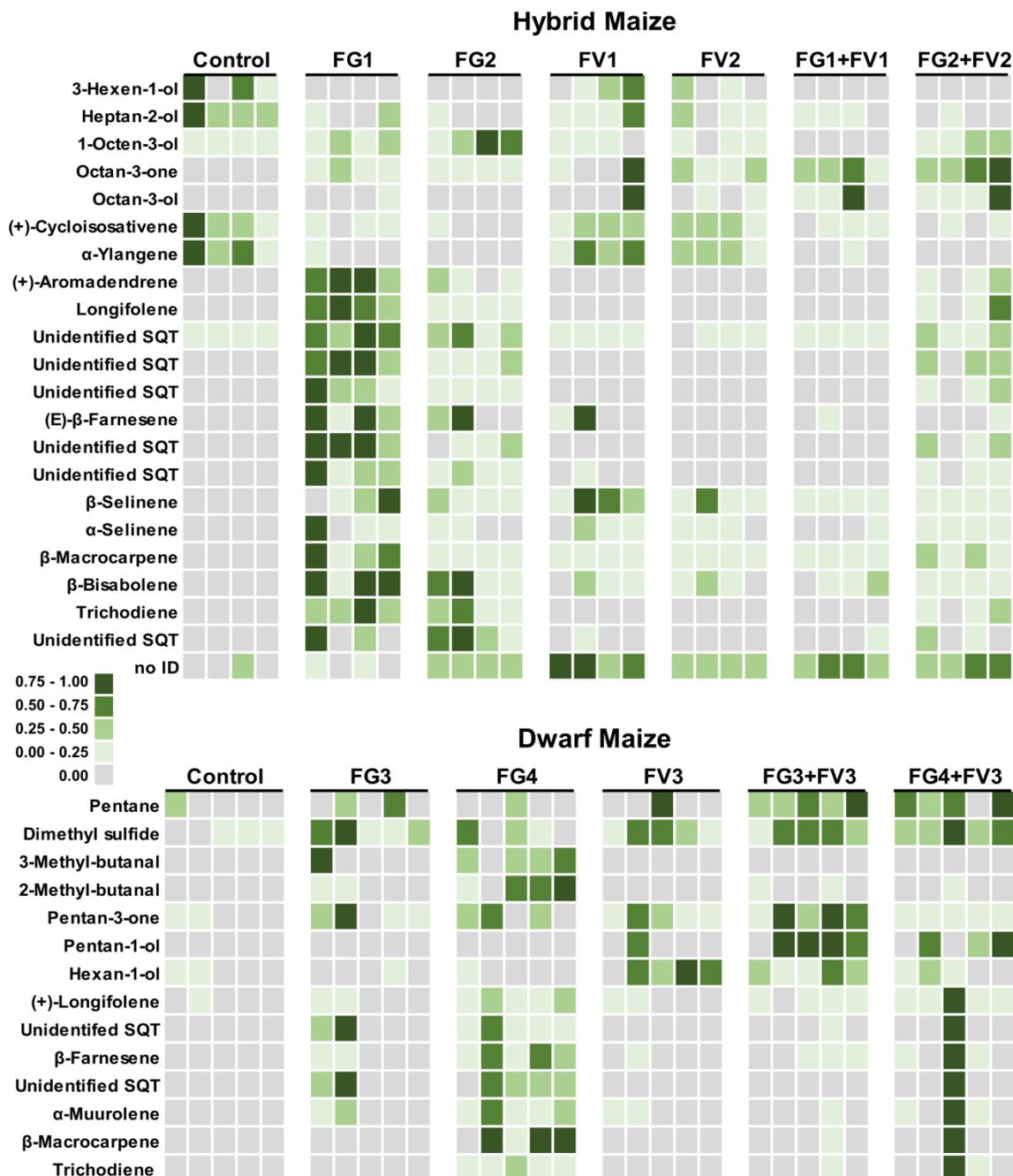


Figure 5. Heatmap representing volatiles that are regulated in dwarf and hybrid maize upon infection with *Fusarium*. Squares correspond to the concentration of single volatiles emitted from independent ears for each treatment - dwarf maize, $n = 5$ replicates per treatment; hybrid maize, $n = 4$ replicates per treatment. Squares have been colour coded to represent volatile concentrations (normalized from zero to one). The heatmap illustrates that volatiles are differentially regulated by single inoculations or co-inoculations of *Fusarium* strains. Treatments: Control = uninfected ears; FV = *F. verticillioides*; FG = *F. graminearum*. Refer to Table 1 for details about strain numbers. For hybrid maize, part of the data (control and single inoculations with FG1 & 2 and FV1 & 2) has already been described in Becker et al, 2014. The data is shown here for consistency with dwarf maize and for allowing the comparison to co-inoculations.

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Chapter 4: Role of trichothecenes and fumonisins in interaction between *Fusarium graminearum* and *F. verticillioides* *in vitro*

Abstract

Trichothecenes and fumonisins are mycotoxins produced by *F. graminearum* and *F. verticillioides*, respectively. In plants they might also act as phytotoxins or as metabolites involved in interference/competition for microbes colonizing the same niche. Here a bioassay in synthetic solid medium was developed to study the interaction between *F. graminearum* and *F. verticillioides*, two species that are commonly encountered in maize ears. Specifically, mycotoxin producing strains (*F. verticillioides*, *F. graminearum* deoxynivalenol chemotype and *F. graminearum* nivalenol chemotype) and their non-mycotoxin producing mutants were tested in confrontational assays and their respective growth was evaluated. The results showed that the growth of *F. graminearum* strains (wild type and mutants) was generally faster and resulted in denser mycelia than *F. verticillioides* strains (wild type and mutants). Although fumonisins produced by *F. verticillioides* have been reported to have antimicrobial properties, this was not apparent from the bioassays. One explanation for this could be that the concentration of fumonisins produced by *F. verticillioides* was below the one that would affect the growth of *F. graminearum*. Quantifying the concentrations of toxins in the assays and determining the inhibitory concentrations with synthetic toxins might shed more light on the possible interaction mechanisms.

Keywords: *Fusarium graminearum*, *Fusarium verticillioides*, *in vitro* interaction, fumonisins, trichothecenes, antagonism

Introduction

Microbial interaction types vary from neutralism, mutualism to antagonism between microorganisms, which inhabit same ecological niche and eventually affect the structure of the microbial community (Wicklow, 1981; Pan and May, 2009). Nutrients are basic

resource for which, microbes compete each other's. Microbes employ their biological machinery to produce antibiotic compounds and other secondary metabolites inside the environment for better growth (Wicklow, 1981; Janisiewicz et al., 2000; Xu et al., 2007; Jonkers et al., 2012).

In addition to their role in plant pathogenesis, mycotoxins were proposed as a biochemical component that might play an important ecological role within the microbial community (Duffy et al., 2004; Karlovsky, 2008). Several studies addressed mycotoxin production in context of microbial interactions and its competitive effect among microorganisms *in vitro*; such as aflatoxins (Cuero et al., 1988) zearalenone (Cuero et al., 1988; Utermark and Karlovsky, 2007), trichothecene type A T-2 toxin (Ramakrishna et al., 1996), fusaric acid (Notz et al., 2002; Bacon et al., 2004), and gliotoxin (Coleman et al., 2011).

Trichothecenes B (Deoxynivalenol and 3-acetyldeoxynivalenol) produced by *F. graminearum* are known as potent phytotoxins (Wang and Miller, 1988). Similarly, fumonisins produced by *F. verticillioides* is known as a phytotoxic compound at low concentrations (Lamprecht et al., 1994). Moreover, fumonisin B1 showed an inhibition effect on yeast (*Saccharomyces cerevisiae*) (Wu et al., 1995), filamentous fungi (Keyser et al., 1999), but not against several gram-positive and gram-negative bacterial species (Becker et al., 1997).

Interaction between *F. graminearum* (trichothecenes-producing, trichothecenes non-producing) strains and *F. verticillioides* (fumonisin-producing, fumonisin non-producing) strains was performed through dual culture technique on synthetic media to evaluate the effect of trichothecenes and/or fumonisin produced by one species on another. In this confrontation assays, mycelial growth of both species was monitored and evaluated.

Materials and Methods

Inoculums of wild type mycotoxigenic fungi; *F. graminearum* deoxynivalenol chemotype (DON+), *F. graminearum* nivalenol chemotype (NIV+), *F. verticillioides* producing fumonisins (FUM+) and their mycotoxins-deficient mutants; *F. graminearum* deoxynivalenol-deficient (DON-), *F. graminearum* nivalenol-deficient (NIV-), *F. verticillioides* FUM-deficient (FUM-) (**Table 1**), were grown separately in liquid mung

bean medium (Bai and Shaner, 1996) as modified by Becker et al. (2014) for better sporulation. Fungal cultures were filtered through sterilized cotton wool and centrifuged at 6000 g for 5 min. The supernatant was removed and precipitated spores were resuspended in sterilized tap water. Fresh harvested spores were counted with Thoma chamber (0.0025 mm²) and were adjusted for a final concentration of 10⁵ spores per 1 mL sterilized water.

Two synthetic media were used in this assay, potato dextrose agar (PDA, 4 g potato extract, 20 g glucose, 15 g agar, per Liter, pH. 5.7) and bio-maize malt agar (BMM; 25 g maize-kernels for liquid extract, 8 g malt extract, 20 g agar, per Liter, pH. 6.4). Petri dishes (90 mm) were filled with 20 mL of medium, then medium plates were inoculated either with single *Fusarium* species/strain (Single cultures; FUM+, FUM-, DON+, DON-, NIV+, NIV-) or with two *Fusarium* species/strains (confronted cultures; FUM+/DON+, FUM+/DON-, FUM+/NIV+, FUM+/NIV-, FUM-/DON+, FUM-/DON-, FUM-/NIV+, FUM-/NIV-). In single cultures, 50 µl of spore suspension (10⁵ mL⁻¹) was placed at the center of the agar plates, and 50 µl of sterilized water (spore-free) was used as negative control. In confronted cultures, 50 µl spore suspension (10⁵ mL⁻¹) of each of two fungal species/strains were placed 2.5 cm apart from each other on the medium in Petri plates (Rodriguez-Estrada et al., 2011). Single and confronted cultures were performed in 5 replicates per treatment. Petri plates of single and confronted cultures were sealed with Parafilm®M and incubated for 10 days at 25 °C in darkness.

Results and discussion

The confrontation assays with either mycotoxin-producing or non-producing strains of *F. graminearum* and *F. verticillioides* were tested on PDA and BMM media *in vitro*. In single cultures on PDA medium, the colonies of *F. graminearum* strains (wild type and mutants) and *F. verticillioides* strains (wild type and mutants) expanded in a circle shape on the medium. Growth of *F. verticillioides* strains (wild type and mutants) was generally slower than *F. graminearum* strains (wild type and mutants) in both single and dual cultures. After 10 days of plates' inoculation with *Fusarium*, single cultures of *F. graminearum* strains (wild type and mutants) were fully covered the plate with dense fungal mycelia compared to single cultures of *F. verticillioides* strains (wild type and mutant) which loosely filled the plates (**Figure 1**).

In all confronted cultures on PDA medium with eight different combinations of *F. graminearum* strains (wild type and mutants) and *F. verticillioides* strains (wild type and mutants), *F. graminearum* mycelia grew fast and reached *F. verticillioides*' colonies earlier and occupied all empty surrounded spaces. This is why, after 10 days *F. graminearum* colonies in all confronted cultures were shaped as crescents with colored pigmentation (**Figure 1**).

Moreover, confronted cultures with all different *Fusarium* species/strains combinations did not show obvious inhibition zones or clear pattern of mycelium imbrication between two species/strains of any *Fusarium* species/strains combinations (**Figure 1**). These results led to the conclusion that the growth of *Fusarium* species/strains inside the plates was only dependent on free and available medium substrate.

Same observations of interaction between *Fusarium* species/strains were recorded from confrontation assay on BMM medium as well.

The ability to produce deoxynivalenol, nivalenol and fumonisins by wild type *Fusarium* species/strains did not visually affect the growth of any encountered fungal species/strain *in vitro*. Keyser et al., (1999) reported that fumonisin B1 (FB1) showed a chemical antagonism against several fungal species including *F. graminearum* using agar-diffusion method on PDA plates. Remarkably, higher concentrations (≥ 5 mM) of FB1 were required to display the antifungal effect against *Fusarium* species and *Aspergillus flavus* in particular, comparing with less concentrations (0.5 – 5 mM) used against other genera (Keyser et al., 1999). This can explain the result of neutral interaction between *F. verticillioides* and *F. graminearum* in our visual observations, where the first species might produce a tiny amount of fumonisins in the growth medium inside the plates. Further quantification of the toxins and determining the inhibitory concentrations with synthetic toxins in the assays are required to shed more light on the possible interaction mechanisms.

Tables**Table 1. Fungal strains of *F. graminearum* and *F. verticillioides***

Strain	Code	Abbreviation	Source
<i>F. graminearum</i> DON	FG 2311 WT	DON+	
<i>F. graminearum</i> DON-deficient ^a	FG 2311#2899	DON-	Wilhelm Schäfer, Hamburg University, Hamburg, Germany
<i>F. graminearum</i> NIV	FG 06 WT	NIV+	
<i>F. graminearum</i> NIV-deficient ^b	FG 06#7	NIV-	
<i>F. verticillioides</i> WT	M-3125	FUM+	Robert Proctor, National Center for Agricultural Utilization Research/ U. S. Department of Agriculture Peoria, Illinois, USA
<i>F. verticillioides</i> FUM-deficient ^c	GfA2364	FUM-	

^a Mutant strain of FG 2311 with disrupted *Tri5* gene (Maier et al., 2006)

^b Mutant strain of FG 06 with disrupted *Tri5* gene (Maier et al., 2006)

^c Mutant strain of M-3125 with disrupted *FUM1* gene (Proctor et al., 1999)

Figures

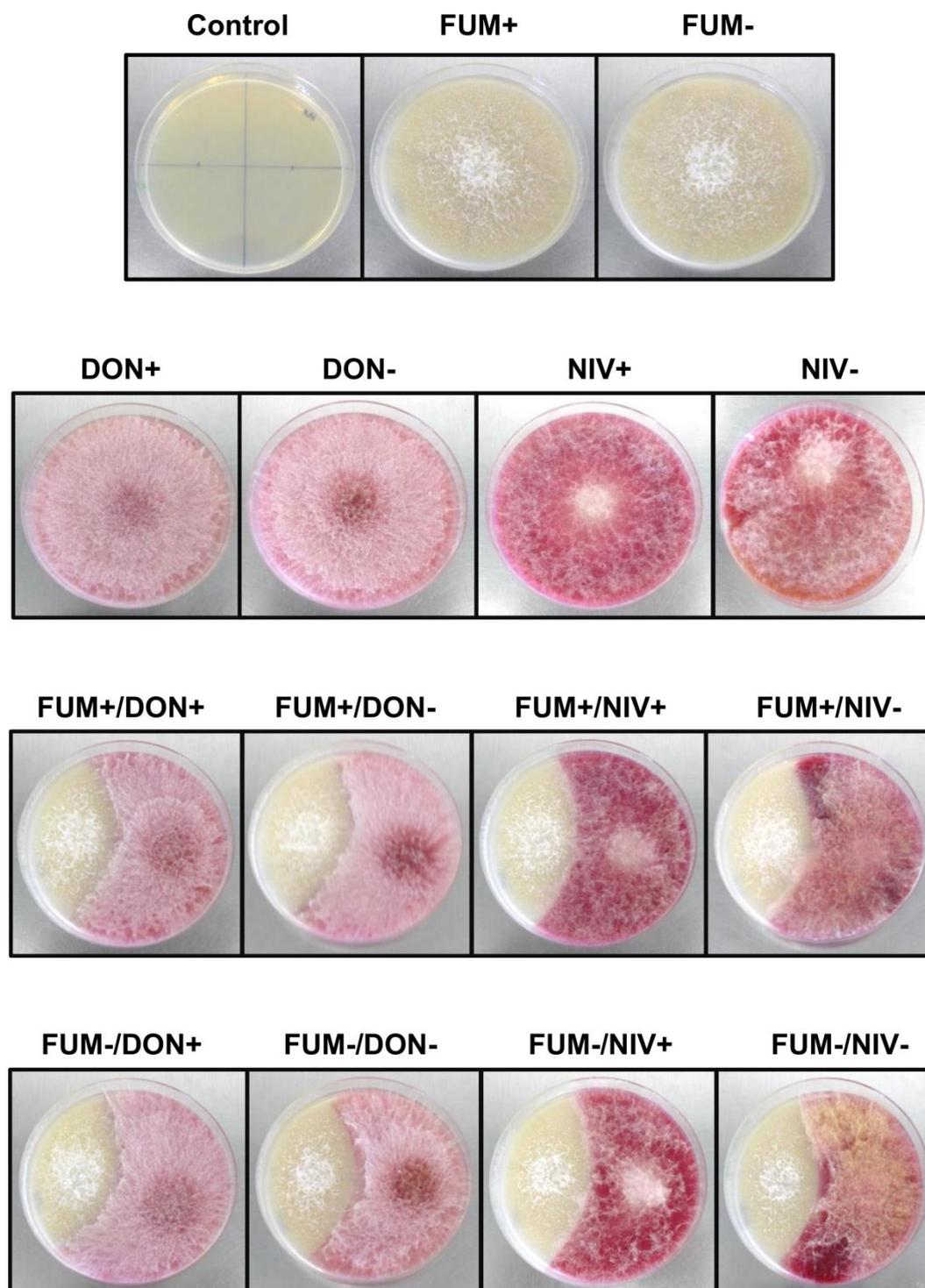


Figure 1. Growth of *F. graminearum* and *F. verticillioides* strains in single and dual cultures on PDA medium, 10 days post inoculation. FUM+: *F. verticillioides* wild type, FUM-: *F. verticillioides* fumonisins-deficient, DON+: *F. graminearum* deoxynivalenol chemotype, DON-: *F. graminearum* deoxynivalenol-deficient, NIV+: *F. graminearum* nivalenol chemotype, NIV-: *F. graminearum* nivalenol-deficient.

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Chapter 5: General discussion

Plant diseases are considered as a detrimental factor in the sectors of crop production and food processing. Crop loss due to microbial infestation is not only the loss in quantity and quality of the yield, but the loss extends to the food deterioration with microbial toxins, that are in many cases carcinogenic to human and animals, this is the case with secondary metabolites produced by fungi known as mycotoxins. Maize crops over the globe are often infected with several mycotoxigenic *Fusarium* spp. at same time, increasing the risks related to multiple mycotoxin contamination of maize (Logrieco et al., 2002; Doohan et al., 2003), and causing ear rot disease in open fields. *Fusarium graminearum* and *Fusarium verticillioides* are most frequently isolated species from maize infected plants under different climatic zones due to their divergent ecological requirements. Ecological requirements vary from warm/humid climate (favoured by *F. graminearum*) to hot/dry climate (favoured by *F. verticillioides*), which might determine the most dominant *Fusarium* species in a certain region (Vigier et al., 1997; Munkvold, 2003). Indeed it is not uncommon that in some years one ear rot causing fungus dominates over the other pathogens. Seasonal fluctuation in *Fusarium* species epidemics has long been reported in the open fields (Schaafsma et al. 1997; Reid et al. 1999) and climatic conditions have been suggested to explain these fluctuations. Our data, however, provides along with the findings of Picot et al. (2012) an additional source of effects of this phenomenon by demonstrating the potent interaction among *Fusarium* species *in planta* with a direct effect on disease severity, fungal biomass and toxin accumulation (**Chapter 2**).

Since it was suggested that mycotoxins might control the interaction between *F. graminearum* and *F. verticillioides* (Munkvold, 2003; Picot et al., 2012), there was no clear evidence whether major mycotoxins produced by both specie would be responsible for tuning this interaction. Generally speaking, secondary metabolites produced by microbes can be considered biochemical indicators for the fitness of microbes under various environments. Microbial secondary metabolites enable their producer to expand and defend themselves in different ecological niches against other competitors (Fox and Howlett, 2008).

The anti-microbial function of fumonisin produced by *F. verticillioides*, was demonstrated *in vitro* (Wu et al., 1995; Keyser et al., 1999; Becker et al., 1997). Our recent results from confrontation assay of *F. verticillioides* and *F. graminearum* in dual cultures did not show antagonistic interaction between both *Fusarium* species, regardless the ability of fungal strains to produce mycotoxin (wild type strain) or not (disrupted mycotoxin biosynthesis mutant) (**Chapter 4**). We suggested that concentrations of the fumonisin produced by the wild type strain might not be present in a sufficient concentration in the synthetic medium to exhibit an antagonistic effect, because similar observations from *F. verticillioides* and *F. graminearum* interaction on maize grain medium has been reported (Velluti et al., 2000a,b). Moreover the confrontation assay showed faster growth and more occupied space on the synthetic medium by *F. graminearum* compared to *F. verticillioides* differing from the results we obtained from the interaction between *F. graminearum* and *F. verticillioides* *in planta* (**Chapter 2**).

Primary infection of maize ears with *F. verticillioides* is very likely to happen naturally firstly through the systemic movement within the plant and vertical transmission "from kernel to kernel" (Bacon and Hinton, 1996), then *F. graminearum* with means of wind, rains or insects infecting the kernels. This we simulated by maize ear co-inoculated with *F. verticillioides* prior to *F. graminearum* in our experiments. The co-inoculation treatments are always in favor of *F. verticillioides* (wildtype or mutant) regardless fumonisin production in terms of fungal growth and disease severity compared to the single inoculation with *F. verticillioides*, while a significant inhibition to *F. graminearum* has been reported (**Chapter 2**).

The phytotoxicity of major mycotoxins produced by *F. verticillioides* and *F. graminearum* (fumonisins and trichothecens, respectively) has been proven (Wang and Miller, 1988; Lamprecht et al., 1994). Trichothecenes produced by *F. graminearum* are known to act as virulence factors to their host plant (Desjardins et al 1996; Proctor et al., 2002, Maier et al., 2006). Nishiuchi et al. (2006), showed that susceptible *Arabidopsis* plants subjected to deoxynivalenol concentrations above 5 μM inhibited protein synthesis in *Arabidopsis* cells, concluding that *F. graminearum* might exploit deoxynivalenol as an inhibitor for defence responses during disease development in *Arabidopsis* (Nishiuchi et al., 2006). Similar conclusions were drawn earlier in maize and wheat using leaf discs and kernel sections exposed to deoxynivalenol (Casale and Hart, 1988; Miller and Ewen, 1997). On the other hand, evidence that *F. verticillioides*

is a competitive endophyte against fungi in many genera (e.g. *Aspergillus flavus* and *Ustilago maydis*) *in vivo* (Zorzete et al., 2008; Lee et al., 2009) and *in vitro* (Cuero et al., 1988; Ramakrishna et al., 1996; Rodriguez-Estrada et al., 2011). Besides, the reports about the competitive interaction of *F. verticillioides* towards *F. graminearum* *in vivo* in maize ears (Reid et al., 1999; Picot et al., 2012) and *in vitro* (Keyser et al., 1999). Taking all together, it was hypothesized that trichothecenes (deoxynivalenol and nivalenol) produced by *F. graminearum* have the function of blocking the defence responses in maize plants and might cause the burst in the growth of *F. verticillioides*, and/or fumonisin produced by *F. verticillioides* might play an antagonistic role in the inhibition of *F. graminearum* in maize ears.

Our results from mixed infections of *F. verticillioides* and *F. graminearum* (deoxynivalenol and nivalenol) chemotypes in different combinations and different sequence of infection using mycotoxin-producing and non-producing strains, suggest that production of either trichothecenes or fumonisin does not influence the high growth rate of *F. verticillioides* and growth inhibition of *F. graminearum* throughout the fungal interaction course *in planta*. The interaction mechanism is more complex and might involve additional secreted fungal metabolites, or specific biochemical changes within the host plant cells upon the invasion of certain *Fusarium* species. Rodriguez Estrada et al., (2011) confirmed the antagonism between *F. verticillioides* and *Ustilago maydis* *in vitro*, suggesting that fumonisin produced by *F. verticillioides* in the solid medium during this interaction inhibited the growth of *Ustilago maydis*. In a further metabolome and transcriptome study of the interaction between *F. verticillioides* and *U. maydis* *in vitro* by Jonkers et al., (2012), they found that fusaric acid was produced in large amounts by *F. verticillioides* co-cultivated with *U. maydis* in liquid medium rather than fumonisin, concluding that fusaric acid is an important compound that is responsible for this antagonistic effect of *F. verticillioides* against *U. maydis* *in vitro*. Even though we did not quantify fusaric acid in our experiments, it seems likely that *F. graminearum* might be more resistant to fusaric acid produced by *F. verticillioides* than *U. maydis* since, the dual cultures did not show an antagonistic response between *F. verticillioides* and *F. graminearum* *in vitro* (**Chapter 4**), it also highlights the specificity of toxins/metabolites as competitive factors.

Our data showed at specific instance of interaction between *F. verticillioides* and *F. graminearum* deoxynivalenol chemotype, that *F. graminearum* was significantly inhibited in biomass and the developed symptoms on maize ears compared to the single

F. graminearum deoxynivalenol chemotype infection. While, this was not the case with *F. graminearum* nivalenol chemotype co-inoculated with *F. verticillioides*. The inhibition effect on *F. graminearum* was less pronounced compared to single *F. graminearum* nivalenol chemotype infection. In both instances, the results were conclusive regardless of the ability of the fungal strain used to produce its respective mycotoxin or not. (**Chapter 2**)

Although we confirmed that nivalenol production increases disease severity caused by *F. graminearum* nivalenol producing strain, compared to *F. graminearum* nivalenol non-producing strain in consistence with Maier et al. (2006), the co-inoculation with *F. graminearum* nivalenol non-producing strain with *F. verticillioides* resulted in an increased disease severity compared to single *F. graminearum* nivalenol non-producing strain. This confirms that nivalenol is a weak virulence factor in maize ears, especially the fungal growth of both *F. graminearum* nivalenol producing and *F. graminearum* nivalenol non-producing was comparable in the single inoculations (**Chapter 2**).

F. verticillioides single infections resulted in very mild symptoms on maize ears, with a comparable growth whether it was a wildtype (fumonisin producer) or mutant (fumonisin-deficient), verifying that fumonisin is not virulence factor in maize ear infections in line with (Desjardins and Plattner, 2000; Desjardins et al., 2002), (**Chapter 2**). In roots and seedling infection, the role of fumonisin produced by *F. verticillioides* was varied. On one hand fumonisin is not required for the infection in maize seedlings (Dastjerdi and Karlovsky, 2015), on the other hand fumonisin is required for the development of foliar disease symptoms on maize seedlings (Williams et al., 2007; Glenn et al., 2008). Whereas, Arias et al., (2012) suggested that the production of fumonisins may favour *F. verticillioides* development on maize seedlings only at high concentrations.

In contrast to *F. verticillioides*, single infections with *F. graminearum* trichothecenes-producing strains (Deoxynivalenol chemotype and Nivalenol chemotype) and non-producing strains had resulted severe maize ear symptoms and an abundance of mycelia mass. Our results also showed that *F. graminearum* deoxynivalenol chemotype and its mutant were more aggressive than the *F. graminearum* nivalenol chemotype and its mutant with respect to symptom development, fungal DNA quantification and trichothecene accumulation-(only by wild type strains). This supports the results of Miedaner et al., (2010), who examined different isolates of *F. graminearum* and *F. verticillioides*, for their severity on maize inbred lines in the open field. They ranked the

ear rot severity according to the isolates from the highest to the lowest as follows; *F. graminearum* deoxynivalenol producers, *F. graminearum* nivalenol producers, and *F. verticillioides* (Miedaner et al., 2010). The higher aggressiveness of *F. graminearum* deoxynivalenol chemotype than *F. graminearum* nivalenol chemotype has also been reported in winter rye (Miedaner et al., 2000). Unlike our results, Maier et al., (2006) found no difference in virulence of the NIV and DON chemotypes on the cobs of inbred maize lines, even though the same strains were used in both studies. The differences among maize varieties used in the two studies may explain the disparities. Apparently, Gaspe Flint, used in our experiments, was more susceptible to *F. graminearum* strain FG 2311 (deoxynivalenol chemotype) than the inbred line A188 used by Maier et al. (2006). Different susceptibility in maize varieties to *F. graminearum* deoxynivalenol chemotype had no effect on the results because deoxynivalenol non-producing strain was as aggressive as the deoxynivalenol producing strain in both studies. Moreover, the pattern of interaction of *F. graminearum* and *F. verticillioides* *in planta* was found to be the same in all maize cultivars tested so far (Reid et al., 1999; Picot et al., 2012) as well as in Gaspe Flint variety that we adopted in our study (**Chapter 2**). Contrary, the stem-base infection assay in maize seedlings with isolates of *F. graminearum* nivalenol chemotype were found to be more pathogenic than *F. graminearum* deoxynivalenol chemotype (Carter et al., 2002). This explains that the aggressiveness of both *F. graminearum* chemotypes vary in different host plant varieties and different infected tissues.

The ecological function of the mycotoxins (deoxynivalenol, nivalenol and fumonisin) was examined thoroughly in this study throughout the complex infection of maize ears with the two species of *F. graminearum* and *F. verticillioides*. Here we demonstrated that the accumulation of mycotoxins under study is more likely to be a representative of the fungal growth in infected kernels, rather than to be competitive metabolites which modulate the interaction between *Fusarium* species in maize ears.

Volatile organic compounds have been implemented as a characteristic tool to understand the complex interaction within the living organisms (plant-plant, plant-insect, and plant-microbe) (Dudareva et al., 2006). These are considered as important indicators to evaluate the plant health and food quality. Thus, plant/microbe-emitted volatiles have been incorporated into several agricultural applications as reviewed in (Beck, 2012; Oms-Oliu et al., 2013) including detection of plant disease infestation, integrated pest management, agricultural commodities and food quality evaluation.

Rapid and precise plant disease assessment and pathogen detection are fundamental to plant disease management and control (Schaad et al., 2003). Highly sensitive, reliable and rapid disease detection methods in an early stage of host infection can guarantee precise intervention to halt the disease development. Despite the high sensitivity of direct detection methods involving serological and molecular techniques, they are considered as invasive techniques with a requisite limit of detection correlated to the lowest concentration of pathogen in the samples (Nezhad, 2014). VOCs profiling has been used frequently during the last decades as indirect detection method for plant disease based on the changes in plant physiology upon the exposure to the pathogen, and as a non-invasive technique to monitor plant health status under laboratory and semi-field conditions (Sankaran et al., 2010; Aksenov et al., 2013; Martinelli et al., 2015).

De Boer and Lopez, (2012) proposed grower-friendly methods of monitoring pathogens under the criteria of cost effectiveness, marketability, simplicity and robustness. Similar to the idea of lab-on-a-chip (Fair, 2007), this advanced approach still face a lot of challenges regarding sampling, open field conditions and detector developments (De Boer and Lopez, 2012)

Plant volatiles play signaling role for defense response activation, or direct inhibitors against the pathogen (Dudareva et al., 2006). Chemical substances that are frequently induced by the host plant upon microbial infection regardless of the pathogen and plant species, include; (*Z*)-3-hexenol, methyl salicylate, (*E*)- β -ocimene, linalool, (*E*)- β -farnesene, (*E*)-4,8-dimethylnona-1,3,7- triene, and (*E,E*)-4,8,12-trimethyl- 1,3,7,11-tridecatetraene (Jansen et al., 2011). Emission of VOCs can be used to characterize the crop pest or disease. The herbivore-attacked plants emit various volatile blends above the ground (Takabayashi et al., 1995; Turlings et al., 1998; Arimura et al., 2004; Delphia et al., 2007; Sufang et al., 2013) or below the ground (Tapia et al., 2007; Lawo et al., 2011), demonstrating the specificity in volatile blends emitted from different plant invaded by different insects at different developmental stages. Moreover, herbivore-induce plant volatiles (HIPVs) can prime the defense response in healthy plants against future herbivore attacks (Ramadan et al., 2011; Ali et al., 2013). Furthermore, microbial plant infestation was monitored by the change in volatile profiles emitted by the host plants, infected tomato plants with *Botrytis cinerea* released quantities of mono- and sesquiterpenes in higher concentrations than healthy plants (Jansen et al., 2009). Chickpea plants infected with *ascochyta rabiei* produced higher 1-

penten-3-ol and cis-3-hexen-1-ol compounds in correlation with *Ascochyta* blight disease severity (Cruz et al., 2012).

Moreover, Girotti et al., (2012), introduced trichodiene volatile as a marker for *Fusarium* head blight disease on wheat cultivars at early stages of the infection. Instead of single compound detection, a blend of compounds (>40 VOCs) in plant/fungus interaction of *Eucalyptus globulus*/*Teratosphaeria nubilosa*, posed reliable disease markers based on the whole chromatographic profile (Hantao et al., 2013). The foliar infection of barley and wheat plants with *Fusarium* spp. induced eleven VOCs compared to non-infected plants (Piesik et al., 2013). Moreover, leaf and root infection of maize plants with *Fusarium* spp. induced several VOCs in high quantities upon fungal infection (Piesik et al., 2011). In addition, disease volatile marker blends were emitted from maize ears infected with *Fusarium* spp. including major sesquiterpenes markers (+)-longifolene, farnesene, macrocarpene and trichodiene (Becker et al., 2013; Becker et al., 2014; Sherif et al., 2016). Further studies also showed that VOCs identification can also allow bacterial and viral plant disease diagnosis (Deng et al., 2004; Prithiviraj et al., 2004; Mauck et al., 2010; Spinelli et al., 2012; Cellini et al., 2016). The individual or blended VOCs was studied in plant-plant communication and the specificity of volatile blend was demonstrated in this regard (Pichersky and Gang, 2000; Ueda et al., 2012) besides the role of volatiles in species-specific microbial interaction (kai et al., 2007; Thorn et al., 2011). This highlights that volatile compound identification introduces a very promising and specific diagnostic tools in crop disorders that should be developed for future open field application.

In our study (**Chapter 3**) we investigated the volatile profiles of maize ears simultaneously infected with two *Fusarium* species in maize to understand how far the fungal co-infection modulates volatile emission from infected plants, and to what extent the *in planta* fungal interaction could be monitored via VOCs profiling. The study adopted two maize varieties, field hybrid maize and experimental dwarf maize, which were infected with a combination of fungal strains belonging to the species of *F. verticillioides* and *F. graminearum*. Besides the dual strains co-infection treatments, single strain infections have been performed, in addition to non-infected reference treatments

Our data reported volatile disease biomarkers of ear rot disease in maize as a result of the infection with *Fusarium* spp. in accordance with Becker et al., (2014). Both maize variety hybrid (field variety) and dwarf maize (experimental variety) shared common

volatile markers that were composed mainly of sesquiterpenoids; β -macrocarpene, (+)-longifolene, β -farnesene and trichodiene, suggesting they are robust markers for *Fusarium* ear rot disease detection.

The principle component analysis (PCA) and similarity clustering tree were applied in order to visualize the relationship between the combination of pathogenic *Fusarium* species and potential volatile markers emitted from infected maize kernels. Our results show a clear distinction of emitted volatiles accordingly to contamination degree with fungal biomass and disease severity. PCAs within different *Fusarium* species combinations on both hybrid and dwarf maize proved the variability among volatile markers in single and mixed treatments, regardless the host variety. This variation of volatile markers in mixed infection treatments highly correlated to the *F. graminearum* and *F. verticillioides* interaction demonstrating that the volatile profile of maize ears infected with two *Fusarium* strains was dependent on the most competitive strain. Based on cluster analysis of the volatile profiles of infected maize ears it was apparent that the volatile profile of maize ears reflect disease severity regardless of the presence of one or more fungal pathogens (**Chapter 3**). The results were consistent with early observations which highlighted the effect of stress (either biotic or abiotic) severity on the change in emitted blends of volatiles (Jansen et al., 2009; Hakola et al., 2006; Niinemets, 2009)

Our results indicate that disease severity correlates with the oxylipin 9-hydroxy octadecadienoic acid (9-HOD) that has been considered as a biomarker for aflatoxin-resistance in maize lines (Wilson et al., 2001). In single and mixed *Fusarium* spp. infections, the linear correlation was higher in single inoculations ($R^2=0.67$, $p<0.05$) compared to mixed inoculations ($R^2=0.23$) suggesting that fungal competition might somehow compromise the plant response. This gives an additional interpretation regarding mycotoxin-independent interaction between *F. graminearum* and *F. verticillioides in planta* which has been demonstrated in **Chapter 2**, and affirms the complexity of maize/*Fusarium* pathosystem which demands further study to unravel this complex network of interactions.

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Summary

Maize (*Zea mays* L.) is an important cereal crop which provides staple food for humans and livestock. Maize cultivation is threatened by numerous microbial diseases under field conditions. Fungal pathogens belonging to genus *Fusarium* can infect roots, stalks and ears during growing season causing what so-called fusarioses. *Fusarium* diseases cause a huge reduction in the yield besides plant material contamination with mycotoxins (fungal metabolites which are toxic to vertebrates). In nature, maize plants are commonly infected by more than one pathogenic *Fusarium* species.

F. graminearum Schwabe [teleomorph = *Gibberella zea* (Schwein) Petch] and *F. verticillioides* (Saccardo) Nirenberg [synonym = *F. moniliforme* J. Sheldon, teleomorph = *G. moniliformis* Wineland] are major causal agents of *Gibberella* ear rot and *Fusarium* ear rot on maize, respectively. The interaction between *F. graminearum* and *F. verticillioides* on maize ears has been reported, however the role of mycotoxins in this interaction is questionable. The main goal of this work was to elucidate the pathological and ecological functions of major *Fusarium* mycotoxins, which might serve as virulence factor during disease development or might be involved in the competitive interactions among fungal species.

These hypothetical functions were studied throughout fungal interaction course on live maize ears (*in vivo*) or on Petri dishes (*in vitro*) using wildtype fungal strains belong to *F. graminearum* (produces trichothecenes; nivalenol, deoxynivalenol) and *F. verticillioides* (produces fumonisins) and their mutant strains impaired in synthesis of nivalenol, deoxynivalenol and fumonisins.

Maize ears were either infected with a single *Fusarium* species or co-infected with two species (concurrently and sequentially) under controlled greenhouse conditions. Disease severity, fungal biomass and mycotoxins accumulation were monitored on infected ears. Among all fungal strains “wildtypes and mutants” that were compared in single inoculation experiment, the strains belong to *F. verticillioides* and *F. graminearum* nivalenol chemotype were less aggressive than strains belong to *F. graminearum* deoxynivalenol chemotype. Moreover, nivalenol non-producing mutant strain of *F. graminearum* caused fewer symptoms than the nivalenol-producing strain, though the biomass of the strains in infected kernels was unaffected.

Regardless of the ability of *F. graminearum* to produce nivalenol or deoxynivalenol and *F. verticillioides* to produce fumonisins, the results indicated that growth of *F. verticillioides* was stimulated by co-infection with *F. graminearum*. The growth of *F. graminearum* deoxynivalenol-producing strain and its mutant was suppressed in co-inoculation with *F. verticillioides* regardless of the ability of the latter to produce fumonisins. Similarly, disease symptoms caused by deoxynivalenol-producing strain of *F. graminearum* and its mutant were reduced in mixed inoculation with *F. verticillioides* regardless of the ability of *F. verticillioides* to produce fumonisins. The results demonstrated that fumonisins and trichothecenes were not involved in fungal competition between *F. graminearum* and *F. verticillioides* on maize ears.

Dual cultures of *F. verticillioides* and *F. graminearum* on synthetic medium did not show inhibition zones between both *Fusarium* species regardless the ability of fungal strains to produce mycotoxins. However, *F. graminearum* occupied more space and grew faster in dual cultures than *F. verticillioides*.

Furthermore, to understand how competitive interactions between both fungal species *F. graminearum* and *F. verticillioides* influence plant volatile blends of infected maize ears, solid phase microextraction-GC/MS was used for the detection of volatile organic compounds (VOCs) emitted by infected (single or mixed fungal infection) and uninfected maize ears. Multivariate analysis (PCA) was used to compare complex volatile profiles from infected and non-infected maize ears. The results showed that volatile profiles in mixed infections were modulated by the most competitive fungal strain. Moreover, volatile profiles reflected disease severity by either single or mixed fungal infections. The data also reported volatile biomarkers of maize ear rot disease caused by *Fusarium* species that were mainly composed of sesquiterpenoids and other compounds, highlighting their potential in precision agriculture and disease monitoring.

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Publications & Workshops

Publications

Sherif M., Becker E-M., Herrfurth C., Feussner I., Karlovsky P., and Splivallo R. (2016). Volatiles emitted from maize ears simultaneously infected with two *Fusarium* species mirror the most competitive fungal pathogen. *Frontiers in Plant Science*. 7:1460. doi: 10.3389/fpls.2016.01460

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Sherif M., Pfohl K., and Karlovsky P. (2015). Aggressiveness of *Fusarium graminearum* chemotypes producing nivalenol or deoxynivalenol in maize. 37th Mycotoxin Workshop. Society for Mycotoxin Research. Bratislava - Slovakia. Book of Abstracts: pp. 95.

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Workshops & Conferences

24 - 27 Aug. 2015

XVIII. International Plant Protection Congress (IPPC). Mission possible: Food for all through appropriate plant protection, Berlin - Germany. Oral Presentation

01 - 03 June 2015

37th Mycotoxin Workshop. Society for Mycotoxin Research, Bratislava - Slovakia.
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