Analysis of toxigenic fungi and their mycotoxins in biotic interactions

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Contents

Chapter 1: Research in fungal chemical ecology

Introduction

Filamentous fungi play a central role in microbial flora and are responsible for a wide range of important functions in ecosystems all over the world. Fungi are important decomposers of leaf litter and have effects on biogeochemical cycling, soil tilth and structure. Furthermore, some filamentous fungi colonize plant tissue and form parasitic and symbiotic relationships, while others use the natural resources in the rhizosphere, on the rhizoplane and also in the phyllosphere [1]. Fungi are usually associated with the production of secondary metabolites, which show a broad range of structural diversity and biological activities. Secondary metabolites are natural products, mostly of low molecular weight and often bioactive. In contrast to primary metabolites, secondary metabolites are not required for growth, development and reproduction and their distribution is taxonomically restricted [2]. Secondary metabolites produced by fungi include polyketides, non-ribosomal peptides, terpenes and indole alkaloids. They can show antibiotic, phytotoxic or also insecticidal activities. Metabolites harmful to humans and animals are called mycotoxins and are associated with ingestion of foods, animal feeds, and forages [3].

The biological role of secondary metabolites is often difficult to define. Some metabolites are involved in pathogenetic processes while others play a role in an extraordinary diversity of biotic interactions such as those between fungi, plants or microorganisms occupying the same ecological niche. Toxigenic fungi are better protected against other organisms and thus have an advantage which allows them to survive in their ecological niches [4, 5].

It is necessary to estimate the amount of fungal biomass as well as to identify the secondary metabolites produced in order to understand ongoing processes in complex fungal biotic interactions and to determine the putative role of secondary metabolites in these processes. However, analytical techniques for accurate and sensitive quantification of species-specific fungal biomass as well as their mycotoxins from biological systems have only been available for a few years. Although numerous analytical methods have already been established, methods for the detection and quantification of diverse specific analytes are still to be

developed.

The role of fungal secondary metabolites in biotic interactions

Fungal secondary metabolites in plant-fungus interactions

Infection of plants by fungi poses an enormous problem in food production and food security. Worldwide harvests of crop plants are endangered because of plant diseases or pests which lead to losses of at least 10% of the global harvest [6].

Infection by fungal plant pathogens occurs via several pathways such as seeds, roots, stems or aerial plant tissues, including flower and fruit [7]. Species belonging to the genera *Aspergillus, Fusarium,* and *Penicillium* are the most common fungi associated with the contamination of crop plants with mycotoxins. They infect many different field crops including wheat, maize, rice, barley and other cereal grains as well as peanuts, tree nuts, grapes, coffee and cotton [8]. Several of these mycotoxins serve as phytotoxins or phytoaggressins that are active against plants [7]. Most phytotoxins are organic acids, cyclic terpenoids, polyketides, and cyclic polypeptides. Phytotoxins differ in the way they act but the main consequences of their activities are damage to the cell membrane as well as abnormalities and biochemical changes in plant cells. In general effects of phytotoxins are wilting and growth suppression, as well as induction of chlorosis, necrosis, and spotting of aerial portions [9]. The biological function of phytotoxins in fungal plant pathogenesis is diverse. Some phytotoxins are pathogenicity factors and are required for plant infection while others act as virulence factors and are responsible for the emergence of symptoms in infected plant tissue [10].

Phytotoxins are divided into host-specific and non-host-specific. Even low concentrations of host-specific toxins are able to affect plants of a genotype susceptible to the pathogen, that is, plants which have genes encoding molecular targets of the toxins [9, 11]. Varieties resistant to the pathogen are not affected even by relatively high concentrations of the toxin. Furthermore, mutants of the pathogen lacking the gene responsible for toxicity are usually non-virulent [10]. Overall, non-host-specific phytotoxins act as virulence factors, while most of the host-specific phytotoxins act as pathogenicity factors [11].

Only a few host-specific phytotoxins are known as yet. Among these, some are produced by the genus *Alternaria*. To give an example, tomato plants of the genotype *asc*/*asc* are susceptible to the host-specific toxin AAL-toxin produced by *A. alternata* f. sp. *lycopersici*, whereas plants of the genotype *Asc*/*Asc* are less sensitive to this toxin. The phytotoxin is structurally related to fumonisins, a class of mycotoxins, which is among other fungal species mainly produced by the important maize pathogen *Fusarium verticillioides* [12]. Furthermore, it is also thought that destruxin B, produced by *A. brassicae,* acts as a host-specific virulence factor in *Brassica* species [13]. The majority of fungal phytotoxins are non-host-specific and can affect a broad spectrum of plants [10]. Deoxynivalenol is the most prominent non-host-specific virulence factor produced during infection and colonization of wheat by *F. graminearum*. Although the mycotoxin does not seem to be necessary for initial infection by the fungus, it supports the spread from one spikelet to another [14]. Similar effects of deoxynivalenol in maize have also examined [15].

In addition to fungal plant pathogens, most plants in natural ecosystems are colonized by mycorrhizal fungi or fungal endophytes belonging to diverse taxa. Fungal endophytes grow from plant roots into the rhizosphere without causing symptoms. The interaction can affect the plant's ecology, fitness, and evolution as well as the interactions of plants with plant pathogens [16]. Associations between plant endophytes and their hosts are diverse and the types of interaction can range from symbiosis to mutualism. Endophytes use their host plants for fungal survival. Although most endophytes do not cause symptoms, latent pathogens can be isolated from symptomless plants at harvest [17]. Endophytic fungi can protect the plant against plant pathogens and pests such as nematodes, bacteria, insects and fungi. The protection provided by the endophyte is either directly by the production of toxic secondary metabolites in, for example, fungus-fungus interaction [18] or indirectly by the production of substances that induce plant defense mechanisms [19, 20]. The fungal species *F. verticillioide*s appears as one of the most important fungal species colonizing maize plants. The fungus occurs as a fungal pathogen [21] but also infects maize plants as a symptomless endophyte on maize [22]. Endophytic growth of the fungus is associated with infection of the seeds or the roots through which the fungus grows systemically up to the cob [23]. This endophytic interaction can positively influence yield and vegetative growth. It was discussed that the type of relationship between *F. verticillioides* and the maize plant is mainly influenced by abiotic or biotic conditions [22].

Infection with *F. verticillioides* usually lead to contamination with fumonisins. The role of fumonisins in virulence of the fungal species remains still unclear. Desjardins and Plattner observed that *F. verticillioides* strains not producing fumonisins infected maize kernels and caused ear rot as effectively as fumonisin-producing strains [24]. Opposite results were obtained by Glenn *et al.* [25] who found that strains of *F. verticillioides* were not pathogenic on maize seedlings because of mutations of the FUM1 gene. Furthermore, a distinct population of *F. verticillioides* is pathogenic on banana, but lack genes in the FUM cluster. These strains were not able to cause disease symptoms on maize seedlings while fumonisin-producing transformants of these strains were pathogenic on maize seedlings. The authors formulate the hypothesis that seedling disease is strongly dependent on the maize genotype and the amount of fumonisins produced by the *F. verticillioides* strains.

Fungal secondary metabolites in fungus-fungus interactions

Plant-fungus interactions have being studied extensively but the function of toxins in fungus-fungus interactions has rarely been addressed. Smallest niche differentiation in time or localization can lead to the coexistence of many different fungal plant pathogens, while others are in direct interaction with each other [26]. Interactions between fungal intra- and interspecies are mainly characterized by competition for resources such as nutrients or space. Antagonism between fungi in nature has been demonstrated in virtually every type of fungal ecosystem [7]. The ability of a fungal species to compete for a host depends on many factors, such as environmental conditions, especially temperature and humidity, and fungal growth rate [27–29]. Rapidly colonized plant tissues may preclude colonization by other pathogens as has been suggested for *F. graminearum* in wheat. Under conditions which are not favorable for the growth of the fungus other pathogens may successfully invade the plant tissue [26].
Additionally, the production of toxic metabolites can increase competitiveness towards other fungal species. Several *Fusarium* species produce the mycotoxin zearalenone, which inhibits or reduces growth of filamentous fungi. It has been found that zearalenone may help *Fusarium* spp. to reduce or inhibit the growth of many fungi and therefore to protect a substrate colonized by zearalenone-producing *Fusarium* spp. The mycoparasite *Gliocladium roseum* produces a zearalenone-specific lactonase which catalyzes the hydrolysis of zearalenone, followed by a spontaneous decarboxylation. Due to the detoxification of

zearalenone, the growth of *G. roseum* is not affected by zearalenone [30].

The best-known competing fungi are species belonging to the genus *Trichoderma* spp. which have a high reproductive capacity, the ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients and the capacity to modify the rhizosphere. The fungi suppress the growth of other fungal species indirectly by competing for nutrients and space, modifying the environmental conditions, and promoting plant growth and plant defensive mechanisms. However, the strongest antagonistic effects are also achieved directly by producing several toxic metabolites active against fungi, thus inhibiting the colonization by competing fungi, and by exerting mycoparasitic actions against several genii of filamentous fungi. Different cell-wall degrading enzymes, mostly chitinases, glucanases and proteases, are involved in parasitic processes. Due to all of these properties listed, *Trichoderma* spp. are ubiquitous in any habitat and at high population densities [31].

Antagonistic fungal species may also have an impact on the toxin production of fungi. It has been shown that *Trichoderma* spp. suppress the production of fumonisins [32] as well as the deoxynivalenol production of several *Fusarium* spp. [8]. The production of aflatoxins by *Aspergillus* spp. has also been found to be reduced in interactions with different fungal species [33].

However, there are no investigations on mechanistic approaches in interactions between inter- and intraspecific fungal species. The production of antifungal metabolites may be involved in interactions and give the fungus a competitive advantage.

Fungal secondary metabolites in insect-fungus interactions

Diverse interactions ranging from antagonistic to symbiotic occur between filamentous fungi and insects. In plant-endophyte interactions, the production of insecticidal metabolites plays an important role in preventing insect feeding on the host plant, resulting in an enhanced resistance of the plant to herbivores [16]. Furthermore, the metabolites of saprophytic fungi in soil are often discussed as putative resistance mechanisms against invertebrate fungivores. Many invertebrates use saprophytic fungi in soil as an important food source. It is suggested that fungivore feeding on fungal hyphae leads to a chemical defense reaction of the fungi, consisting of an increase in the synthesis of secondary metabolites which act against fungivores [34].

Furthermore, even competitive strategies have been determined in interactions between insects and fungi. Saprophytic fungi of the genus *Aspergillus* and insects use the same nutritional resources. Secretion of toxic metabolites, such as aflatoxin B1, which are toxic for insects, protects the substrate from insect feeding [35].

Entomopathogenic fungi infect insects and use insect tissue as a nutrient resource for growth. It has been suggested that toxic secondary metabolites play an important role as virulence factors in infection [35]. A wide assortment of secondary metabolites are produced by the entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae*, including the hexadepsipeptides beauvericin and destruxins, respectively. The toxins have strong insecticidal activity against a broad spectrum of insect pests. Both fungal species have been applied as commercial biocontrol agents against pests [36]. The toxicity of both toxins is mainly attributed to their activity as membrane carriers by forming ionophoric lipophilic complexes [37].

Quantitative analysis of species-specific fungal biomass in plant material

Real-time PCR is a powerful method for the quantification of species-specific fungal biomass in a complex matrix and permits the accurate differentiation between closely-related species. Especially in plant pathology, accurate identification and quantification of fungal biomass is essential for virtually all areas, from fundamental research on the biology of pathogens to disease development and control or the variation of fungal plant pathogen populations. DNA-based diagnostics allow the specific differentiation even between closely-related fungal species due to the selection of species-specific nucleic acid sequences used for identifying the fungal pathogen [38]. The research on plant diseases caused by more than one fungus, in particular, requires analytical techniques for the identification and quantification of fungal biomass on a species level.

For example, *Gibberella* and *Fusarium* ear rot on maize are two diseases, each caused by a range of closely-related *Fusarium* species [21]. Quantitative differentiation between the closely-related fungi on a species level are required for the determination of fungal colonization, their contribution to the fungal disease as well as interactions between the species.

Real-time PCR combines the principle of conventional PCR with the real-time analysis of

reaction kinetics due to a specific fluorescent signal. The most frequently used fluorescent dye is SYBR Green I, which emits fluorescent light when intercalated into double-stranded DNA. During the real-time analysis the fluorescence is proportional to the amount of total DNA in the reaction. On the basis of a linear calibration curve, the technique permits the quantification of detected DNA constructed with external standards [39]. Melting curve analysis is usually performed immediately after PCR, confirming the identity of the amplified DNA fragments due to their specific melting temperatures.

Optimization of the reaction process includes adjusting quantities of the components in the reaction mixture as well as adjusting cycle length and annealing temperature. These parameters mainly influence the building of primer-dimers as well as efficiency, specificity and sensitivity of the assays [40]. In order to describe the quality of a real-time PCR assay, the performance characteristics sensitivity, specificity, limit of detection (LOD) and limit of quantification (LOQ) are usually determined. However, the methods commonly accepted in chemical analysis for determining LODs and LOQs are unsuitable for real-time PCR [41, 42] and the values are usually only estimated empirically.

Quantitative analysis of mycotoxins in plant material

High-performance liquid chromatography (HPLC) followed by UV and fluorescence is the most frequently and widely used method for the quantitative analysis of mycotoxins. However, there has been an increase in the use of liquid chromatography coupled with mass spectrometry in the last decade. This makes the simultaneous detection and quantification of toxins in complex matrices possible and mass spectrometry is nowadays the method of choice in mycotoxin analysis [43]. Mass spectrometry is especially advantageous for the analysis of metabolites with low ultraviolet absorbance or native fluorescence. Additional time-consuming sample preparation steps such as the derivatization of the mycotoxins are required in order to be able to analyze the compounds mentioned above with UV fluorescence or absorbance detection. For example, fumonisins lack a useful chromophore or fluorophore; the specific detection of fumonisins with fluorescence or UV detection methods thus involves time consuming derivatization with o-phthaldialdehyde [44].

Mass spectrometry offers new perspectives for the sensitive, selective, and accurate analysis of several mycotoxins in one sample. The approach of tandem mass spectrometry (MS/MS),

in particular, makes the determination of the specific fragmentation pattern of compounds possible, providing the maximum level of confidence in analyte identification [45].

The high selectivity in MS/MS analysis leads to the reduction of tedious sample preparation and time-consuming clean up efforts. The samples are prepared in a uniform way and mycotoxins of diverse polarities can often be analyzed simultaneously in multi-mycotoxin methods [46–48]. Nevertheless, extraction of mycotoxins from complex matrices can lead to matrix interferences, resulting in suppressed or less frequent enhanced signals of the target compounds. Matrix effects are caused by the co-elution of matrix compounds interfering in the ionization and evaporation process of the analytes. MS/MS methods are as sensitive to ion suppression as single LC-MS methods [49]. Although sample preparation efforts can be reduced, chromatographic methods require suitable solvent extraction in order to release the mycotoxins from the sample matrix and to minimize the amounts of co-eluted compounds. The choice of the extraction solvent depends both on the physicochemical properties of the sample matrix and on the mycotoxins and must be adjusted as appropriate. In multi-analyte methods, compromises in the choice of extraction solvent have usually to be made [43]. The suitability of extraction solvents for toxins and matrices as well as the degree of ion suppression are usually obtained by determining the matrix effect, the recovery of the extraction procedure, and the overall process efficiency [50].

Additionally, performance characteristics of the analytical method are usually obtained in order to support the comparability of a method. Typical performance characteristics in quantitative MS/MS methods are the LOQ and LOD, linearity, precision, repeatability, selectivity and robustness [43].

Objective of the study

Understanding biotic interactions of filamentous fungi and the putative role of secondary metabolites in these interactions requires experimental studies based on specific and accurate analytical techniques. Overall, the object of the study was the development and use of analytical detection methods for fungal biomass and secondary metabolites, as well as the identification and investigation of toxic secondary metabolites involved in diverse fungal biotic interactions. Five major objectives of this study were:

1. to develop and validate analytical methods for the simultaneous determination of the six hexadepsipeptides beauvericin, enniatin A, A1, B and B1 and destruxin A in asparagus, potato, maize, tomato, rice and wheat with HPLC-ESI-MS/MS using an ion trap mass spectrometer.

2. to develop a method for the determination of the validation parameter limit LOQ and LOD in real-time PCR assays and its application on real-time PCR assays for *F. verticillioides* and *F. proliferatum* DNA in maize kernels which have been optimized in previous works.

3. to evaluate interactions between *F. verticillioides* and both chemotypes of *F graminearum* in maize ears with regard to infection rate, growth and mycotoxin accumulation. Furthermore, we examine the possibility that global warming will cause an increase in fumonisin content of maize grain in moderate climatic areas such as northern parts of Germany.

4. to determine secondary metabolites putatively involved in a chemical defense response of *A. nidulans* against grazing of the fungivore *F. candida.*

5. to determine the toxin production by *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2; a possible biocontrol agent against the parasitic weed *Striga hermonthica*; additionally, to evaluate the possible risk from mycotoxin transfer in the plant to mature sorghum grains grown from Foxy-2-coated sorghum seeds.

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Chapter 2: HPLC-ESI-MS/MS method for simultaneous determination of the depsipeptides beauvericin, enniatins and destruxin A in vegetables and cereals

Abstract

A sensitive method for the simultaneous determination of the six hexadepsipeptides beauvericin, enniatin A, A1, B and B1 and destruxin A in asparagus, potato, maize, tomato, rice and wheat has been developed. Analysis was carried out by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using an ion trap. Specific mass spectra on the basis of sodium adduct fragmentation were determined.

Several solvents based on acetonitrile, methanol and acetone were tested for extraction efficiency, for the recovery of the mycotoxins as well as for matrix effects. Furthermore, full scan analyses covering the whole gradient were carried out in order to examine amounts of co-eluted matrix components. The new solvent combination acetonitrile/isopropyl alcohol/water (70:15:15) led to high efficiency rates and low matrix effects. Limits of quantification and limits of detection ranged from 1-12 ng g^{-1} and 0.3-4 ng g^{-1}, respectively. In addition further examinations concerning defatting of samples were carried out resulting in a compromise between toxin losses and defatting efficiency by using n-hexane with sample matrix in methanol/water (75:25).

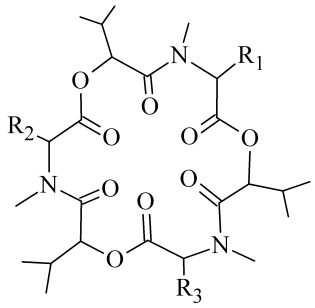
Introduction

Fungal species are often associated with the production of biologically active secondary metabolites such as mycotoxins. Their level of toxicity as well as their mode of action vary greatly, resulting in specific or non-specific effects on insects, bacteria, plants as well as humans and animals [1–3]. On the one hand, detection of harmful mycotoxins can assist food control and preventive strategies for mycotoxicoses [4], on the other hand, the detection and examination of specific toxic compounds sustains the finding of microorganisms useful in

their function as biocontrol agents [5–7].

Beauvericin, the enniatins A, A1, B and B1 and destruxin A are cyclic hexadepsipeptides produced by filamentous fungal species. Beauvericin and the homologous enniatins A, A1, B and B1 are mainly produced by several *Fusarium* species and occur in a wide range of host plants [8], whereas beauvericin was first described in *Beauveria bassiana* [9]. They consist of D-α-hydroxy-isovaleric acids, alternating with amino acid residues linked by peptide and ester bonds (Fig. 1). The three aromatic amino acid residues in beauvericin are N-methyl-phenylalanines [9], whereas enniatins A and B differ in their composition of amino acid residues of N-methyl-valine and -isoleucine [10, 11]. Destruxin A is a secondary metabolite mainly produced by *Metarhizium anisopliae* [12] and belongs to a large family (A-E), whereas destruxin A, B and E are most frequently observed. The metabolite is composed of an α-hydroxy acid and residues of the five amino acids proline, isoleucine, methylvaline, methyl-alanine, and beta-alanine [7].

Beauvericin and enniatins



	R_1	R_2	R_3
Beauvericin	$CH_2C_6H_5$	$CH_2C_6H_5$	$CH_2C_6H_5$
Enniatin A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
Enniatin A1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
Enniatin B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
Enniatin B1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$

Destruxin A

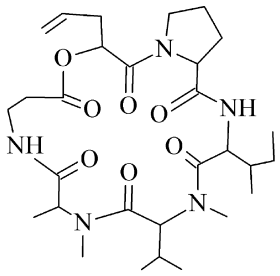


Figure 1. Simplified chemical structures of beauvericin and enniatins (left) and destruxin A (right)

The structurally related cyclic hexadepsipeptides exhibit ionophoric properties, which means

that they are able to transport ions through the membrane into the lipophilic phase. Ionophoric complexes usually consist of one cation and one ionophore [13]. The toxicity of beauvericin and enniatins is mainly based on their ability to incorporate into membrane structures and act as membrane carriers by forming ionophoric lipophilic complexes and affecting ionic homeostasis. Beauvericin and enniatins can form stable and lipophilic complexes with several cations or neutral molecules like sodium, potassium, rubidium, caesium, tellurium, calcium, strontium, barium, and ammonium [11]. It has been proven that the bioactivity of destruxin A is also linked to its ability to form complexes with cations, especially calcium, and to transport them across liposomal membranes, affecting the cellular calcium balance and leading to membrane depolarisation [13].

There are currently no reports of mycotoxicoses caused by the consumption of these mycotoxins, but only very limited data are available concerning the toxicity of beauvericin and enniatins to animals and humans [11]. Both mycotoxins possess a range of biological activities including antiinsectan, antimicrobial and cytotoxic [9, 14, 15]. Destruxins differ but also overlap in their biological activities. Destruxin A-E have insecticidal activities [16–18], destruxin B has additional phytotoxic activities [19] and destruxin A, B and E have shown antiviral and immuno-depressant activity in insect cells [7]. As a consequence, the entomopathogenic fungus *Metarhizium anisopliae* is one of the most frequently applied species amongst fungal biocontrol agents for the control of different insect pests [20].

HPLC-ESI-MS/MS methods based on the identification of ionized metabolites on their specific fragmentation products allows reliable and sensitive detection and quantification of metabolites in plant material. Some LC-MS/MS detection methods for beauvericin and enniatins have been described for grain [21, 22] sweet pepper [23], cassava flour, peanut cake and maize [24–26]. By contrast, no LC-ESI-MS/MS method for the detection of destruxin A has as yet been described.

This study was carried out to develop and validate a specific, fast and reliable method based on HPLC-ESI-MS/MS for the simultaneous detection of the cyclic hexadepsipeptides beauvericin, enniatins A, A1, B and B1 as well as destruxin A extracted from asparagus, maize, rice, potato, tomato and wheat.

Materials and Methods

Reagents and materials

For sample preparation methanol, acetonitrile, acetone, isopropyl alcohol, cyclohexane, n-hexane and dichloromethane, all HPLC grade were purchased from VWR International (Zaventem, Belgium). For analysis acetonitrile, methanol, ammonium acetate, sodium acetate and acetic acid, all LC-MS grade were supplied by Merck (Darmstadt, Germany).

Analytical mycotoxin standards

Mycotoxin standards of beauvericin, enniatin A, enniatin A1, enniatin B, enniatin B1 and destruxin A were obtained from Sigma Aldrich in powder form (1 mg). Individual stock solutions were prepared by redissolving the powder in acetonitrile resulting in a concentration of 1 mg mL^{-1}. A standard mixture with equal concentrations of each mycotoxin was prepared.

Plant material

Fresh asparagus, potatoes, tomatoes as well as rice and wheat-meal were bought at local markets in Göttingen, Germany. Maize kernels were obtained from fields in Göttingen, Germany. Fresh tomatoes, potatoes and asparagus were cut into pieces, frozen at -80 °C and freeze dried. Maize cobs were dried at 55 °C for one week and kernels were milled. Rice and wheat-meal were used as purchased.

Mycotoxin extraction

For the evaluation of an adequate extraction solvent, 5 mL of different solvent combinations, containing the mycotoxin mixture with a final concentration of 1 µg g^{-1}, were added to 500 mg ground samples. The samples were shaken over night and then centrifuged at 4800 x g for 10 min. An aliquot of 800 µL of the supernatant was evaporated to dryness at 40 °C using a vacuum concentrator. After redissolving the residue thoroughly in 400 µL

methanol the same volume of water was added. The samples were mixed and stored at -20 °C until analysis commenced. Three replicates were prepared of each kind of meal (potato, asparagus, wheat, maize, rice and tomato) and extraction solvent.

Optimization of defatting step

Defatting tests were carried out by adding 10 µL of mycotoxin stock solution (10 µg mL^{-1}) and 10 µL of plant oil to reaction tubes containing 990 µL methanol/water in ratios of 85:15, 75:25 and 50:50. The samples were mixed thoroughly. Afterwards, 1 mL of n-hexane, cyclohexane or octane were added to the samples. One sample of each variant was colored by adding the red dye Sudan III to stain the fat. The samples were mixed thoroughly and centrifuged for 10 min at 4800 x g. For mycotoxin analysis aliquots (200 µL) of the methanol/water phase were transferred to new reaction tubes. The samples were stored at -20 °C until analysis commenced. Three repetitions were prepared of each variant.

HPLC-MS/MS analysis

Mycotoxin separation and analysis were carried out using a high pressure liquid chromatography system equipped with an autosampler (ProStar 410, Varian, Darmstadt, Germany), a binary pump system (ProStar 210, Varian, Darmstadt, Germany), a degasser (Degassit, MetaChem Technologies) and a column oven (Jetstream 2 plus, Techlab, Germany) coupled to an electrospray ionization (ESI) source followed by an ion trap mass spectrometer (500 MS, Varian, Darmstadt, Germany). Mycotoxin separation was carried out by high pressure liquid chromatography using a reverse phase column Kinetex C18 (50.0 x 2.1 mm, particle 2.6 µm) coupled with a C18 security guard cartridge (4 mm × 2 mm i.d., both from Phenomenex (Aschaffenburg, Germany) maintained at a temperature of 40 °C. An aliquot of 10 µL of the sample was used for injection. The mobile phase consisted of water with 5% acetonitrile (A) and methanol (B), both containing 0.01 mM sodium acetate and 7 mM acetic acid. The binary gradient used was: 0-3 min from 40 to 80% B, 3-8 min from 80 to 98% B, 8-11 min at 98% B, 11-11.5 min from 98 to 40% B and finally from 11.5-16.5 at 40% B. The flow rate was set to 0.2 ml min^{-1}. ESI was operated in positive mode for all analytes with the

following settings: spray chamber temperature 50 °C, nebulizing gas (nitrogen) 50 psi, drying gas (nitrogen) 25 psi at 350 °C, shield voltage 600 V, needle voltage 5000 V, trap damping gas (helium) 0.8 mL min^{-1}. The detector was operated in standard mode with 15.000 Da sec^{-1}. Control of the system was carried out using Varian MS workstation 6.9.1.
Full scan mode was carried out in positive mode scanning for mass range *m/z* 100-2000, with a scan speed of 15.000 Da sec^{-1}.

Validation parameters

For validation of the extraction process and the repeatability of the method the responses of pure standards, spiked matrix and spiked supernatant were used to evaluate matrix effects (ME), recovery of the extraction procedure (RE) and process efficiency (PE), as described by Matuszewski *et al.* [27]. The analysis of the pure analytical standard (100 ng mL^{-1}) for the determination of the mean peak area was repeated ten times. ME, RE, and PE values were calculated as follows:

$PE\ (\%) = 100 \times \text{Peak area}_{\text{spiked samples}} / \text{Mean peak area}_{\text{pure standards}}$
$ME\ (\%) = 100 \times \text{Peak area}_{\text{matrix-matched standards}} / \text{Mean peak area}_{\text{pure standards}}$
$RE\ (\%) = 100 \times \text{Mean peak area}_{\text{spiked samples}} / \text{Mean peak area}_{\text{matrix-matched standards}}$

For method validation specificity, linearity, limit of quantification (LOQ) and limit of detection (LOD) were evaluated. Artificially spiked samples in concentrations of 0.1, 0.5, 1, 5, 10 and 50 ng g^{-1} target compounds were subjected to the extraction procedure using acetonitrile/isopropyl alcohol/water (70:15:15). Each sample was analyzed three times. LOQ and LOD were calculated on the basis of the standard deviation of response (peak area) and the slope of the calibration line [28] and were defined as

$$LOQ = \frac{10*\sigma}{S} \quad \text{and} \quad LOD = \frac{3.3*\sigma}{S}$$

σ: standard deviation of the response
S: slope of the calibration line.

Linearity was examined by analyzing the relationship between response and concentration from the calibration curves. Additionally to the correlation coefficients the response factor was calculated by plotting the relative responses versus the analyte concentration.

Results and discussion

Optimization of the HPLC-MS/MS analysis

Specific masses of the precursor ions and fragmentation products, the radio frequency loading (RF loading) and the capillary voltage were determined and optimized by the direct infusion of all six mycotoxins each in positive ionization (Table 1).

Table 1. MS/MS parameters for the determination of beauvericin, enniatins and destruxin A.

Toxin	Molecular weight	Precursor ions	Product ions (*m/z*)			RF loading	Capillary voltage
	(g mol^{-1})	(*m/z*)	(1)	(2)	(3)	(%)	(V)
Beauvericin	783.95	806.4 $[M+Na]^+$	645.5*	545.5	384.5	90	140
Destruxin A	577.71	600.4 $[M+Na]^+$	572.6*	528.5	344.4	75	140
Enniatin A	681.90	704.5 $[M+Na]^+$	577.6*	477.4	350.6	85	155
Enniatin A1	667.88	690.5 $[M+Na]^+$	563.6	463.6	350.4*	90	130
Enniatin B	639.80	662.4 $[M+Na]^+$	549.5*	449.6	336.4	80	150
Enniatin B1	653.90	676.6 $[M+Na]^+$	563.5*	463.4	336.4	85	140

*used as quantifier

All mycotoxins showed greatest sensitivity in positive mode with sodium adducts as the highest signals. The addition of a cation to a solvent system is a common way to control complex formation and to exclude other complexes. Without the addition of excess cations to the solvent system, uncontrolled complexes can occur and the quantification of the mycotoxins becomes inaccurate due to different amounts of the cations among the samples [22]. In previous publications, the addition of ammonium acetate or ammonium formate led to the formation of ammonium adducts for beauvericin and enniatins and resulted in high sensitivity of the methods [21, 26]. However, in the current study the fragmentation of ammonium adducts as precursor ions, resulting from the addition of ammonium acetate to the mobile phase of concentrations up to 1 mM, led to low signal intensities of the fragments. In

most methods reported ammonium adduct fragmentation of one or more of these mycotoxins was carried out on triple quadrupol mass spectrometers [22, 23, 26]. Only Sewram *et al.* also used an ion trap to fragment the protonated ion of beauvericin and found MS/MS to be 500 times less sensitive than MS [24]. Furthermore, Uhlig and Ivanova fragmented the ammonium adduct only to the protonated form [21]. There is no other publication describing fragmentation of these toxins with an ion trap. The reason for low sensitivity is not known but it cannot be ruled out that the fragmentation mechanism is responsible for the discrepancies in fragmentation attempts.

The combination between capillary voltage and RF loading can mainly influence the intensity of analyte response, but sodium adducts were stable over a wide range of capillary voltage (50-200 V) and RF loading (50-250%). Sodium adducts proved to be robust, stable and sensitive in tandem mass spectrometry. To guarantee the occurrence of sodium in the solvent system, 0.01 mM sodium acetate was added to the binary solvent system. Three daughter ions were detected for each toxin (Table 1, Fig. 2).

To obtain high repeatability of the method the number of data points per chromatographic peak of all analytes were increased by separating the chromatogram into two different segments with destruxin A in segment one and beauvericin and enniatins in segment two. Additionally, single microscans with a scan time of 2.82 sec $scan^{-1}$ were used for beauvericin and enniatins and for destruxin A two microscans were averaged per data point with 3.38 sec $scan^{-1}$.

The Kinetex C18 column was chosen as the stationary phase in liquid chromatography to obtain a swift and, nevertheless, good separation of beauvericin, enniatins and destruxin A (Fig. 3). All four enniatins were completely separated, and only beauvericin co-eluted slightly with enniatin B1 and enniatin A1, but this is of minor importance as the compounds showed different mass transitions.

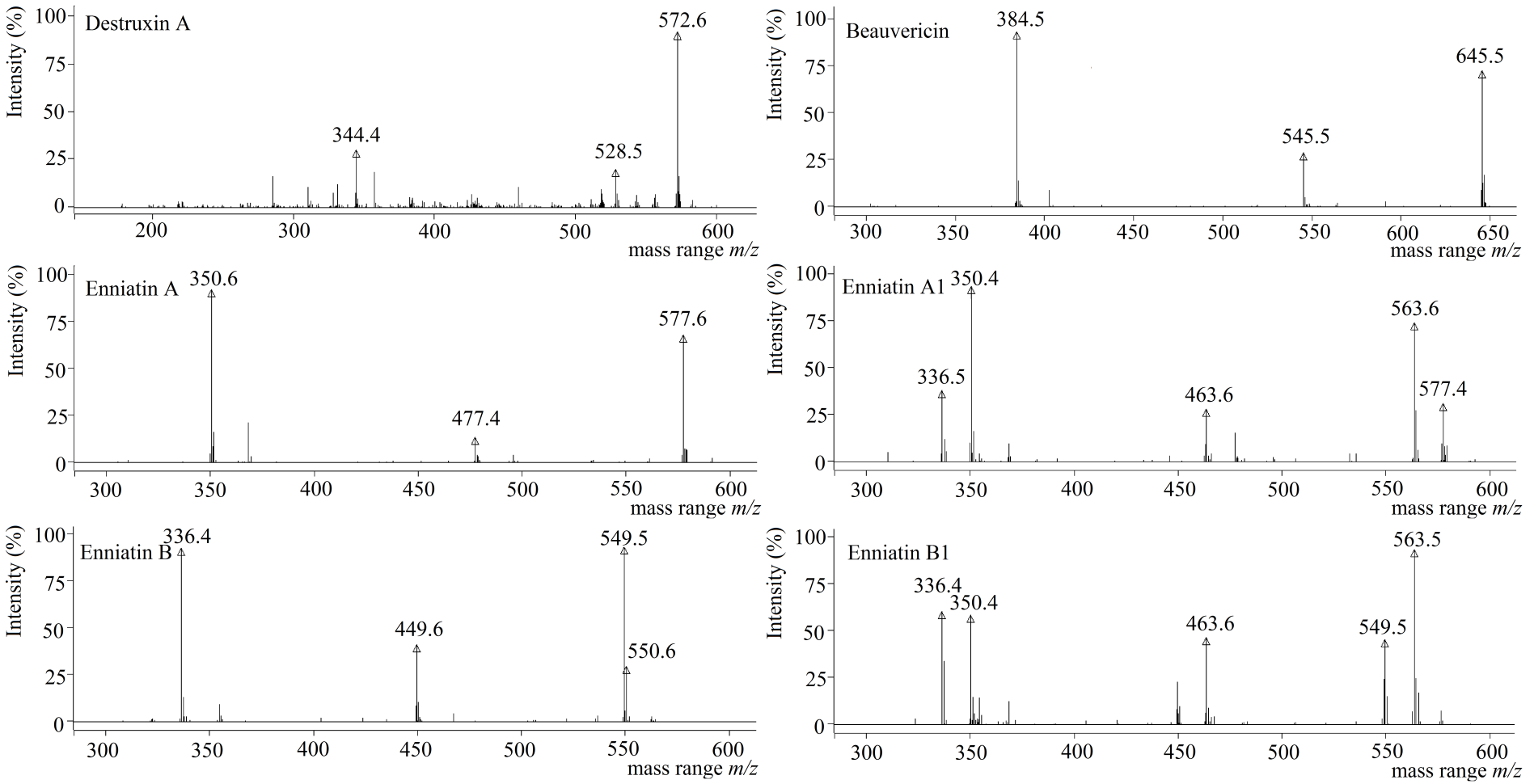


Figure 2. Mass spectra of examined mycotoxins obtained by HPLC-MS/MS analysis of pure standards in positive mode.

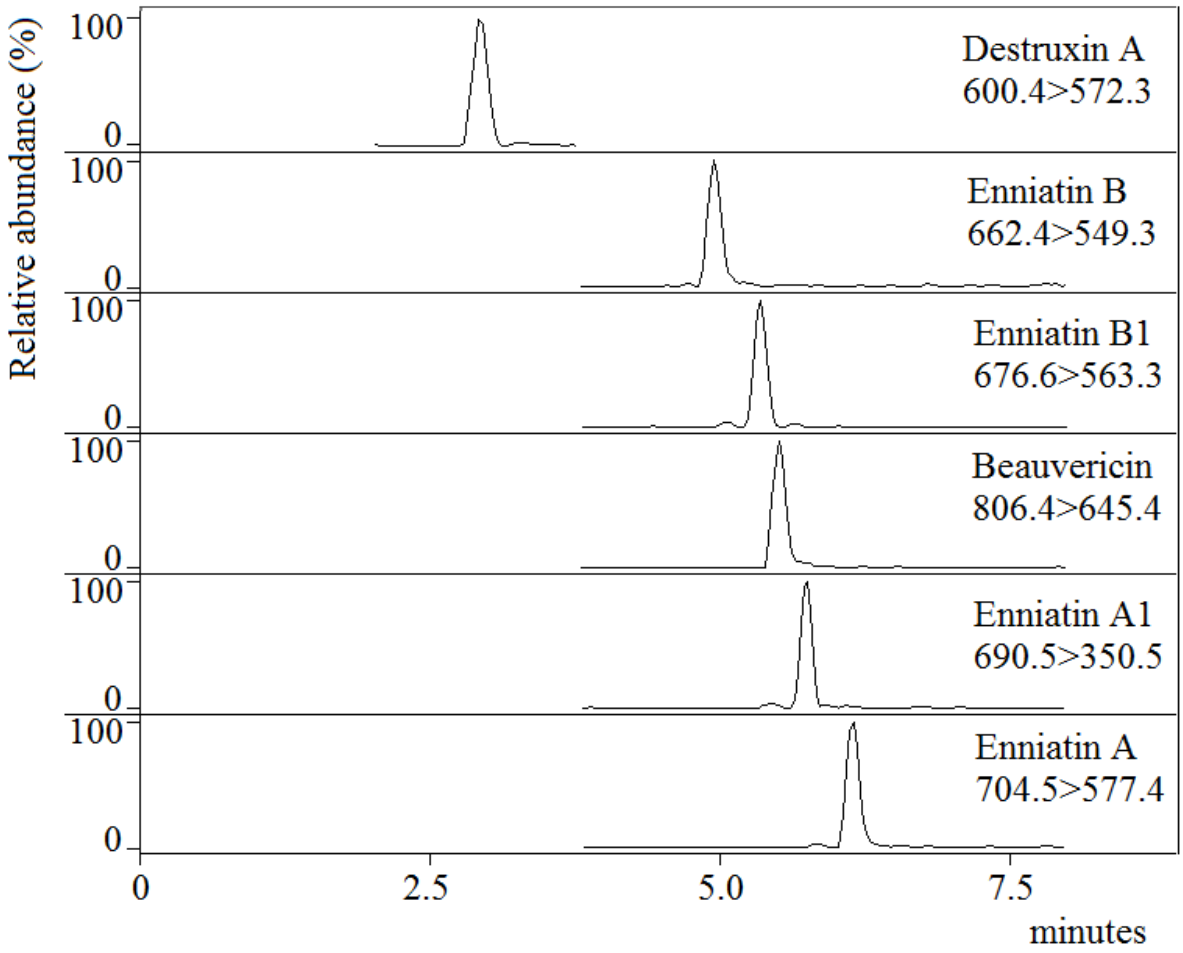


Figure 3. Selected chromatograms of the quantifier ions obtained from HPLC-ESI-MS/MS analysis of beauvericin, destruxin A and enniatin A, A1, B and B1 extracted from rice samples spiked with 50 ng g^{-1} of each mycotoxin.

Selection of the extraction solvent

Extraction and preparation of samples for mycotoxin analysis have to be conducted with care and with consideration of the chemical characteristics of each single analyte. Depending on the matrix, the extraction solvent influences the recovery and extraction efficiency but also the amount and composition of the co-extracted compounds according to their chemical properties such as polarity and acidity. They are caused by the co-elution of the compounds which interfere in the ionization and evaporation process of the analytes during analysis [29]. Different extraction solvent compositions based on acetonitrile, methanol and acetone were

tested for the extraction of beauvericin, enniatin A, A1, B and B1 as well as destruxin A on asparagus, maize, rice tomato, potato and wheat. The following solvents were used: acetonitrile/water (84:16), acetonitrile/isopropyl alcohol/water (70:15:15), methanol/isopropyl alcohol/water (80:5:15), acetone/water (80:20), methanol/water (75:25), acetonitrile/ acetone/water (80:6:14), acetone/water/acetic acid (80:19:1), acetonitrile/water/acetic acid (84:15:1). Full scan analysis in positive mode was carried out to compare the amounts of co-eluted components depending on the solvent composition and matrix. It must be borne in mind that only ionizable components were detectable. Huge discrepancies in the quantities of eluted components occurred depending on the matrices and extraction solvents (Fig. 4). Both methanol based extraction solvents led to high amounts of matrix components in potato, tomato and wheat at earlier retention time in the first half of the gradient. In the case of tomato both acetone based extraction solvents led to similar results. Less matrix compounds were detected in the rest of the matrices with only slight differences between the extraction solvents. Acetonitrile based solvent mixtures led to the lowest amounts of detectable matrix components.

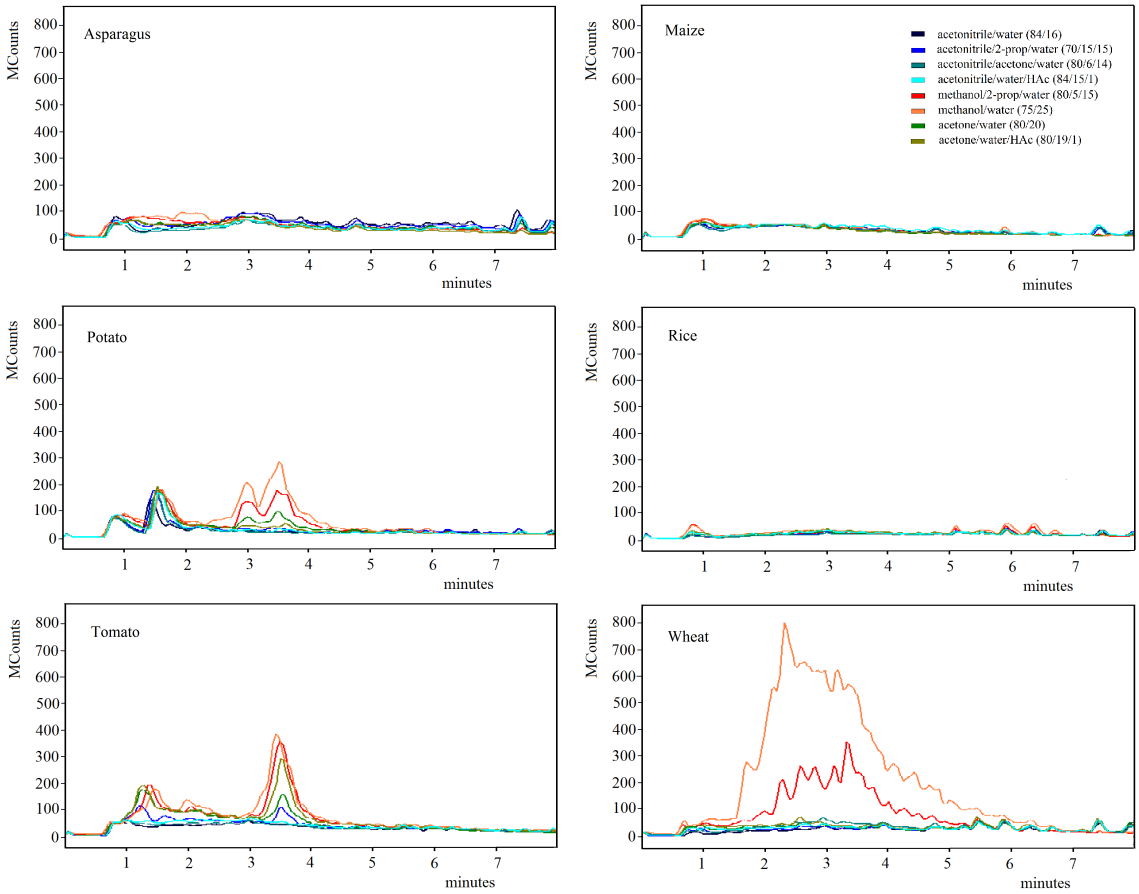


Figure 4. Overlaid chromatograms of spiked samples extracted with different solvent compositions and analyzed from *m/z* 100-2000 in positive mode

Furthermore, recovery of the extraction procedure, process efficiency and matrix effects were evaluated in order to determine the most suitable extraction solvent (Table 2). The recovery signals of the mycotoxins obtained with the extraction solvents were highly dependent on the matrices. The worst efficiencies were observed with methanol/isopropyl alcohol/water (80:5:15) and methanol/water (75:25), especially in asparagus, maize, tomato and wheat often with very low efficiencies of <50%. Moreover, extraction with both acetone-based mixtures led to highly variable results depending on mycotoxin and matrix. In most cases, the results of beauvericin and enniatins were greatly compromised, whereas the extraction of destruxin A often differed. In contrast to the other mycotoxins in maize, very high extraction efficiencies were achieved for destruxin A with almost all solvent mixtures. However, none of the solvent mixtures tested led to sufficient values for destruxin A (<65%) in the case of tomato.

Acetonitrile/isopropyl alcohol/water (70:15:15), acetonitrile/water (84:16) and acetoni-

trile/acetone/water (80:6:14) achieved comparably high efficiency rates for all mycotoxins in all matrices except destruxin A in tomato (only 63-64%). In comparison, addition of acetic acid (1%) to acetonitrile/water often resulted in lower but still acceptable values. Lower efficiency rates were mainly caused by inhibiting matrix effects, which can be seen in the reduced signal intensity of the analytes in spiked supernatants. Finally, acetonitrile/isopropyl alcohol/water (70:15:15) was chosen as a suitable solvent resulting in very good recovery rates, low matrix effects (Table 2) and relatively low amounts of co-eluted matrix components over a range of different matrices.

Table 2. Process efficiency (PE), matrix effects (ME) and recovery (RE) of the extraction procedure of beauvericin, enniatins and destruxin A in different cereals and vegetables; SD: Standard deviation; - indicates: not determined

	Beauvericin			Destruxin A			Enniatin A			Enniatin A1			Enniatin B			Enniatin B1		
	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)
Asparagus																		
ACN/ H_2O (84:16)	96±29	108±9	90	90±2	106±7	85	109±4	113±7	97	110±2	117±4	94	99±9	101±4	99	96±1	94±5	102
ACN/2-PrOH/H_2O (70:15:15)	93±3	108±9	87	85±2	91±1	93	101±7	89±7	113	90±9	95±10	95	93±6	90±11	104	86±2	78±11	110
ACN/Acetone/H_2O (80:6:14)	112±5	106±3	106	90±7	100±15	90	104±12	106±13	99	103±7	110±3	94	91±6	99±9	92	96±10	88±6	108
ACN/ H_2O/HAc (84:15:1)	93±11	99±5	95	85±7	89±1	96	103±7	110±11	94	98±9	98±8	99	95±8	96±2	99	96±6	99±7	97
MeOH/H_2O (75:25)	16±0	18±2	93	51±11	60±4	85	39±3	40±3	96	41±6	42±2	98	44±3	48±4	91	41±4	42±3	97
MeOH/2- PrOH/H_2O (80:5:15)	34±20	37±7	92	65±3	79±4	82	56±12	66±2	85	56±9	76±2	73	65±18	72±3	90	60±10	60±2	101
Acetone/ H_2O (80:20)	71±7	69±15	103	75±6	72±8	104	87±15	90±6	98	84±2	77±3	110	77±1	77±3	100	78±11	77±8	101
Acetone/H_2O/ HAc (80:19:1)	78±6	47±0	165	83±14	87±8	95	94±10	78±7	120	86±4	84±9	102	80±6	83±3	97	92±7	67±5	138
Maize																		
ACN/ H_2O (84:16)	77±9	95±5	82	106±7	96±5	110	86±9	83±6	103	103±5	86±3	120	95±6	71±3	134	96±1	91±8	105
ACN/2-PrOH/H_2O (70:15:15)	82±10	83±15	98	113±11	94±8	121	84±12	73±12	115	86±8	84±16	102	96±5	74±13	130	92±2	78±19	119
ACN/Acetone/H_2O (80:6:14)	83±14	123±7	68	104±6	102±13	101	83±6	86±5	96	98±9	104±10	94	96±8	110±8	88	96±3	110±7	87
ACN/ H_2O/HAc (84:15:1)	84±11	116±13	73	81±9	104±7	78	77±9	100±2	77	84±11	104±1	80	82±9	112±5	73	77±2	109±6	71
MeOH/H_2O (75:25)	37±4	90±12	42	105±3	104±8	101	44±6	70±7	63	83±5	87±9	96	87±4	88±8	98	81±6	92±15	88
MeOH/2- PrOH/H_2O (80:5:15)	88±6	136±11	65	104±4	109±4	95	89±5	90±9	100	53±6	121±10	44	43±4	103±7	42	50±7	113±7	45
Acetone/ H_2O (80:20)	36±12	-	-	108±4	-	-	39±7	-	-	42±12	-	-	47±7	-	-	44±6	-	-
Acetone/H_2O/ HAc (80:19:1)	87±7	118±33	74	96±5	114±10	84	76±12	105±18	72	43±3	108±23	39	95±3	71±18	134	96±8	91±18	105
Potato																		
ACN/ H_2O (84:16)	89±8	86±5	103	92±4	102±9	90	91±11	107±7	84	92±11	114±4	81	83±9	103±12	80	82±4	98±3	83
ACN/2-PrOH/H_2O (70:15:15)	97±1	97±9	101	96±5	103±7	93	94±2	112±6	81	93±5	114±13	81	94±8	91±17	104	84±6	93±4	91
ACN/Acetone/H_2O (80:6:14)	85±12	105±2	81	92±4	108±8	86	101±10	108±2	93	99±6	115±5	86	92±7	99±2	92	85±4	108±13	79
ACN/ H_2O/HAc (84:15:1)	96±5	86±7	111	94±10	98±8	95	98±6	96±7	102	93±4	91±3	102	96±8	96±5	100	88±8	96±8	91
MeOH/H_2O (75:25)	75±11	68±7	110	84±2	92±4	92	91±1	82±3	89	79±3	85±15	93	77±6	79±4	99	73±9	71±7	103
MeOH/2- PrOH/H_2O (80:5:15)	85±9	80±7	106	97±6	83±6	117	89±7	83±2	110	95±6	95±9	100	87±1	82±3	107	82±4	76±8	108
Acetone/ H_2O (80:20)	78±17	82±12	95	90±18	91±2	98	91±4	87±8	99	85±17	67±8	127	71±16	83±4	86	75±13	77±8	98
Acetone/H_2O/ HAc (80:19:1)	86±10	79±10	109	91±6	102±10	89	91±2	107±10	85	91±5	106±10	86	84±4	101±7	83	86±6	80±9	107

Table 2. (continued)

	Beauvericin			Destruxin A			Enniatin A			Enniatin A1			Enniatin B			Enniatin B1		
	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)	PE±SD (%)	ME±SD (%)	RE (%)
Rice																		
ACN/ H_2O (84:16)	104±12	111±3	94	89±8	107±2	83	104±10	105±12	99	94±6	111±7	85	97±6	116±4	84	96±3	107±7	90
ACN/2-PrOH/H_2O (70:15:15)	102± 4	118±3	86	88±2	105±18	84	100±4	105±1	95	90±7	121±2	74	92±5	123±9	75	98±5	105±7	93
ACN/Acetone/H_2O (80:6:14)	101±5	108±3	94	87±4	106±8	82	101±14	104±7	97	96±2	116±6	82	98±5	118±9	83	97±6	105±4	93
ACN/ H_2O/HAc (84/15:1)	88 ±11	93±5	95	82±8	85±3	97	90±15	84±8	108	87±7	80±3	109	81±13	88±4	92	87±13	99±14	88
MeOH/H_2O (75:25)	90 ±6	94±25	96	87±3	135±34	64	82±6	73±13	112	84±4	107±8	78	86±3	125±30	69	92±10	118±13	78
MeOH/2- PrOH/H_2O (80:5:15)	93 ±2	119±29	78	84±9	105±39	80	81±6	89±13	92	86±5	94±21	92	87±6	113±24	77	90±10	108±29	84
Acetone/ H_2O (80:20)	91 ±4	83±38	110	86±4	94±9	91	85±6	78±14	109	90±5	90±11	100	87±12	94±4	93	82±11	91±11	91
Acetone/H_2O/ HAc (80:19:1)	97 ±5	103±26	94	90±2	100±6	90	101±6	98±9	103	92±5	106±6	87	95±18	110 ± 4	86	92±12	107±6	86
Tomato																		
ACN/ H_2O (84:16)	94±2	97±4	96	65±6	74±16	88	106±10	101±16	105	99±7	114±9	86	96±13	104±10	93±	99±7	95±12	104
ACN/2-PrOH/H_2O (70:15:15)	83±9	90±7	92	63±4	63±6	99	100±12	108±13	93	83±10	104±11	80	96±11	96±4	100±	81±5	89±13	91
ACN/Acetone/H_2O (80:6:14)	98±6	98±12	100	64±2	74±6	87	100±9	103±77	97	101±4	107±18	94	105±3	97±9	108±	99±16	95±6	105
ACN/ H_2O/HAc (84:15:1)	104±8	-	-	65±0	-	-	108±4	-	-	104±13	-	-	111±1	-	.	99±2	-	-
MeOH/H_2O (75:25)	40±3	37±2	108	48±4	52±5	91	44±3	44±5	101	43±4	46±8	92	43±3	43±5	98±	40±6	37±5	109
MeOH/2- PrOH/H_2O (80:5:15)	46±4	51±5	91	50±6	63±5	79	50±0	65±6	78	53±2	68±8	77	56±1	64±3	87±	50±4	62±2	81
Acetone/ H_2O (80:20)	78±3	61±2	128	64±3	57±2	114	91±6	83±4	110	84±4	73±1	116	87±7	72±5	121±	55±48	73±6	76
Acetone/H_2O/ HAc (80:19:1)	79±3	77±4	103	61±1	82±16	74	90±1	93±2	96	81±0	88±7	92	86±2	98±4	88±	80±5	76±3	106
Wheat																		
ACN/ H_2O (84:16)	99±6	122±10	81	94±5	107±6	87	78±8	104±16	75	89±11	108±1	82	95±4	124±8	77	86±6	98±5	87
ACN/2-PrOH/H_2O (70:15:15)	104±1	122±2	85	92±5	107±10	86	95±11	109±5	88	89±8	115±14	77	93±8	135±10	69	84±5	97±3	86
ACN/Acetone/H_2O (80:6:14)	99±3	103±17	96	87±5	91±6	96	95±4	114±13	83	91±2	101±11	90	100±9	105±7	95	84±6	84±2	100
ACN/ H_2O/HAc (84:15:1)	87±9	107±8	81	82±6	81±7	102	84±8	99±9	85	84±3	87±7	96	86±8	109±19	79	77±2	97±8	79
MeOH/H_2O (75:25)	69±4	69±7	99	26±3	20±3	133	61±6	75±5	81	77±6	86±6	89	79±2	102±5	78	73±3	81±1	90
MeOH/2- PrOH/H_2O (80:5:15)	57±12	65±10	87	81±8	70±19	116	62±7	84±2	74	68±9	90±8	77	84±6	101±13	83	71±9	83±5	85
Acetone/ H_2O (80:20)	97±13	83±4	117	85±4	79±1	108	90±6	91±11	99	82±2	82±9	101	92±3	96±4	95	77±1	87±6	89
Acetone/H_2O/ HAc (80:19:1)	90±2	102±13	89	77±4	86±10	90	89±8	98±11	91	87±12	98±21	89	91±7	107±5	85	74±4	91±7	82

Sample defatting

Fat is usually removed prior to analysis to avoid compounds that interfere during quantitative analysis and also to protect the HPLC column from damages. However, defatting of sample extracts containing beauvericin with n-hexane proved to be a critical reason for relative losses of up to 62% during sample preparation [30]. Ediage *et al.* received sufficient results by using methanol/water (85:15) with dichloromethane/n-hexane (30:70), formulating the hypothesis that the high solvent content prevents losses of the toxins to the dichloromethane/n-hexane phase [25]. However, visual assessment of defatting efficiency by staining fats showed that the suggested solvent combinations resulted in insufficient defatting efficiency in maize matrix, making the step of no use (Fig. 5).

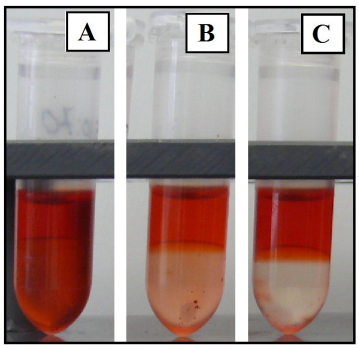


Figure 5. Maize extracts in methanol/water A) 85:15, B) 75:25 and C) 50:50 and dichloromethane/n-hexane (30:70). Fat is colored with the dye Sudan III.

Further investigations on defatting samples for analysis in HPLC were carried out testing n-hexane, cyclohexane and octane (Fig. 6). The fat contents of the healthy plant material tested varies greatly, ranging usually from approximately 0.1-5% and for high oil maize varieties up to 9%. Therefore, the experiment was conducted independently of these matrices and their fat contents by the artificial spiking of plant oil with a final concentration of 0.5% which corresponds to approximately 10% fat content in plant material. Only in the lowest concentration of methanol (50%) phase separation with octane was obtained, but almost no toxin was found (Fig. 6). Destruxin A was detected with sufficient recoveries in all other solvent combinations. In contrast, beauvericin and enniatins resulted in high losses (60-80%) when using n-hexane and cyclohexane in methanol/water (50:50). Recoveries increased with

solvent concentrations as also Ediage *et al*. [25] obtained, but again defatting efficiency decreased with insufficient results at the highest concentration. Defatting with n-hexane in methanol/water (75:25) showed the best compromise between defatting efficiency and recoveries. For enniatins a loss of about 20% was detected, while almost no reduction was obtained for destruxin A and beauvericin. Although these results provide an opportunity to defat samples in future analysis the step was omitted out for method validation in the current study.

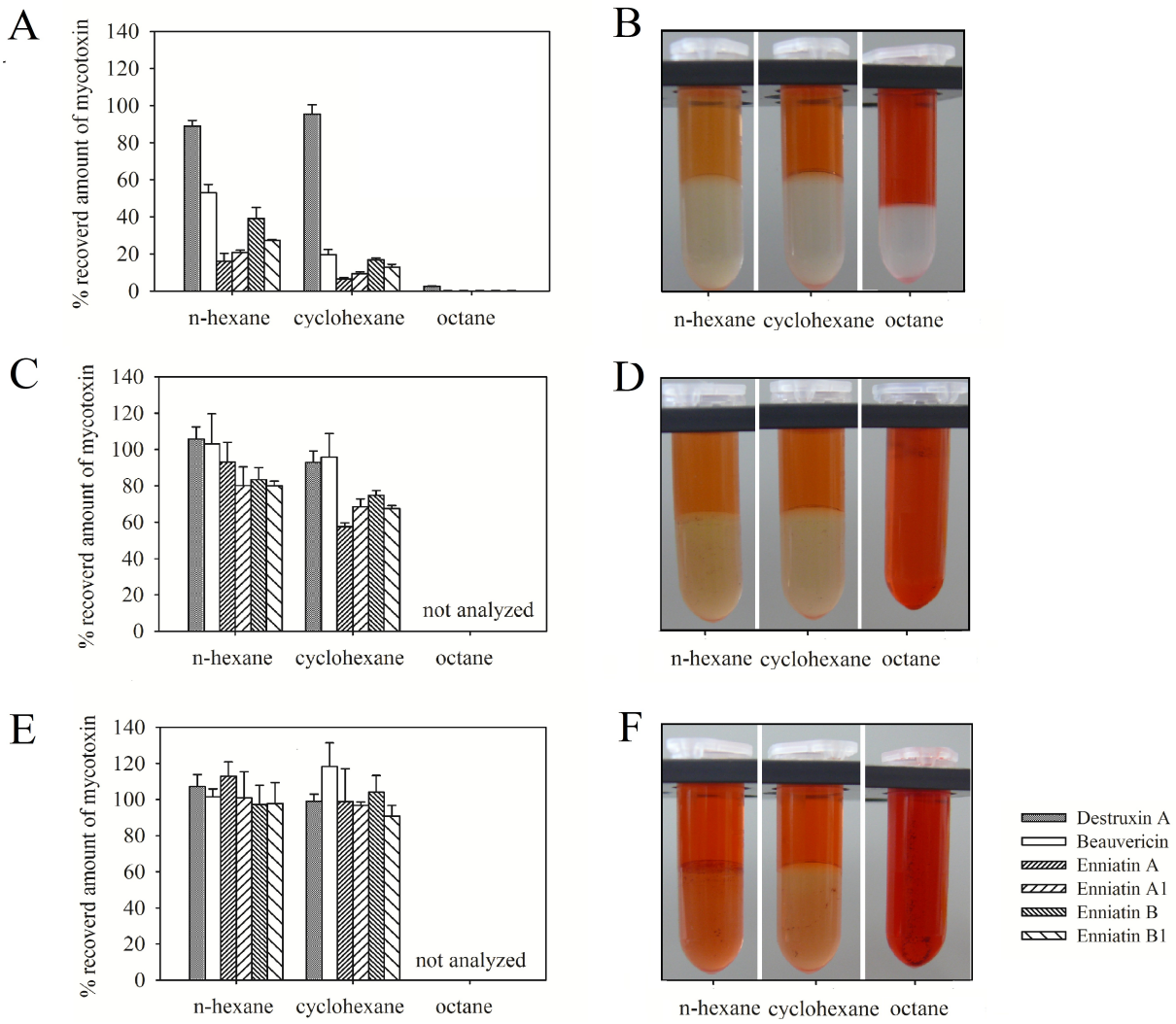


Figure 6. Relative amounts of mycotoxins and corresponding samples colored with Sudan III in methanol/water A/B) 50:50, C/D) 75:25 and E/F) 85:15 after defatting with n-hexane, cyclohexane and octane. The samples were compared to not defatted controls

Method validation

For the validation of the optimized method the parameters specificity, linearity, LOQ and LOD were determined. Meal from all six matrices was spiked with destruxin A, beauvericin and enniatins in triplicate at six concentration from 0.1 to 50 ng g^{-1} and extracted with acetonitrile/isopropyl alcohol/water (70:15:15).

The retention time and three specific ion transitions were used for the identification and confirmation of the specific compound. The fragmentation of enniatins results partly in the occurrence of the same daughter ions but differences in that mass of the precursor ions and retention times allow specific determination. Chromatographic separation of the mycotoxins via HPLC coupled with tandem mass spectrometry resulted in a highly specific analytical method. Retention time, the precursor ion and three daughter ions fulfill more identification points than stipulated by the Commission Decision 2002/657/EC [31], describing the performance criteria for analytical methods.

A common method for the calculation of LOQs and LODs in mass spectrometry bases on the calculation of the signal-to-noise ratio. However, tandem mass spectrometry in ion trap provides very low background noise close to zero. The use of these low noise values for the calculation the signal-to-noise ratios is not meaningful. The LOQs and LODs were calculated on the basis of the residual standard deviation and the slope of the calibration line. LOQs and LODs ranged from 1 to 12 ng g^{-1} and 0.3 to 4 ng g^{-1}, respectively (Table 3). Because of the slight natural contamination of potato and asparagus with beauvericin and wheat with enniatin B and B1 no method limits could be determined. Overall, the method proved to be very sensitive for measuring the six hexadepsipeptides in the tested plant matrices.

Table 3. Method limits of quantification and detection.

	LOQ (ng g^{-1})						LOD (ng g^{-1})					
	Aspa-ragus	Maize	Potato	Rice	Tomato	Wheat	Aspa-ragus	Maize	Potato	Rice	Tomato	Wheat
Beauvericin	-	3.5	-	6.4	2.3	3.6 -	-	1.1	-	2.1	0.8	1.2
Destruxin A	11.0	4.9	1.0	4.9	8.4	8.5	3.6	1.6	0.3	1.6	2.8	2.8
Enniatin A	12.3	2.3	5.7	4.4	3.8	2.8	4.1	0.8	1.9	1.4	1.2	0.9
Enniatin A1	5.6	10.4	2.7	3.3	5.1	3.5	1.4	3.4	0.9	1.1	1.7	1.1
Enniatin B	7.3	5.0	1.2	7.4	8.0	-	2.4	1.7	0.4	2.4	2.7	-
Enniatin B1	6.6	2.4	6.4	4.6	6.1	-	2.2	0.8	2.1	1.5	2.0	-

For evaluation linearity, the calibration line was combined with a response factor plot. Response factor plots with data points obtaining an equivalent response at each concentration form a straight line with a zero slope [32]. In all plant matrices and mycotoxins the plot of the response factor versus analyte concentration revealed an upward movement in the lower concentrations of 0.1 up to 1 ng g^{-1} resulting in a deviation from linearity (Fig. 7a-f). Values of LOQ corresponded to concentrations of beginning deviation from linearity so that accurate quantification of the mycotoxins was ensured.

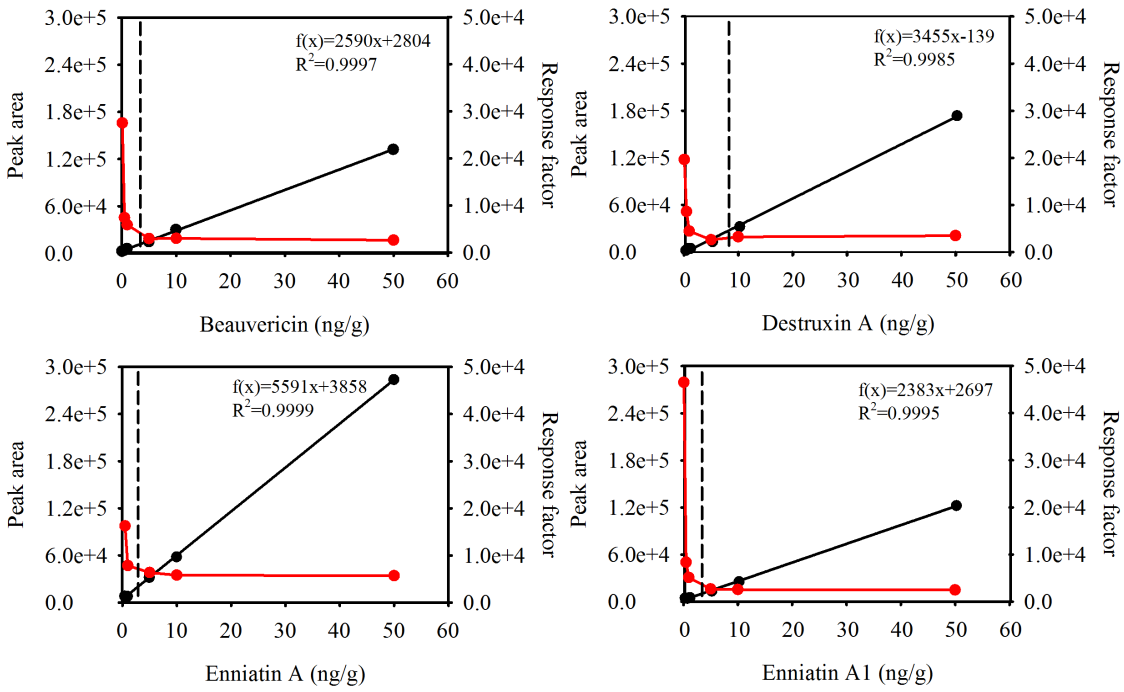


Figure 7a. Regression line (black line) and response factor plot (red line) of beauvericin, destruxin A, enniatin A, enniatin A1, enniatin B and enniatin B1 in wheat. The dotted line represents the limit of quantification of the specific assay.

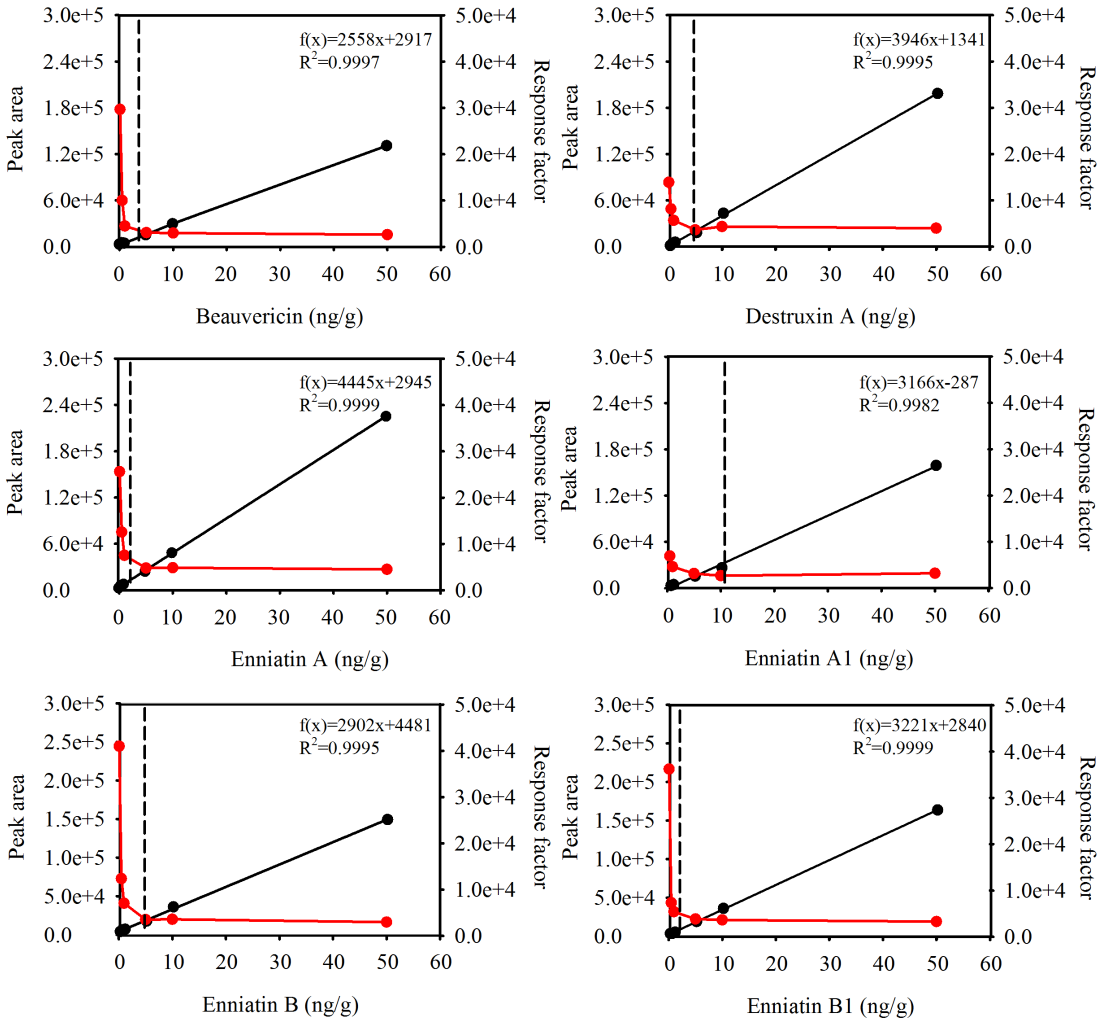


Figure 7b. Regression line (black line) and response factor plot (red line) of beauvericin, destruxin A, enniatin A, enniatin A1, enniatin B and enniatin B1 in maize. The dotted line represents the limit of quantification of the specific assay.

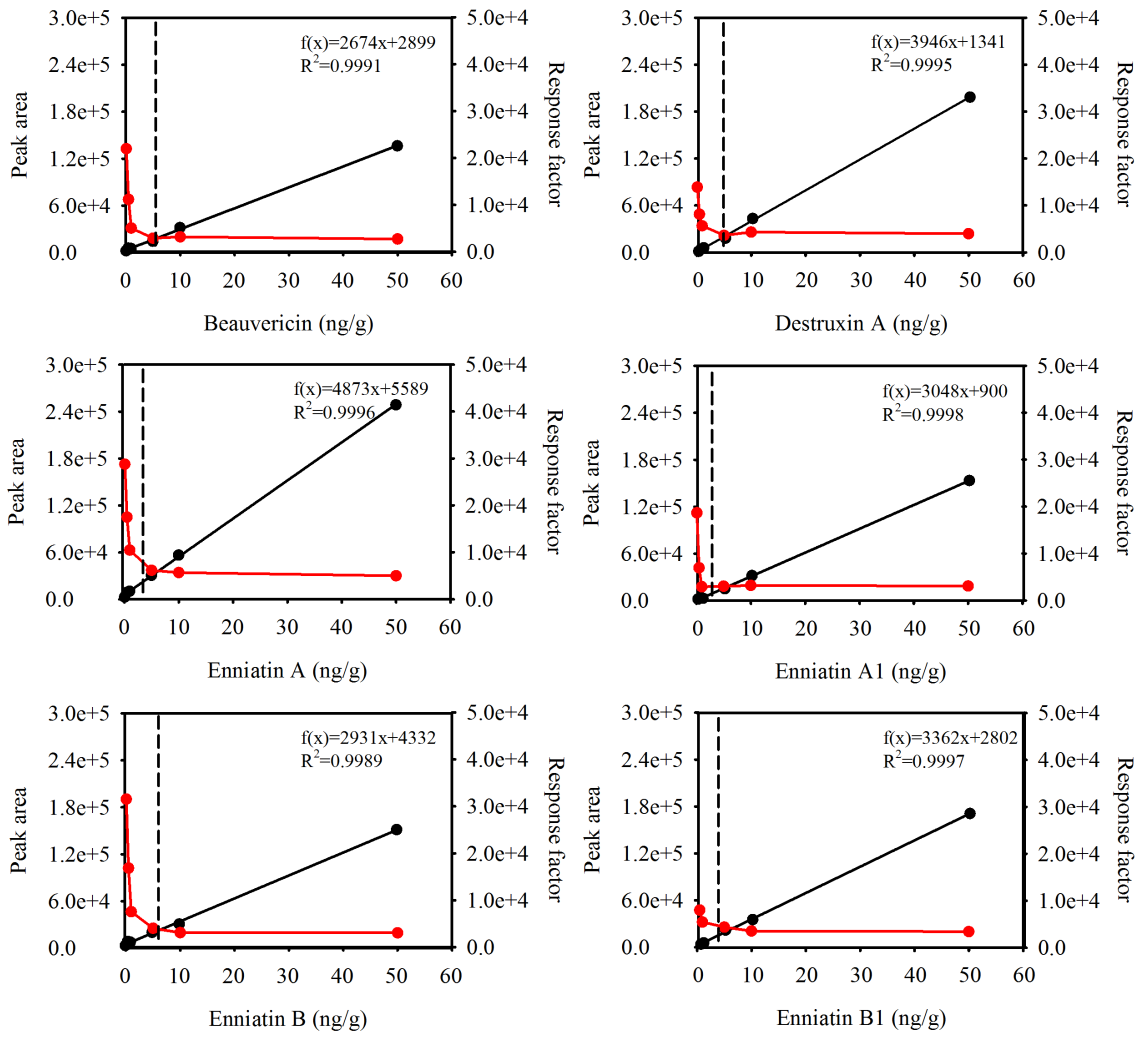


Figure 7c. Regression line (black line) and response factor plot (red line) of beauvericin, destruxin A, enniatin A, enniatin A1, enniatin B and enniatin B1 in rice. The dotted line represents the limit of quantification of the specific assay.

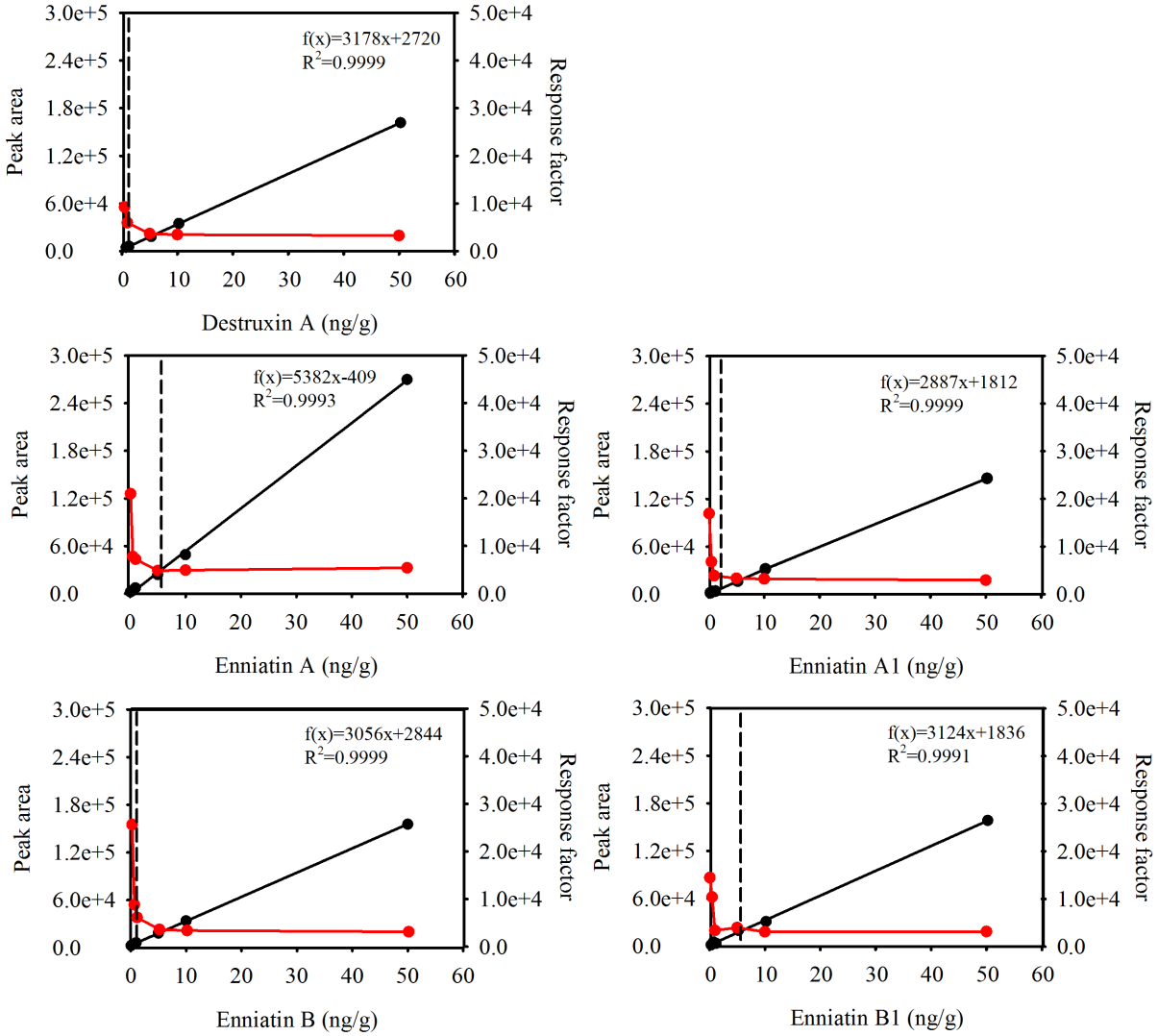


Figure 7d. Regression line (black line) and response factor plot (red line) of destruxin A, enniatin A, enniatin A1, enniatin B and enniatin B1 in potato. The dotted line represents the limit of quantification of the specific assay.

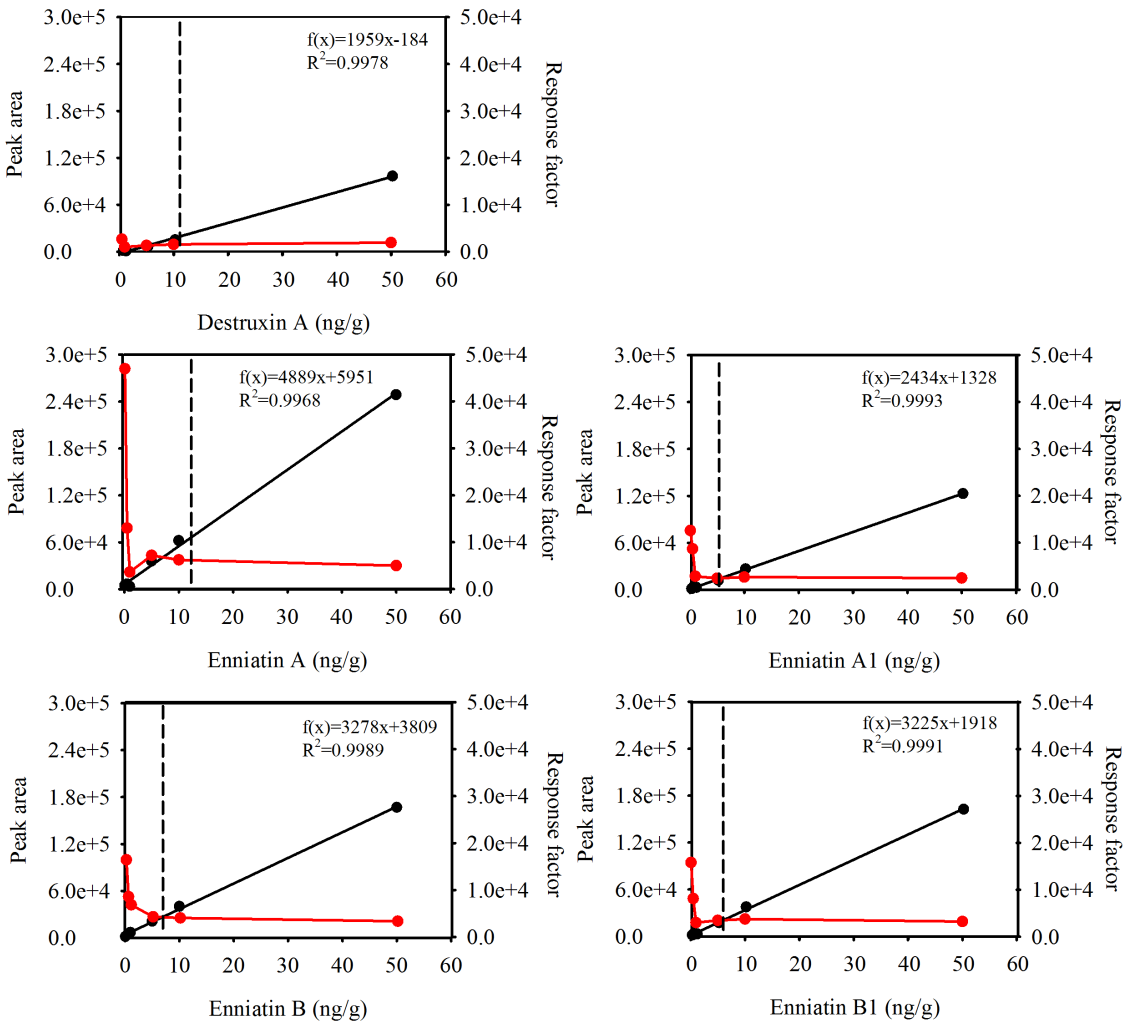


Figure 7e. Regression line (black line) and response factor plot (red line) of destruxin A, enniatin A, enniatin A1, enniatin B and enniatin B1 in asparagus. The dotted line represents the limit of quantification of the specific assay.

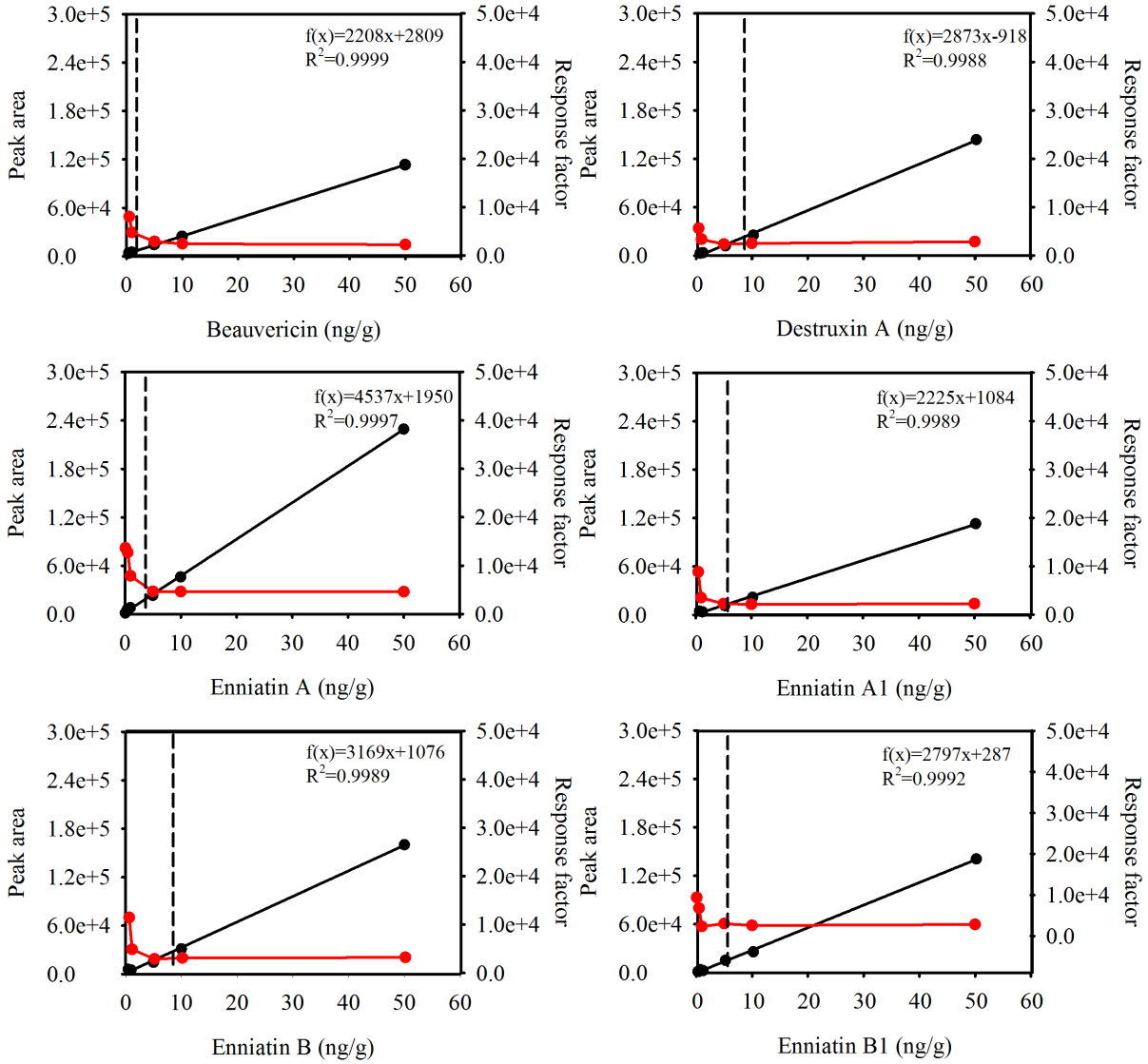


Figure 7f. Regression line (black line) and response factor plot (red line) of destruxin A, enniatin A, enniatin A1, enniatin B and enniatin B1 in tomato. The dotted line represents the limit of quantification of the specific assay.

The method was developed for the simultaneous detection of the six hexadepsipeptides beauvericin, destruxin A and enniatins A, A1, B and B1 in six different matrices. The extraction mixture acetonitrile/isopropyl alcohol/water (70:15:15) was found to extract the mycotoxins with high recovery rates, low matrix effects and with low amounts of co-eluted components over the whole HPLC gradient. This study is the first report of tandem mass spectrometry analysis of these six hexadepsipeptides using ion trap based on sodium adduct

fragmentation.

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Chapter 3: Determination of the LOQ in real-time PCR by receiver operating characteristic curve analysis: application to qPCR assays for *Fusarium verticillioides* and *F. proliferatum*

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Abstract

Real-time PCR (qPCR) is the principal technique for the quantification of pathogen biomass in host tissue, yet no generic methods exist for the determination of the limit of quantification (LOQ) and the limit of detection (LOD) in qPCR. We suggest using the Youden index in the context of the receiver operating characteristic (ROC) curve analysis for this purpose. The LOQ was defined as the amount of target DNA that maximizes the sum of sensitivity and specificity. The LOD was defined as the lowest amount of target DNA that was amplified with a false-negative rate below a given threshold. We applied this concept to qPCR assays for *Fusarium verticillioides* and *Fusarium proliferatum* DNA in maize kernels. Spiked matrix and field samples characterized by melting curve analysis of PCR products were used as the source of true-positives and true-negatives. On the basisi of the analysis of sensitivity and specificity of the assays, we estimated the LOQ values as 0.11 pg of DNA for spiked matrix and 0.62 pg of DNA for field samples for *F. verticillioides.* The LOQ for *F. proliferatum* were 0.03 pg for spiked matrix and 0.24 pg for field samples. The mean LOQ values correspond to approximately eight genomes for *F. verticillioides* and three genomes for *F. proliferatum*. We demonstrated that the ROC analysis concept, developed for qualitative diagnostics, can be used for the determination of performance parameters of quantitative PCR.

Keywords Real-time PCR - *Fusarium verticillioides* - *Fusarium proliferatum* - Receiver operating characteristic - Limit of detection - Limit of quantification

Introduction

Real-time PCR (qPCR) is the standard analytical method for quantifying pathogen biomass in the tissue of host organisms. Standard performance parameters of an analytical method are the limit of detection (LOD) and the limit of quantification (LOQ). The LOD is defined as the lowest amount of the analyte detectable in a single reaction. The LOQ is the lowest amount of analyte that can be quantified. The methods commonly used in chemical analysis for determining LOD and LOQ values [1 - 3] are unsuitable for qPCR.

We suggest that the LOD and LOQ can be determined by use of receiver operating characteristic (ROC) curve analysis, which is a method used to evaluate the sensitivity and specificity of diagnostic tests. ROC is based on a comparison of the outcome of a series of assays ("positive" and "negative") with the "true" status of the samples. The "true" status is either evaluated with a well-established test, which is called the "gold standard", or known *a priori* because the samples were prepared by spiking negative matrix with the target analyte. The central concept in ROC curve analysis is the cutoff point. The cutoff point is a threshold value of the analytical signals below which samples are regarded as negative and above which samples are regarded as positive. The ROC curve is a plot of the sensitivity (genuinely positive samples that are detected as positive, "true positives") against 1- specificity (negative samples that are detected as positive, "false positives") for different cutoff points [4]. In qPCR, the cutoff point is the threshold cycle above which a sample is considered to be negative. If a cycle number is chosen as a cutoff point, the fraction of positive samples that reached the threshold of fluorescence intensity before this cycle is the "true-positive fraction". The fraction of negative samples that reached the threshold of fluorescence intensity before this cycle is the "false-positive fraction". If a higher cycle number is chosen as a cutoff point, more samples are likely to be rated as positive, increasing the sensitivity. At the same time, the false-positive rate is likely to grow and the specificity to decrease. An optimal cutoff point corresponds to the desired trade-off between true-positive and false-negative rates. To balance the demands for sensitivity and specificity of a diagnostic assay, i.e., to determine the optimal cutoff point, the Youden index is often used [23].

Using artificially prepared, spiked samples for estimating an optimal cutoff value guarantees that the assignment of samples to true positives and true negatives is correct. The drawback is that the properties of matrix spiked with target DNA may differ from the properties of

samples obtained from the field. The optimal cutoff point determined with the help of spiked samples may therefore differ from the optimal cutoff point for field samples. In the current research, we investigated this dilemma by assigning field samples to true positive and true negative by melting curve analysis. We then compared cutoff values derived for field samples with those obtained for spiked matrix. As a model system, we used the fungal plant pathogens *Fusarium verticillioides* and *Fusarium proliferatum* in maize kernels.

Fusarium species are among the most important pathogens of maize worldwide. Infection with *Fusarium* spp. reduces grain yield and quality [5], and infected grain, when used for the production of food and feedstuff, is often contaminated with mycotoxins that endanger the health of consumers and livestock [6]. Illness of farm animals and less frequently of humans caused by *Fusarium* mycotoxins has regularly been reported [7 - 9].

Fusarium species cause two types of ear rot in maize: red ear rot (*Gibberella* ear rot) caused by *Fusarium* spp. belonging to the *Discolor* section, and pink ear rot (*Fusarium* ear rot or ear mold) caused by species of the *Liseola* section. *Fusarium* species isolated from cobs exhibiting pink ear rot symptoms are usually *Fusarium verticillioides*, *F. proliferatum*, and *F. subglutinans* [5]. Apart from beeing found in maize [10] and asparagus [11], *F. proliferatum* has been found in wheat [12], sorghum [13], and rice [14], but only infection of the first two crops is considered economically relevant. *F. verticillioides* and *F. proliferatum* are producers of fumonisin mycotoxins. Fumonisins B1 (FB1) and B2 (FB2) are the most abundant fumonisins in maize, and levels of FB1 are generally higher than those of FB2 [15]. FB1 causes leukoencephalomalacia in horses and pulmonary edemas in swine [16], and it is very likely that FB2 and B3 have the same effects. Although toxicologically relevant amounts of fumonisins in maize are occasionally found in food products in countries with a highly developed agriculture, serious health impacts of fumonisin contamination are thought to occur in areas with suboptimal growing and storage conditions and a high maize consumption [17]. Indeed, levels of FB1 and FB2 in maize used as staple food in South Africa correlated with the incidence of esophageal cancer [18]. Beside fumonisins, *F. verticillioides* produces the mycotoxins fusaric acid and fusarins, whereas *F. proliferatum* was reported to produce mycotoxins beauvericin, enniatins, fusaproliferin, and moniliformin [19].

The relationship between the development of symptoms, the amount of fungal biomass in the plant tissue, and the production of mycotoxins is incompletely understood. Ramirez et al. [20] found that fumonisin contamination and the level of infection for *Fusarium* species of the

Liseola-section did not correlate. In contrast, Pascale et al. [21] found that fumonisin contamination was highly correlated with ear rot symptoms after inoculation of maize with *F. verticillioides* or *F. proliferatum*. Clarifying the relationship between the accumulation of fungal biomass in the plant, development of symptoms, and mycotoxin production requires a species-specific method to reliably quantify *F. verticillioides* and *F. proliferatum* biomass in plant tissue.

qPCR is useful for quantifying fungal colonization of crops while distinguishing among species. Species-specific PCR primers have been developed for most *Fusarium* species that cause ear rot [22 - 26].

In this work, we evaluate qPCR assays for quantification of *F. verticillioides* and *F. proliferatum* in maize kernels. Furthermore, we examined the use of the Youden index in the framework of ROC curve analysis for estimating the LOD and LOQ of qPCR assays.

Materials and Methods

Fungal cultures

The fungal strains used in this study are listed in Table 1. Cultures for DNA extraction were grown in 100 ml of potato dextrose broth (24 g l^{-1}; Scharlau, Barcelona, Spain) at room temperature and without shaking. The mycelium was harvested after 14 days by filtration and then freeze-dried.

Table 1. Fungal strains used in this work

Species	**Strain**	**Source**
Fusarium acuminatum	ICARDA 93803	F
Fusarium acuminatum	ICARDA 92099	F
Fusarium acuminatum	ICARDA 93682	F
Fusarium acuminatum	ICARDA 93831	F
Fusarium avenaceum	Fa95	C
Fusarium avenaceum	Fa23	E
Fusarium avenaceum	Fa21	E
Fusarium avenaceum	Fa39	E
Fusarium avenaceum	Fa5-2	E
Fusarium avenaceum	Fa7	E
Fusarium concolor	Fconc1	E
Fusarium concolor	Fconc2	E
Fusarium crookwellense	BBA 63558, DSM 8704	D
Fusarium crookwellense	BBA 64483	D
Fusarium crookwellense	BBA 64545	D
Fusarium culmorum	Fc15	I [27]
Fusarium culmorum	Fc2	D [27]
Fusarium culmorum	Fc22	I [27]
Fusarium culmorum	CBS 251.52	A
Fusarium culmorum	FcH69	E
Fusarium graminearum	DSM 62217	B [27]
Fusarium graminearum	DSM 62722	B [27]
Fusarium graminearum	DSM 64848	B [27]
Fusarium graminearum	DSM 67638	B [27]
Fusarium graminearum	DSM 4528	B [27]
Fusarium graminearum	DSM 1096	B
Fusarium oxysporum	FO 125	E
Fusarium oxysporum	SAGW 124	E
Fusarium oxysporum	Foxy121	E
Fusarium oxysporum	Foxy436	E
Fusarium oxysporum	Foxy119	E
Fusarium oxysporum	Foxy6	E
Fusarium poae	DSM 62376	B
Fusarium poae	FP 2	I
Fusarium poae	Fpoae 369	E
Fusarium poae	Fpoae 365	E
Fusarium poae	Fpoae 517	E
Fusarium proliferatum	DSM 764	B
Fusarium proliferatum	DSM 840	B

Table 1 (continued)

Species	**Strain**	**Source**
Fusarium proliferatum	DSM 62267	O
Fusarium proliferatum	DSM 62261	O
Fusarium proliferatum	DSM 63267	O
Fusarium proliferatum	FPRO1	N [23]
Fusarium proliferatum	FPRO2	N [23]
Fusarium proliferatum	FPRO3	N
Fusarium proliferatum	FPRO4	N
Fusarium proliferatum	FPRO5	N
Fusarium proliferatum	FPRO8	N
Fusarium proliferatum	FPRO9	N
Fusarium proliferatum	FPRO11	N
Fusarium proliferatum	FPRO12	N
Fusarium proliferatum	D00502	G [12, 40]
*Fusarium sacchari (*former *subglutinans)*	B03852	G [40, 41]
*Fusarium sacchari (*former *subglutinans)*	B03853	G [41]
Fusarium solani	Fsol1	E
Fusarium subglutinans	B00278	G [12]
Fusarium subglutinans	B00281	G [12]
Fusarium subglutinans	B01722	G [40]
Fusarium subglutinans	B01728	G [40]
Fusarium subglutinans	B038J	G
Fusarium subglutinans	B03819	G
Fusarium subglutinans	B03820	G
Fusarium subglutinans	B03821	G
Fusarium subglutinans	B03828	G [40]
Fusarium subglutinans	E02192	G [12]
Fusarium tricinctum	FT1	E
Fusarium tricinctum	FT2	E
Fusarium tricinctum	FT3	E
Fusarium verticillioides	1.51	M [23]
Fusarium verticillioides	EJAB,21/1BA	L [23]
Fusarium verticillioides	FRC M-7358	K [42]
Fusarium verticillioides	FRC M-7362	K [42]
Fusarium verticillioides	FRC M-7367	K [42]
Fusarium verticillioides	FRC M-7370	K [42]
Fusarium verticillioides	FRC M-7437	K [42]
Fusarium verticillioides	FRC M-7363	K [42]
Fusarium verticillioides	FRC M-8114	J [39, 42]
Fusarium verticillioides	FV 234/1	P [39]

Table 1 (continued)

Species	Strain	Source
Fusarium verticillioides	1.34	M [23]
Fusarium verticillioides	F01377	G [12, 40]
Fusarium verticillioides	A00102	G [12]
Fusarium compactum	ICARDA 93823	F
Acremonium chrysogenum	AC1	E
Acremonium chrysogenum	AC2	E
Acremonium longisporum	AL	E
Acremonium ochraceum	AO	E
Acremonium polychromum	AP	E
Alternaria alternata	A 4.1.1	E
Cladosporium herbarum	CH 3	C
Cladosporium herbarum	CH 4	E
Drechslera sorokiniana	D 3.1	E
Microdochium nivale	GN 7	I
Microdochium nivale	GN 25	I
Microdochium nivale	GN 35	I
Microdochium nivale	GN 36	I
Pseudocercosporella herpotrichoides	C39A	E
Pseudocercosporella herpotrichoides	PHA 20/3	C
Rhizoctonia cerealis	INRA 161	H
Rhizoctonia cerealis	SAGW J7	E
Rhizoctonia cerealis	SAGW J5	E
Septoria nodorum	7n/II/2	E
Ustilago maydis	DSM 3121	B

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DNA isolation from pure fungal cultures grown in liquid media

A variant of the cetyltrimethylammonium bromide method as described by Brandfass adn Karlovsky [27] was used, and the quality and quantity of DNA was estimated by electrophoresis in 0.8 % (w/v) agarose gels (Cambrex, Rockland, ME, USA) prepared in

40 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM EDTA, pH adjusted to 8.5 with acetic acid. The electrophoresis was carried out at 4 V cm^{-1} for 90 min. The gel was stained with ethidium bromide (2 mg l^{-1}) and documented with a digital imaging system (Vilber Lourmat, Marne la Vallee, France). The densitometry was performed using Multi Analyst-Software (BioRad, Hercules, CA, USA). The concentration of fungal DNA was calculated by comparing a dilution series with defined amounts of DNA of lambda phage (methylated, from *Escherichia coli* host strain W3110).

DNA extraction from maize field samples

Maize kernels were dried at 60 °C for 24 h and ground in a cross hammer mill (Cross Beater Mill SK 1; bottom sieve 1 mm; Retsch, Haan, Germany). The DNA extraction from 1 g of maize meal was carried out following an upscaled protocol for DNA extraction from plant material as described by Brandfass and Karlovsky [28]. The quality and concentration of DNA were determined by agarose gel electrophoresis as described above. Total DNA from 1 g of starting material was dissolved in 200 µl of 10 mM Tris, 1 mM EDTA, pH adjusted to 8.0. The DNA solution was diluted tenfold, and 1 µl was used as template for each reaction.

Primers

The primers used for *F. verticillioides* were VER1 (CTTCCTGCGATGTTTCTCC) and VER2 (AATTGGCCATTGGTATTATATATCTA), which were designed by Mulè et al. [25] on the basis of the coding sequence of the calmodulin gene; these primers amplify a DNA fragment of 587 bp. The primers used for *F. proliferatum* were Fp3-F (CGGCCACCAGAGGATGTG) and Fp4-R (CAACACGAATCGCT TCCTGAC), which were designed by Jurado et al. [26] on the baisi of the intergenic sequence of the ribosomal RNA gene cluster; these primers amplify a DNA fragment of 230 bp.

qPCR assays

The optimized conditions for qPCR assays were as follows: The reaction mixture for *F. verticillioides* (25 µl) contained reaction buffer amended with NH_4 [67 mM Tris-HCl, 16 mM

$(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, pH 8.8 at 25 °C; Bioline, Luckenwalde, Germany], 2.5 mM $MgCl_2$, 0.1 mM of each of the four deoxynucleoside triphosphates (dNTPs; Bioline, Luckenwalde, Germany), 0.3 µM concentration of each primer, 0.75 U of *Taq* DNA polymerase (BIOTaq, Bioline, Luckenwalde, Germany), 10 nM fluorescein (used for the calculation of well factors, see below), 0.1x SYBR Green I (Invitrogen, Karlsruhe, Germany), and 1 µl of template DNA.

The reaction mixture for *F. proliferatum*-specific PCR was identical except for the following components: 2 mM $MgCl_2$, 0.6 µM concentration of each primer, and 0.4 U of *Taq* DNA polymerase.

qPCR was performed in an iCycler thermocycler (BioRad, Hercules, CA, USA). The amplification for *F. verticillioides* consisted of an initial denaturation at 95 °C for 1.5 min, during which the well factors were collected (compensation for differences among optical properties of individual wells), followed by 40 cycles of 50 s denaturation at 94 °C, 50 s annealing at 62 °C, and 1 min elongation at 72 °C. The final elongation step was performed for 7 min at 72 °C. Fluorescence was measured in each cycle during the annealing phase. Melting curve analysis was performed after each PCR: Samples were heated to 95 °C for 1 min, cooled to 55 °C for 1 min, and heated to 65 °C, and subsequently the temperature was ramped from 65 °C to 95 °C in steps of 0.5 °C every 10 sec. Fluorescence was measured at each step.

The PCR for the quantification of *F. proliferatum* DNA was performed according to the following protocol: initial denaturation for 1.5 min at 95 °C; followed by 35 cycles with 35 s at 95 °C, 30 s at 64 °C, and 30 s at 72 °C, with fluorescence measurement during the annealing step of each cycle; and a final elongation of 5 min at 72 °C. The melting curve analysis was performed as described above.

Calibration curves and PCR efficiency

Dilution series were prepared containing purified fungal DNA in amounts of 0.05, 0.15, 0.5, 1, 5, 10, and 50 pg mixed with maize DNA. For *F. proliferatum*, two additional standards (1.5 and 15 pg of fungal DNA) were used. Every set of standards was analyzed ten times. Standard curves were generated by plotting threshold cycle (Ct values) against the logarithm of starting DNA quantities. The slopes of the standard curves were used to calculate the reaction

efficiency *E* of PCR assays, using the following equation:

$E = 10^{(-1/slope)} - 1$

These samples were also used as spiked positive samples for ROC curve analysis (see later).

Specificity of PCR primers

The specificity of both PCR assays was determined with DNA extracted from pure cultures of 81 fungal isolates (14 *Fusarium* species and 20 isolates of 12 other fungal species, Table 1). Samples were classified as positive when the melting point was identical with the melting point of the standard with a tolerance of 0.5 °C.

Sensitivity, specificity, ROC curves, and optimal cutoff points

ROC curve analysis was used for estimating the performance of qPCR assays [29]. ROC curves were constructed as plots of sensitivity versus 1 - specificity for a set of positive and negative samples. Sensitivity is the fraction of true-positive samples that score positive. Sensitivity was calculated for each PCR cycle by dividing the number of true-positive samples with equal or lower Ct value by the total number of true-positive samples. Specificity is the fraction of true-negative samples that score negative. Specificity was calculated for each PCR cycle by dividing the number of true-negative samples with higher or equal Ct value by the total number of true-negative samples. ROC curves show the relationship between sensitivity and specificity. They facilitate visual evaluation of the performance of an assay. The area under a ROC curve can be regarded as an aggregate quality indicator for a diagnostic assay.

The Youden index *J* is defined as [29]

$J = Se + Sp - 1,$

where Se is sensitivity and Sp is specificity.

The optimal cutoff point is the PCR cycle with the highest value of the Youden index:

Optimal cutoff point = $\max_{ct} \{J\}$.

Samples with a threshold cycle higher than the chosen cutoff point are classified as negative,

whereas samples with threshold cycle lower than the cutoff point are classified as positive [30]. ROCs, areas under ROC curves, and Youden indices were calculated with the ROC module of the package "Sigma Plot 11.0" (Systat Software, San Jose, CA, USA). The same software was used to generate graphics.

Determination of LOQ and LOD

The LOQ was determined as the amount of DNA corresponding to the threshold cycle at which the sum of specificity and sensitivity of the assay was maximized. For this purpose, the Youden index *J* was calculated for each PCR cycle. The cycle for which *J* reached the maximum was selected as the optimal cutoff point. The LOQ was then determined as the amount of DNA corresponding to the optimal cutoff point in the calibration curve.
The LOD was determined as the amount of DNA corresponding to the threshold cycle at which at most 5% of true-positive samples scored negative (selectivity of 0.95).

Determination of mycotoxin production

Polished rice (25 g) and 35 ml of tap water were autoclaved in 100-ml Erlenmeyer flasks and inoculated with a 100-µl spore suspension of the fungal strains. The cultures were incubated at 25 °C for 2 weeks. A 4-g portion of the colonized substrate (water content 15–20%) was extracted with 40 ml of acetonitrile. A 1-ml volume of the extract was dried in a vacuum, and the residue was dissolved in 1 ml of methanol/water (1:1), defatted with 1 ml of cyclohexane, and diluted 20-times with methanol/water (1:1). High-performance liquid chromatography was performed on a reverse-phase C18 column (Kinetex, 50.0 mm x 2.1 mm, particle size 2.6 µm; Phenomenex) with a gradient of methanol in water with 7 mM acetic acid at flow rate of 0.2 ml min^{-1}. The analytes were ionized by electrospray and detected by tandem mass spectrometry with an ion trap detector (500 MS, Varian, Darmstadt, Germany).

Results

The first amplifications were performed under conditions for end-point PCR as described by Mule et al. [25] and Jurado et al. [26]. To improve the sensitivity, we reduced the reaction

volume to 25 µl and optimized the following: the concentrations of dNTPs, $MgCl_2$, and primers; the activity of *Taq* DNA polymerase; and the cycling parameters for qPCR conditions. For *F. verticillioides*, the most important changes in the conditions for PCR concerned the concentrations of dNTPs and $MgCl_2$, which were increased from 50 to 100 µM and from 1.5 to 2.5 mM, respectively, as compared with the original publication. In contrast, the amount of *Taq* DNA polymerase could be reduced from 1.25 to 0.75 U. An annealing temperature of 62 °C yielded specific products, in contrast to the annealing temperature of 56 °C, which was suggested by the designers of the primers [25]. In the *F. proliferatum* assay, the amount of each primer could be reduced from 0.8 mM to 0.6 mM, teh amount of dNTPs could be reduced from 1 mM to 100 µM, and the amount of *Taq* DNA polymerase could be reduced from 1.0 to 0.4 U per reaction. The annealing temperature was lowered from the recommended temperature of 69 °C [26] to 64 °C.

The optimized conditions were used for the ROC curve analysis with artificially prepared samples and field samples. Artificial negative samples consisted of nontarget DNA and blank plant matrix and artificial positive samples consisted of plant matrix spiked with known quantities of target DNA (0.05-50 pg). A total of 226 artificial samples for *F. verticillioides* assay and 224 samples for *F. proliferatum* assay were used. Field samples originated from monitoring and field trials carried out from 2005 to 2008 in Germany and Italy; 994 field samples for *F. verticillioides* assay and 436 field samples for *F. proliferatum* assay were used. Melting curve analysis was used as the "gold standard" for classification of field samples as positive or negative. Unknown samples generating products with melting temperatures ±0.25 °C above/below the mean melting temperature of the standards and positive controls for a given PCR run were ranked as positive. Over a period of 3 years, the melting temperature among PCR runs fluctuated between 90.0 and 91.5 °C for F. *verticillioides* and between 91.5 and 92.5 °C for *F. proliferatum*. Within a single PCR run, melting temperatures for standards and positive controls were constant within a range of 0.5 °C.

Calibration curves generated with spiked matrix revealed a linear relationship between Ct values and the logarithm of DNA amount down to at least 0.05 pg for *F. proliferatum* and 0.15 pg for *F. verticillioides* (Fig. 1). The average PCR efficiency of the assays was 0.92 for *F. verticillioides* and 0.98 for *F. proliferatum*. The Ct values for *F. proliferatum* DNA were consistently about four cycles lower than the values for the same amount of *F. verticillioides* DNA.

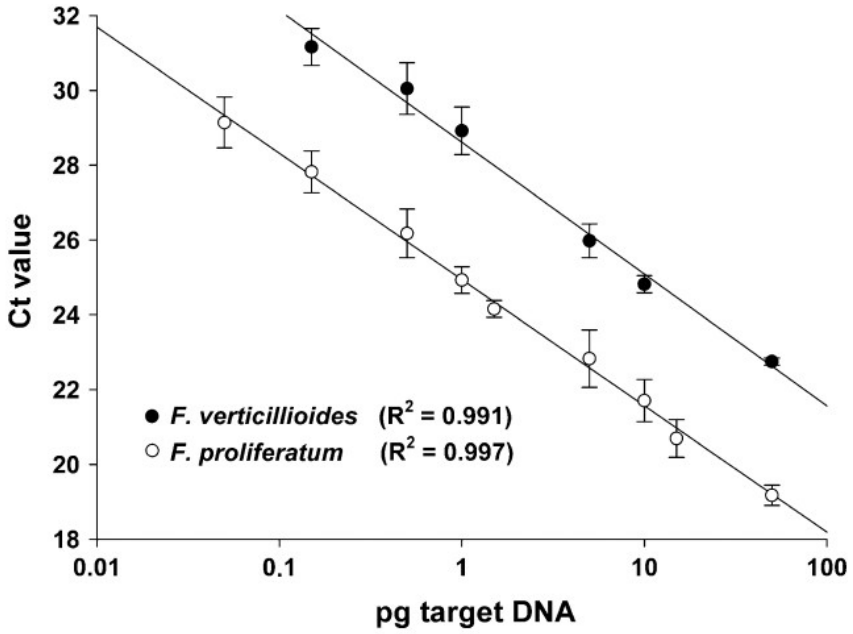

Figure 1. Linear standard curves obtained from dilution series of *F. verticillioides* DNA (*filled symbols*) and *F. proliferatum* DNA (*open symbols*) in a range from 0.05 to 50 pg, with five to 10 replications per quantity. The quantity of 0.05 pg of *F. verticillioides* DNA was excluded from the standard curve because of low reproducibility. The threshold cycle (*Ct*) is plotted against the decadic logarithm of starting DNA quantity in grams. *Error bars* represent standard deviation

With all 13 *F. verticillioides* isolates (formerly *F. moniliforme*) and 15 *F. proliferatum* isolates (Table 1), we obtained PCR products with the expected melting temperatures. As a confirmation of the taxonomic affiliation of these strains, we determined which mycotoxins they produced. Ten strains labeled as *F. verticillioides* and 12 strains labeled as *F. proliferatum* were grown in rice for 2 weeks. With one exception, only *F. proliferatum* strains produced *F. proliferatum*-specific depsipeptide beauvericin (Table 3). Furthermore, neither species produced enniatins, and all strains except one produced fumonisins.

Pure maize DNA and all isolates of 18 nontarget fungal species tested negatively (87 isolates for the *F. proliferatum* assay and 89 isolates for the *F. verticillioides* assay). Samples of nontarget fungal DNA generated no amplification products or unspecific products with melting temperatures lower than those of the target products by at least 4 °C (Fig. 2).

ROC curve analysis was performed for spiked maize matrix and field samples (Fig. 3). For both fungi, the areas under ROC curves were slightly higher for spiked matrix than for field

samples. The ROC curves were used to determine the LOQs of the assays. We defined the LOQ of the PCR assays as DNA amounts that maximized the sum of sensitivity and specificity. The corresponding Ct values (optimal cutoff points) were determined by maximizing the Youden index. Calibration curves (Fig. 1) were used to determine LOQs for both assays using these Ct values.

For a given threshold of the false-positive rate, the LOD was defined as the lowest amount of target DNA that was amplified with a false-negative rate below or equal to this threshold. We selected a maximal acceptable false-negative rate of 5% and then used this threshold to determine the LOD values (Table 2).

Table 2. Performance parameters of qPCR assays

Samples	Positive[a]	Negative[b]	Optimal cutoff point	Sensitivity at optimal cutoff	Specificity at optimal cutoff	LOD (pg)	LOQ (pg)
F. verticillioides							
Spiked matrix	112	114	36	0.96	0.97	0.021	0.11
Field samples	796	198	33	0.85	0.95	---	0.62
F. proliferatum							
Spiked matrix	92	132	30	0.99	0.96	0.016	0.03
Field samples	379	57	27	0.94	0.96	---	0.24

[a]Spiked matrix- number of samples spiked with target DNA; field samples- number of samples that generated products with melting temperatures differing by less than 0.25 °C from target DNA.

[b]Spiked matrix- number of samples consisting of matrix with nontarget DNA only; field samples- number of samples that generated melting curves different from those of target DNA.

Table 3. Production of mycotoxins by selected *Fusarium* strains

	Mycotoxin (μg/g rice culture)					
Strain	Fumonisin B1	Beauvericin	Enniatin B	Enniatin B1	Enniatin A	Enniatin A1
F. verticillioides						
1.51	90	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7358	154	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7362	240	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7367	93	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7370	5.2	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-4737	5.4	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7363	116	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-8114	265	<LOD	<LOD	<LOD	<LOD	<LOD
1.34	53	1.2	<LOD	<LOD	<LOD	<LOD
Fv234/1	114	<LOD	<LOD	<LOD	<LOD	<LOD
F. proliferatum						
DSM 62267	<LOD	518	<LOD	<LOD	<LOD	<LOD
DSM 62261	141	678	<LOD	<LOD	<LOD	<LOD
DSM 63267	29	2.6	<LOD	<LOD	<LOD	<LOD
Fpro1	226	135	<LOD	<LOD	<LOD	<LOD
Fpro2	218	10	<LOD	<LOD	<LOD	<LOD
Fpro3	233	5.7	<LOD	<LOD	<LOD	<LOD
Fpro4	200	424	<LOD	<LOD	<LOD	<LOD
Fpro5	150	277	<LOD	<LOD	<LOD	<LOD
Fpro8	52	2.0	<LOD	<LOD	<LOD	<LOD
Fpro9	75	309	<LOD	<LOD	<LOD	<LOD
Fpro11	27	186	<LOD	<LOD	<LOD	<LOD
Fpro12	26	637	<LOD	<LOD	<LOD	<LOD

Limit of detection values (LOD) values were 5 ng/g for beauvericin, enniatin B, B1, A1, and fumonisin B1, and 10 ng for enniatin A

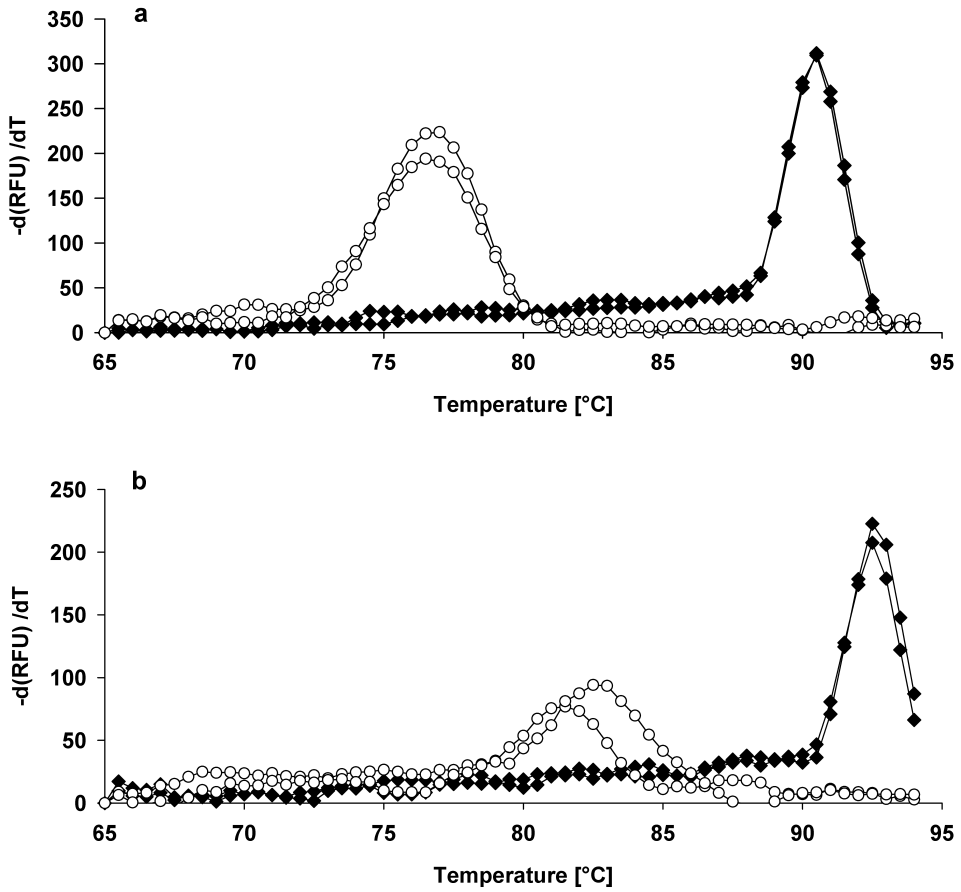

Figure 2. Melting curve analysis of PCR products obtained with primers specific for *F. verticillioides* (a) and *F. proliferatum* (b). *Filled symbols* indicate the negative first derivation of SYBR Green fluorescence for PCR products heated from 65 °C to 94 °C. *Open symbols* indicate melting curves of PCR products of negative controls (water and non-target DNA). *RFU* relative fluorescence units

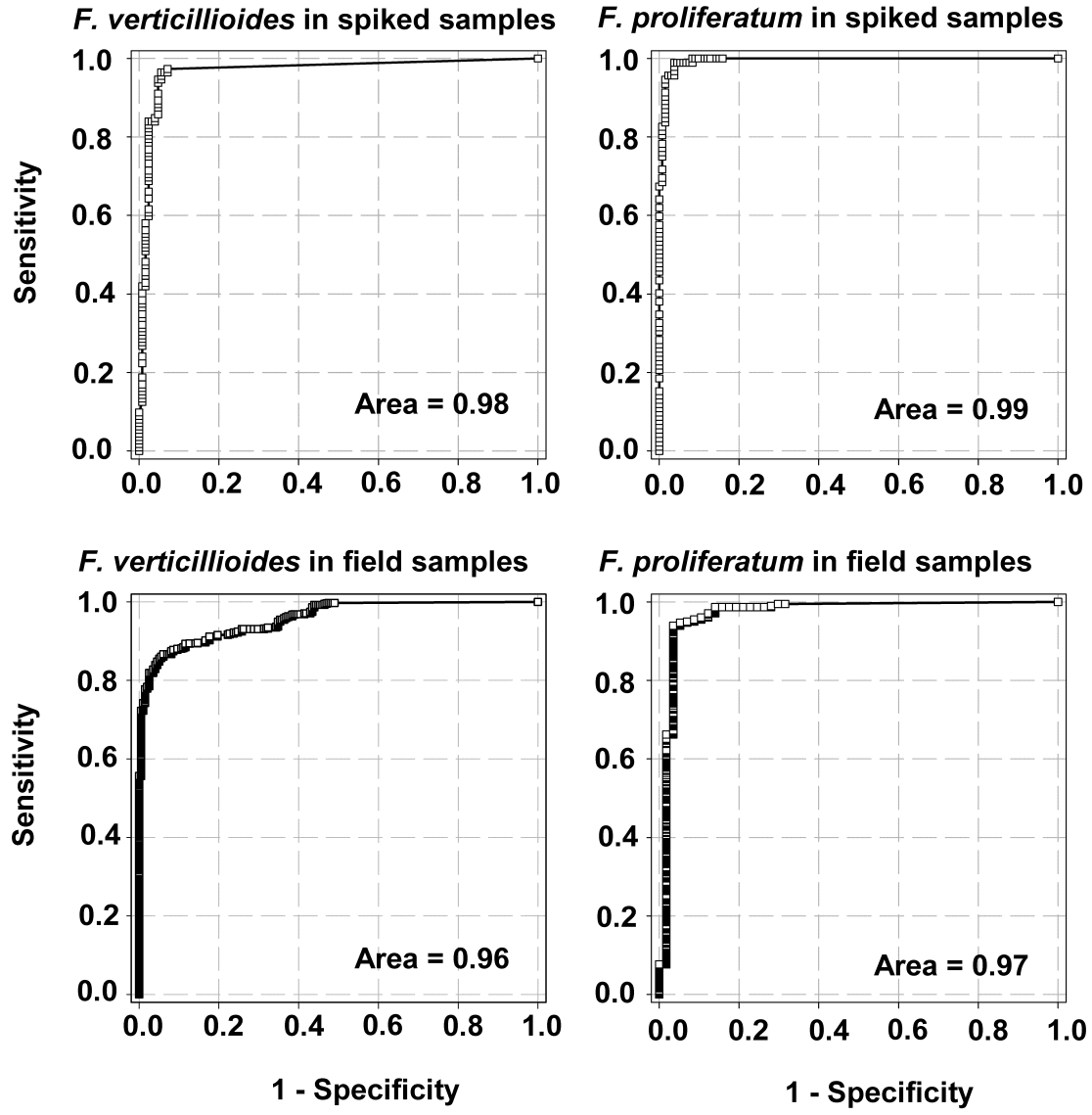

Figure 3. Receiver operating characteristic (ROC) curves for real-time PCR for *F. proliferatum* and *F. verticillioides*. The *upper panels* show the ROC curves resulting from maize flour spiked with *F. verticillioides* DNA (n = 226) and *F. proliferatum* DNA (n = 224). The *lower panels* show the ROC curves for field samples for *F. verticillioides* DNA (n = 994) and *F. proliferatum* DNA (n = 436)

Discussion

Using published PCR primers for *F. verticillioides* [25] and *F. proliferatum* [26], we developed qPCR assays for the quantification of the DNA of these species in maize kernels.

Mule et al. [25] evaluated the specificity of their primers for *F. verticillioides* by testing 21 strains of *F. verticillioides*, 12 strains of *F. proliferatum*, and six strains of *F. subglutinans,* in addition to single isolates of *F. graminearum, F. poae, Aspergillus flavus,* and *Acremonium strictum*. Jurado et al. [26] tested the specificity of primers for *F. proliferatum* against 12 strains of *F. graminearum*, seven strains of *F. culmorum*, five strains of *F. poae*, six strains of *F. sporotrichioides*, and one or two strains of eight other *Fusarium* species and five other fungal species. The use of only one strain of *F. verticillioides* and *F. subglutinans* in the test of primers for *F. proliferatum* [26] appeared insufficient. We therefore extended the specificity tests for both primer pairs with additional 12 isolates of *F. verticillioides,* 15 isolates of *F. proliferatum,* 12 isolates of *F. subglutinans*, 42 isolates of nontarget *Fusarium* species, and 20 isolates of other fungal species. These tests, performed under qPCR conditions, generated positive signals only for the target species. Primer pairs Fp3-F/Fp4-R [26] and VER1/VER2 [25] can therefore be regarded as species-specific in real-time mode for *F. proliferatum* and *F. verticillioides*, respectively.

The qPCR assays described here are suitable for the estimation of *F. verticillioides* and *F. proliferatum* DNA in maize flour with LOQ values of 0.11 pg and 0.032 pg, respectively, which correspond to 3.8 µg and 1.05 µg of DNA per kilogram of flour, respectively. The mean LOQ values for field and spiked samples correspond to 8.5 genomes for *F. verticillioides* and 3.2 genomes for *F. proliferatum*, assuming that the genome size of both species is approx. 40 Mbp. The amount of genomic DNA determined by qPCR can be used as a measure of fungal content in studies of the relationships between *Fusarium* infection, mycotoxin production, and disease symptoms. Relative to classic end-point PCR, the sensitivity of the detection was increased significantly for both *F. verticillioides* and *F. proliferatum*. Furthermore, the costs of the modified assays were reduced because optimized PCR uses less Taq polymerase and a lower concentration of dNTPs than classic end-point PCR.

The Ct values for *F. proliferatum* DNA were consistently lower than those for the same amount of *F. verticillioides* DNA. This observation is reasonable because the primers for *F. proliferatum* were derived from a multicopy sequence [26] whereas the primers for *F. verticillioides* were based on a single-copy calmodulin gene [25]. The difference in the copy number of targets also explains why the *F. proliferatum* assay was more sensitive than the *F. verticillioides* assay.

ROC curve analysis of a dilution series of target DNA and nontarget DNA generated areas

under ROC curves of 0.98 for the *F. verticillioides* assay and 0.99 for the *F. proliferatum* assay, which are close to the optimal value of 1. Occasionally, nontarget DNA caused unspecific amplification. On the basis of cutoff points calculated according to the Youden index (Table 2), the sensitivity was 97% for the *F. proliferatum* assay and 94% for the *F. verticillioides* assay, whereas the specificity was 97% in the *F. verticillioides* assay and 96% in the *F. proliferatum* assay. Therefore, automatic processing of the results based merely on Ct values (without melting curve analysis) is possible. Melting curve analysis is recommended when the content of target DNA approaches LOQ values.

Adejumo et al. [31] compared PCR analysis with an agar plating method for detection of *F. verticillioides* in maize samples from a Nigerian market. They found that only 71% of the maize samples that were positive for *F. verticillioides* by agar plating were confirmed positive by species-specific PCR. Part of this contradiction can probably be explained by the morphological similarity between *F. verticillioides* and *F. proliferatum*, highlighting the difficulty in distinguishing between these species on the basis of morphology. Other work by these authors [32] demonstrated an even greater difficulty in differentiating between *F. verticillioides* and *F. proliferatum* on the basis of morphology: *F. verticillioides* was found to be the dominant species in Nigerian maize, followed by eight other *Fusarium* species, but *F. proliferatum* was not found. It is likely that *F. proliferatum* isolates were confused with *F. verticillioides* in this work and that 29% of isolates morphologically identified as *F. verticillioides* but not confirmed by PCR were *F. proliferatum*. The use of PCR for differentiating *F. proliferatum* from *F. verticillioides* is therefore highly recommended [33].

To confirm the taxonomical affiliation of strains used in this work, we determined the production of beauvericin, enniatins, fumonisins, and moniliformin by 12 isolates each of *F. verticillioides* and *F. proliferatum*. Whereas fumonisins are produced by both *F. verticillioides* and *F. proliferatum*, moniliformin is produced only by *F. proliferatum* [19] and beauvericin is produced by *F. proliferatum* but is not produced or is produced in only low amounts by *F. verticillioides* [34-36]. That *F. proliferatum* produces enniatins was affirmed in an authoritative review [19] but this was rejected in other publications [37,38]. We did not find enniatins in any of the *F. verticillioides* or *F. proliferatum* cultures in the current study.

Our laboratory has extensively used the qPCR assays described here for quantifying *F. verticillioides* and *F. proliferatum*. We have used qPCR to analyze maize kernels artificially infected with *F. verticillioides* or *F. proliferatum*, naturally infected samples from the field,

and maize cobs inoculated with mixtures of *F. verticillioides*, *F. proliferatum*, and other fungal species in the greenhouse.

ROC curve analysis was developed for the assessment of qualitative diagnostic assays. Turechek et al. [44] used ROC curve analysis to compare the performance of PCR primers. Inspired by their work, we used the ROC concept to establish performance parameters for quantitative PCR assays. LOQ and LOD, which are fundamental parameters in analytical chemistry, thus became available for quantitative PCR.

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Author`s contributions

KD optimized the real-time PCR assay for *F. proliferatum* as part of her master thesis. SN optimized the real-time PCR assay for *F. verticillioides* as part of her master thesis.

SN performed ROC curve analysis for *F. verticillioides*. KD performed ROC curve analysis for *F. proliferatum*. SN provided field data. KD performed the mycotoxin analysis. All authors contributed in writing the manuscript and approved the final version of the manuscript.

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Chapter 4: Potential impact of climate change on *Fusarium verticillioides* in interaction with *F. graminearum*

Abstract

The effects of interactions between *F. verticillioides* and *F. graminearum* on maize ears were investigated in field studies and in a climate chamber experiment. Maize ears were inoculated with *F. verticillioides*, *F. graminearum* and a mixture of both and were analyzed for the content of species-specific fungal biomass and mycotoxins. The incidence and amounts of biomass of *F. verticillioides* as well as fumonisin B1 were either increased or not affected by mixed inoculations. By contrast, the incidence and biomass of *F. graminearum* were either not affected or decreased in mixed inoculations.

The effect of temperature on single and mixed treatments was investigated in climate chambers at five defined temperature scenarios in a day/night rhythm. The incidence and amounts of *F. verticillioides* DNA and fumonisin B1 were either not affected or correlated positively with an increased temperature in maize ears. The amount of biomass of *F. graminearum* was not influenced with an increased temperature scenario.

The results indicate that climate warming may favor *Fusarium* ear rot caused by *F. verticillioides*. In addition, interaction with *F. graminearum* rather facilitates the infestation of *F. verticillioides* rather than repressing the fungus. This may result in an increased risk of fumonisin contamination of maize in moderate climate areas.

Introduction

Contamination of maize with mycotoxins endangers the health of consumers and leads to economical losses in countries with legal limits for maximum mycotoxin levels. Worldwide maize ear rot is caused by a wide range of *Fusarium* spp. associated with numerous mycotoxins, arising from the ability of most *Fusarium* species to produce mycotoxins. *Fusarium* spp. rarely occur in isolation but rather in fungal communities, and maize ear rot is characterized by the co-occurrence of different *Fusarium* species [1]. Maize ear rot is divided

into two distinct diseases called *Fusarium* and *Gibberella* ear rot, both characterized by the occurrence of different *Fusarium* spp. and mycotoxins. *Fusarium* ear rot is predominantly associated with the occurrence of *F. verticillioides* as well as *F. proliferatum* and *F. subglutinans,* while *F. graminearum* followed by *F. culmorum*, *F. cerealis* and *F. avenaceum* are predominantly responsible for causing *Gibberella* ear rot [1, 2].

F. verticillioides is a heterothallic fungus, but sexual reproduction is not important for dispersal of the fungus and the epidemiology of the disease. The fungus produces a large number of asexual micro- and macroconidia on crop residues, which are the main source for plant infection by this fungus. *F. verticillioides* survives on crop residues as thickened hyphae in intervals between host plants [2]. *F. verticillioides* infects the maize kernels via several pathways, but silk infection through airborne conidia is the most important [3]. Undamaged maize kernels represents a barrier to the infestation of kernel tissue. The fungus does not form any penetration structures and the mechanism by which the fungus invades intact kernels is not fully understood. The hypothesis has been formulated that under specific environmental circumstances free water may transport conidia of the pathogen along the surface of the silks, then the fungus enter the kernel via an open stylar canal [4]. It has also been proven that insects, including European corn borers (*Ostrinia nubilalis*), sap beetles (*Carpophilus* spp. and *Glischrochilus quadrisignatus*), western flower thrips (*Frankliniella occidentalis*), and corn rootworm beetles (*Diabrotica* spp.), greatly facilitate infection with *F. verticillioides* by wounding plant tissue as well as by transporting spores. After silk infection, the fungus colonizes the kernels and causes the typical white or light pink mold on infected kernels or groups of kernels [2]. Further important infection pathways are via the seeds or the roots through which the fungus grows systemically up to the cob [3]. This systemic transmission of the fungus is often associated with symptomless infection of the plant [5, 6]. The ubiquitous fungus occupies dual roles in the maize plant: on the one hand, *F. verticillioide*s occurs as a fungal pathogen [1]; on the other hand, the fungus infects maize plants as a symptomless endophyte on maize [7]. The fungal-host interaction can be beneficial to maize plants as regards yield and vegetative growth. The hypothesis has been formulated that *F. verticillioides* exists in a symbiotic, mutualistic relationship until external abiotic or biotic conditions lead to a change of the type of relationship [7]. During endophytic, asymptomatic growth, the fungal hyphae were only found in intercellular spaces, but during symptomatic infection hyphae were detected at both intercellular and intracellular sites [8].

Infection with *F. verticillioides* can lead to contamination with fumonisins, of which fumonisin B1 (FB1) is usually predominant and found at the highest levels [9]. Fumonisins are a class of mycotoxins first described by Gelderblom *et al.* in 1988 [10]. In total, 28 fumonisin analogs have been characterized since 1988, and they can be classified as fumonisin A, B, C, and P series, but fumonisin B1, B2 and B3 are the most abundant of them. Fifteen *Fusarium* species have been reported to produce fumonisins [11], with *F. verticillioides* and *F. proliferatum* being the predominant producers worldwide [12]. Apart from *Fusarium* species, only *Aspergillus niger* [13] and *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici* [14] have also been found to produce fumonisins.

Contamination with fumonisins is of particular importance because of the relationship between consumption of fumonisin-contaminated maize and the occurrence of oesophageal cancer in humans [15]. The International Agency for Research on Cancer (IARC) has classified fumonisins (group 2B) as probably carcinogenic to humans. Fumonisins have also been shown to cause equine leukoencephalomalacia, pulmonary oedema in pigs and hepatocarcinogenesis in rats [16]. Fumonisins are structurally similar to sphinganine and their mechanism of action is presumably the inhibition of the enzyme sphinganine N-acyltransferase, resulting in the alteration of cell regulation and the disruption of the *de novo* sphingolipid biosynthesis [17]. This leads to a rapid accumulation of free sphinganine (sometimes also sphingosine) which promote cell death and is mainly responsible for the toxic effects of fumonisins [18, 19]. Since the discovery of fumonisins in 1988, their biological function has been the subject of speculation, but it still remains unclear. Studies concerning the potential role of fumonisin production in plant pathogenicity have led to contradictory results. Desjardins and Plattner observed that *F. verticillioides* strains not producing fumonisins infected maize kernels and caused ear rot as effectively as fumonisin-producing strains [20]. Opposite results were obtained by Glenn *et al.* [21] who found that strains of *F. verticillioides* were not pathogenic on maize seedlings because of mutations of the FUM1 gene. Furthermore, a distinct population of *F. verticillioides* is pathogenic on banana, but lack genes in the FUM cluster. These strains were not able to cause disease symptoms on maize seedlings while fumonisin-producing transformants of these strains were pathogenic on maize seedlings. The authors formulate the hypothesis that seedling disease is strongly dependent on the maize genotype and the amount of fumonisins produced by the *F. verticillioides* strains.

The primary source of inoculum for *F. graminearum* is plant residue, especially from maize

plants. *F. graminearum* forms sexual ascospores in perithecia as well as asexual macroconidia in sporodochia in such residue. As survival structures *F. graminearum* produces chlamydospores, which can survive between host crops [2]. Ascospores are the primary source of infection in wheat, whereas this question remains unresolved with regard to *Gibberella* ear rot [22]. Macroconidia are initially dispersed by water splashes, but both ascospores and macrospores can be carried long distances by air at a later stage. The most important infection pathway for the infection of maize kernels by *F. graminearum* is via the silks [2]. The fungus grows on the surface and inside the silk towards the kernel and penetrates the ovary through the silkcover attachment point or interkernel spaces [23]. *Gibberella* ear rot is characterized by infection beginning at the tip of the ear followed by the development of a reddish mold down the ear [1].

F. graminearum strains are distinguished by which trichothecene B mycotoxins they predominantly produce. The DON-chemotype produce predominantly deoxynivalenol (DON) and its C-3 acetylated derivatives (3ADON) or its C-15 acetylated derivatives (15ADON), whereas the NIV-chemotype predominantly produce nivalenol (NIV) with its acetylated derivative [24, 25]. Trichothecenes in animal food generally lead to weight loss, decreased feed conversion, feed refusal, vomiting, bloody diarrhea, severe dermatitis, hemorrhage, decreased egg production, abortion and death [26]. In addition, *F. graminearum* also produces zearalenone (ZEN). ZEN is a uterotrophic and estrogenic compound leading to hyper-estrogenism in swine and infertility and poor performance in cattle and poultry [1].

Interspecific interactions between *F. verticillioides* and *F. graminearum* have already been investigated *in vitro* and *in planta* under field conditions, but the results have often been contradictory. In mixed inoculations under field conditions, *F. verticillioides* was the predominant species and led to reduced growth of *F. graminearum* [27]. In a recently published field experiment, *F. verticillioides* biomass was even increased in mixed and especially in sequential inoculations, while the biomass of *F. graminearum* was similar or reduced compared to single inoculations [28]. *In vitro* studies, on the other hand, showed the reduced growth of *F. verticillioides* and *F. proliferatum* in co-occurrence with *F. graminearum*, while the latter was affected less [29]. In several *in vitro* and *in planta* studies, *F. verticillioides* dominated other fungal species, such as *Aspergillus* spp. [30] or *Ustilago maydis* [31, 32], but the results were found in interactions with the antagonist *Trichoderma harzianum* [33].

It is predominantly abiotic conditions that influence interspecific interactions between *Fusarium* spp. colonizing the same host plant and may be critical for dominance of *Fusarium* spp. Remarkably, the predominant spectrum of *Fusarium* spp. and their mycotoxins in maize appears to be specific to geographical areas. Climatic conditions, host susceptibility as well as agricultural techniques influence growth, survival, dissemination and, finally, fungal incidence and fungal complex composition [1, 34]. Nowadays, *Fusarium* ear rot caused by *F. verticillioides* predominates in warmer and drier climates, in Europe especially in Italy and Spain, while *Gibberella* ear rot caused by *F. graminearum* is mainly distributed in regions with frequent rainfall and moderate temperatures, especially in central and northern European areas [1, 2]. However, in Germany temperature increases of about +2 °C by 2050 and up to +4 °C by 2100 (1961-1990 base period) are predicted [35]. Similar values are also estimated for global warming, ranging from 1.1-3.5 °C by 2100, depending on the emissions scenario (1980–1999 base period) [36].

The aim of this study was to evaluate interactions between *F. verticillioides* and both chemotypes of *F. graminearum* in maize ears concerning infection rate, biomass production and mycotoxin accumulation. Furthermore, we examine the possibility that global warming will cause an increase in fumonisin content of maize grain produced in northern parts of Germany, and other European countries.

Materials and Methods

Materials for mycotoxin sample preparation and analysis

Methanol (HPLC-grade), isopropyl alcohol (p.a. grade), n-hexane (for synthesis) for mycotoxin extraction were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). For mycotoxin analysis, acetonitrile, methanol, both LC-MS grade, were supplied by Th. Geyer GmbH & Co. KG (Renningen, Germany), and acetic acid (LC-MS grade) was purchased from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany).

Analytical mycotoxin standards

Mycotoxin standards of FB1 and ZEN were obtained from Fermentas (St. Leon-rot, Germany), NIV was purchased from LGC standards (Wesel, Germany) and DON was obtained from Alexis Deutschland GmbH (Grünberg, Germany). All toxins were obtained in powder form. Individual stock solutions were prepared by redissolving them in acetonitrile, which resulted in a concentration of 1 mg mL^{-1}. A standard mixture with equal concentrations of each mycotoxin was prepared.

Fungal strains

In this study, two *F. verticillioides* and two *F. graminearum* isolates were used. The first *F. verticillioides* strain, VP2 (ITEM 10670) [37], was kindly provided by Francesca Cardinale, University of Turin, Italy. The second *F. verticillioides* strain, Fv 234/1 [38], was received from P. Battilani, Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy, via T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany. The *F. graminearum* strain IFA 66 (DON-chemotype) [38] was received from M. Lemmens, Institute of Biotechnology in Plant Production, Tulln, Austria, via T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany. The *F. graminearum* strain Fg71 (NIV-chemotype) was received from T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany. The fungal strains VP2, Fv 234/1, IFA66, Fg71, will be referred to as Fv1, Fv2, FgD and FgN respectively by way of simplification.

Fungal inoculum preparation

Spore suspensions were prepared in a liquid mung bean medium slightly modified from Bai [39]. Mung beans (40 g) were cooked in 1 L of tap water for approx. 20 min and filtered, and the supernatant was autoclaved. Spore suspension was added to 100 mL of mung bean medium in 500 mL of Erlenmeyer flasks and the cultures were grown at 28 °C and shaken at 200 rpm for one week. Spores were filtered through sterile cotton gauze and transferred to 50 mL falcons. After centrifugation at 4800 x g for 10 min, the medium was discarded and the spores in each falcon were redissolved in 3 mL of sterile tap water. The spore suspensions of

each fungal strain were pooled, thoroughly mixed, divided into aliquots of 500 µL and frozen at -80 °C.

The spore densities were determined with a Thoma counting chamber using an optical microscope. Afterwards, three different, defined amounts of spore suspension were added and spread on PDA plates using a Drigalski spatula in order to evaluate the germination rates in three replicates. Prior to inoculation, the desired concentration of the inocula were achieved by diluting the spore suspensions with sterile tap water. The inocula were cooled on ice throughout the entire period of inoculation.

Maize field trials

In 2009, the two maize varieties Amadeo and Kabanas were grown in Relliehausen, about 50 km north of Göttingen, Germany. In 2010, Kabanas was sown in a field in Göttingen. Maize seeds were sown in the middle of April at seed rates of 10 seeds/m^2 and 11.5 seeds/m^2 in 2009 and 2010, respectively. In 2009, seeds were treated with the insecticide Mesurol (Bayer Crop Science, Monheim, Germany). Additionally, 1.21 L ha^{-1} of Calaris (Syngenta, Maintal, Germany) and 15 g ha^{-1} of Cato (DuPont, Neu-Isenburg, Germany), two herbicides, were applied. In 2010, no pesticides were applied.

Inoculation of maize cobs with fungal spore suspension was carried out in two rows in the middle of the maize fields, with equal numbers of plants per row being inoculated during each treatment. Inoculum (mL) containing 1 x 10^4 of conidia per fungus was injected into the silk channel approx. 3 cm above the cob with a self-refilling syringe 6 days after 50% of silk emergence. In 2009, maize cobs were inoculated with a spore suspension of Fv1 and FgN, as single and mixed inoculations in 10 replicates. The cobs were harvested at maturity stage (55 dpi). Ten uninoculated maize cobs of each variety were used as controls, with the kernels of each variety being pooled.

In 2010, maize cobs were artificially inoculated with a spore suspension of *F. verticillioides* (Fv1, Fv2), *F. graminearum* (FgN, FgD) and mixtures of these species (Fv1 + FgN, Fv1 + FgD and Fv2 + FgN, Fv2 + FgD). Each variant was inoculated 30 times. 10 repetitions were harvested each time point 21 dpi, 35 dpi and finally at maturity stage (49 dpi). Additionally, ten uninoculated maize cobs were collected at each harvest time point. Whole cobs were dried at 45 °C for seven days. Corn ears were hand-shelled and the kernels of each ear were pooled,

milled to fine powder and thoroughly mixed.

Meteorological data

Daily temperature (maximum, minimum and mean) and precipitation data were measured at meteorological stations placed in Göttingen and Dassel close to the fields in 2009 and 2010. The data were obtained from the German weather service "Deutscher Wetterdienst".

Disease severity in field experiments

In both years, the disease severity of each cob was rated visually as the percentage of cob surface covered with mycelium (0–100%). No distinction was made between the symptoms of *F. verticillioides* and *F. graminearum* infestation.

Climate chamber experiment

The mini maize variety Gaspe Flint was grown in a greenhouse until inoculation. Inocula (0.5 mL) containing 1 x 10^4 of conidia per fungus were injected into the silk channel approx. 1 cm above the cob with a syringe 5-7 days after silk emergence in each individual plant. Maize cobs were inoculated with spores of Fv1, FgD and FgN as well as mixtures of the species, Fv1+FgD and Fv1+FgN.

For every treatment, separate syringes were used to avoid contamination. Plants were transferred to five climate chambers with different temperature scenarios. The lowest temperature value was calculated by using the mean values of daily maximum and minimum August temperatures in Bad Harzburg, Lower Saxony, Germany from 1970 to 2000. The climate in Bad Harzburg is one of the coolest in Lower Saxony. From one scenario to the next the temperature was increased by 2 °C, while light and relative humidity were kept constant in a day/night rhythm (Table 1).

In each climate chamber, 10 plants of each treatment were inoculated. Three plants per climate chamber were not inoculated and used as controls. After 14 days, the cobs were harvested and dried for seven days at 45 °C. Samples with insufficient fertilization were discarded. Corn ears were hand-shelled and the kernels of each ear were pooled. Kernels and

cobs were milled separately to a fine powder. The experiment was carried out one time.

Table 1. Simulated climate scenarios showing temperature, light and relative humidity for interaction experiments with Gaspe Flint

Temperature scenarios	Temperature °C day/night	Humidity % day/night	Light h day/night
T1	22/13	75/85	13/11
T2	24/15	75/85	13/11
T3	26/17	75/85	13/11
T4	28/19	75/85	13/11
T5	30/21	75/85	13/11

Mycotoxin extraction

For the extraction of mycotoxins in the field samples 40 mL of methanol/isopropyl alcohol/water (80:5:15) were added to 4 g of maize meal, thoroughly shaken over night and centrifuged at 4800 x g for 10 min. Supernatant (1 mL) was evaporated to dryness at 40 °C and the residue was dissolved in 1 mL of methanol/water (1:1). The samples were defatted with 500 µL n-hexane, thoroughly mixed and centrifuged at 14000 x g for 10 min. The lower phase was taken for analysis.

The extraction procedure of samples from the climate chambers was slightly modified and down-scaled by adding 1 mL of methanol/isopropyl alcohol/water (80:5:15) to 100 mg of meal of kernels and cobs. The samples were thoroughly shaken over night and centrifuged at 4800 x g for 10 min. Supernatant (400 µL) was evaporated at 40 °C to dryness and the residue was dissolved in 400 µL of methanol/water (1:1). The samples were defatted with 200 µL of n-hexane, thoroughly mixed and centrifuged at 14000 x g for 10 min. The lower phase was taken for analysis.

Mycotoxin analysis

Mycotoxin separation and analysis was carried out by high pressure liquid chromatography coupled with electrospray ionization and tandem mass spectrometry detection. The mobile phase consisted of water with 5% acetonitrile (A) and methanol (B), both containing 7 mM of

acetic acid. The injection volume was 10 µL and the flow rate was set at 0.2 mL min^{1}. Fumonisin analysis was performed using an ion trap 500 MS (Varian, Darmstadt, Germany) with electrospray ionization in positive mode. For the determination of FB1, mycotoxin separation was carried out using a reverse phase column Kinetex C18 (50.0 x 2.1 mm, particle 2.6 µm) coupled with a C18 security guard cartridge (4 mm × 2 mm i.d., both from Phenomenex, Aschaffenburg, Germany) maintained at a temperature of 40 °C. The binary gradient was performed with the following conditions: 20 sec held at 40% B, 5 min linear gradient from 40% to 98% B, 4 min held at 98% B, 20 sec linear gradient from 98% to 40% B and 5 min held at 40% B. The mass transitions described by Bartók *et al*. were used for FB1 analysis [40].

Separation of NIV, DON and ZEN was carried out on a polar-modified reverse-phase HPLC column (Polaris C18-Ether, 100.0 x 2.0 mm, 3 µm particle size; Agilent, Darmstadt, Germany) kept at 40 °C. The binary gradient was: linear from 10 to 98% B in 7 min, hold 98% B for 5 min, linear from 98% to 10% B in 0.5 min, hold at 10% B for 7.5 min. Tandem mass spectrometry detection in multiple reaction monitoring mode was performed using a triple quadrupole 1200 L (Varian, Darmstadt, Germany) coupled with electrospray ionization in negative mode. Mass transitions were used for the detection of DON and NIV as described by Rasmussen *et al.* [40]. Furthermore, mass transitions described by Trebstein *et al.* [41] were used for the detection of ZEN.

In kernels, the estimated limits of quantification (LOQ) and limits of detection (LOD) based on the signal to noise ratios of 10:1 and 3:1 were 10 µg kg^{-1} and 5 µg kg^{-1} for FB1, 100 µg kg^{-1} and 30 µg kg^{-1} for DON, 300 µg kg^{-1} and 100 µg kg^{-1} for NIV, 20 µg kg^{-1} and 10 µg kg^{-1} for ZEN, respectively.

In cobs, the estimated values for LOQ and LOD for FB1 were 100 µg kg^{-1} and 50 µg kg^{-1}. Due to strong inhibition effects during analysis, DON, NIV and ZEN were not analyzed in cobs. Quantification was carried out using external calibration. Uncontaminated maize kernels and cobs were spiked with defined amounts of pure mycotoxins and processed in the same way as the samples.

DNA extraction

Total DNA was extracted using the CTAB method described by Brandfass and Karlovsky

[42]. For samples from maize fields, the extraction protocol was used with the following modifications: 8 mL of CTAB buffer, 160 µg of proteinase K (from a stock solution of 20 mg mL^{-1}) and 16 µL of mercaptoethanol were mixed and added to 500 mg of meal. After incubation, 6.5 mL of chloroform/isoamyl alcohol (24:1) were added. After centrifugation, 600 µL of the upper phase were transferred to a 1.5 mL tube containing 100 µL of 5 M NaCl and 193.6 µL of PEG 30%. Finally, samples were redissolved in 100 µL of TE buffer. Due to low amounts of meal obtained from the mini maize variety Gaspe Flint, the extraction protocol was scaled down to 100 mg per sample. For analysis, the samples were diluted 1:10 with distilled water. The quality of DNA extraction was controlled by agarose electrophoresis in 0.8 % (w/v) agarose gels (Cambrex, Rockland, ME, USA), prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH adjusted to 8.5 with acetic acid). The electrophoresis was performed at 4 V cm^{-1} for 60 min. After staining with ethidium bromide (2 mg L^{-1}), the gel was documented with a digital imaging system (Vilber Lourmat, Marne la Vallee, France).

Real time PCR analysis of fungal DNA

For species-specific detection and quantification of *F. verticillioides* and *F. graminearum* in field trials, real-time PCR followed by melting curve analysis was performed as described by Nutz *et al.* [43] and Brandfass and Karlovsky [42]. For the quantification of *F. graminearum* slight modifications were made: the amplification mix consisted of Absolute™ Blue QPCR SYBR Green I Fluorescein Mix (Thermo Start TM DNA-Polymerase, 3 mM MgCl2, dNTP Mixture, SYBR Green I, and 10 nM Fluorescein (Abgene Limited, Thermo Fisher Scientific, Epsom, United Kingdom) and 0.3 µM of each primer. The initial denaturation step of the cycling protocol was extended to 15 min at 95 °C. Real-time PCR analysis was performed by using an iCycler thermal cycler (BioRad, Hercules, CA, USA).

For climate chamber experiments, the detection and quantification of *F. verticillioides* and *F. graminearum* DNA was carried out using real-time PCR assays optimized by Dastjerdi *et al.* (unpublished). Real-time PCR analysis was performed by using an iCycler CFX384 (BioRad, Hercules, CA, USA).

Quantification was performed using dilution series of *F. verticillioides* DNA and *F. graminearum* containing purified fungal DNA in amounts of 0.5 pg to 500 or 1100 pg mixed with maize DNA. The lowest standard of 0.5 pg was used as LOQ.

Statistical analysis

Statistical analysis was performed with SigmaPlot version 11.0. (Systat software Inc.). Non-parametric tests were always used since data often did not fulfill requirements for normal distributions.

The Fisher exact test was used to study differences in observations of fungal DNA or mycotoxins between single and mixed inoculations. All samples containing DNA or mycotoxins below LOQ were seen as not infected.

For comparison of the amount of DNA or mycotoxins between the treatments in field experiments, samples associated with a signal less than LOQ were allocated a value of ½ LOQ and samples below LOD were allocated a value of zero.

The Fisher exact test was also used to examine associations between observations of fungal species in mixed inoculated treatments in the greenhouse experiment. All samples containing DNA below LOQ were allocated a value of zero and samples containing DNA above LOQ were allocated a value of one.

Correlations between temperature and amounts of fungal DNA as well as mycotoxins were evaluated with the Spearman Rank Order Correlation. For DNA analysis samples associated with a signal less than LOQ were excluded from the data set so as to obtain differences in fungal biomass exclusively from infected samples. In the case of mycotoxins, all samples were used for analysis. Samples with a signal less than LOQ were allocated a value of ½ LOQ and samples below LOD a value of zero. Within all tests the level of significance was set to P=0.05.

Results

Field trial experiment 2009

Disease symptoms in the field trial experiment of 2009

In 2009, maize cobs were harvested at maturity stage (55 dpi). The visually detectable disease severity was significantly lower in cobs infected with *F. verticillioides* than in samples inoculated with both fungal species. Inoculations with *F. graminearum* did not show

significant differences in comparison to either of the inoculation treatments. These results were obtained in both maize varieties (Fig. 1).

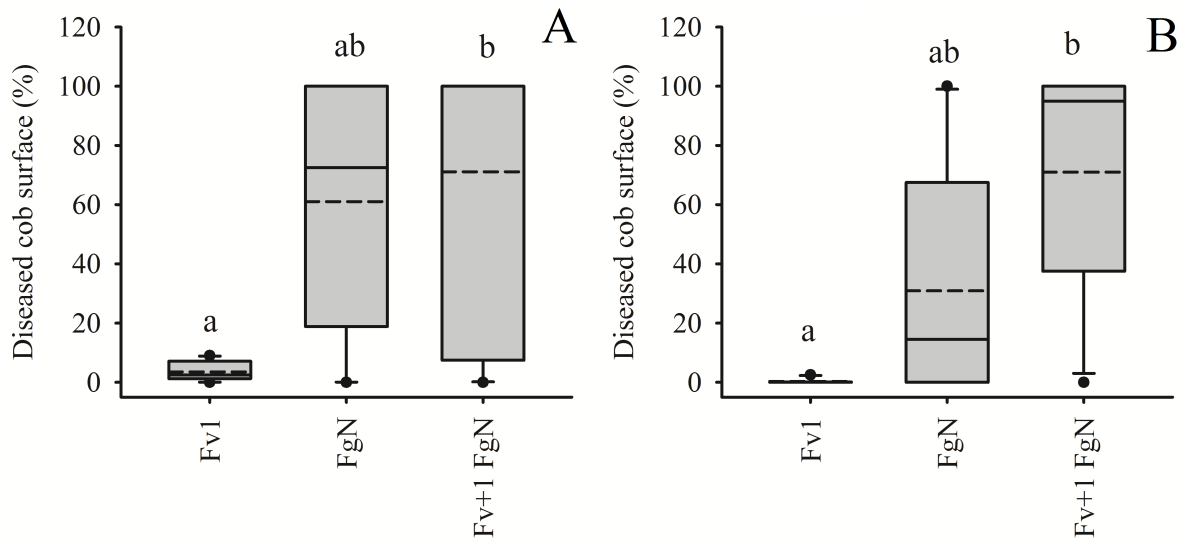

Figure 1. Visual symptoms estimated as percentage of infected cob surface per cob in variety Amadeo (A) and Kabanas (B) in 2009. Fv1: single inoculation with *F. verticillioides* Fv1; FgN: single inoculation with *F. graminearum* FgN*;* Fv1+FgN: mixed inoculations with Fv1 and FgN. The black line (—) and the dotted line (---) in each boxplot represent the median and the mean value, respectively. Different letters represent significantly different values at $P<0.05$ (Kruskal-Wallis One Way Analysis of Variance on Ranks followed by multiple comparison with Tukey test)

DNA in the field trial experiment of 2009

The incidence rates of both fungal species in the treatments were obtained. Comparison between single and mixed inoculation treatments revealed significantly higher infestation rates of *F. verticillioides* in mixed inoculations. This result was obtained in both maize varieties (Table 2). For *F. graminearum* no differences in infestations rates were obtained between single and mixed inoculations.

The amounts of fungal DNA in single and mixed treatments were compared. The amounts of *F. verticillioides* DNA were significant higher in mixed inoculations than in single inoculated treatments in both maize varieties. In contrast, no differences between treatments were determined concerning amounts of *F. graminearum* DNA (Fig. 2). By exclusively comparing the fungal biomass of samples with amounts of DNA above LOQ, no significant differences

between the treatments were determined (S-Table1).

Contamination with *F. graminearum* was found in 30-40% of plants inoculated with *F. verticillioides* in amounts close to or below the LOQ. In the control samples of non-infected plants, either no DNA or values below the LOQ were determined. Additionally, contamination with *F. verticillioides* was found in *F. graminearum* inoculated treatments (60-80%) in amounts of 0.02 ±0.12 µg g^{-1} in Kabanas and 0.62 ±1.08 µg g^{-1} in Amadeo. In the two control samples, amounts of *F. verticillioides* DNA was low, with amounts below LOQ in Kabanas and 0.03 µg g^{-1} in Amadeo (S-Table 1 and 2).

Table 2. Evaluation of the differences between frequencies of samples containing species-specific DNA inoculated either with *F. verticillioides Fv1*, *F. graminearum FgN* or as a mixture of both fungal species. *significant

		No. of plants containing fungal DNA > LOQ (total No. of plants)		
Maize variety	Species-specific DNA	Single inoculation	Mixed inoculation	Fisher exact test (P-value)
Amadeo	*F. verticillioides*	4 (10)	10 (10)	0.011*
	F. graminearum	7 (10)	6 (10)	1.000
Kabanas	*F. verticillioides*	3 (10)	10 (10)	0.003*
	F. graminearum	4 (10)	7 (10)	0.370

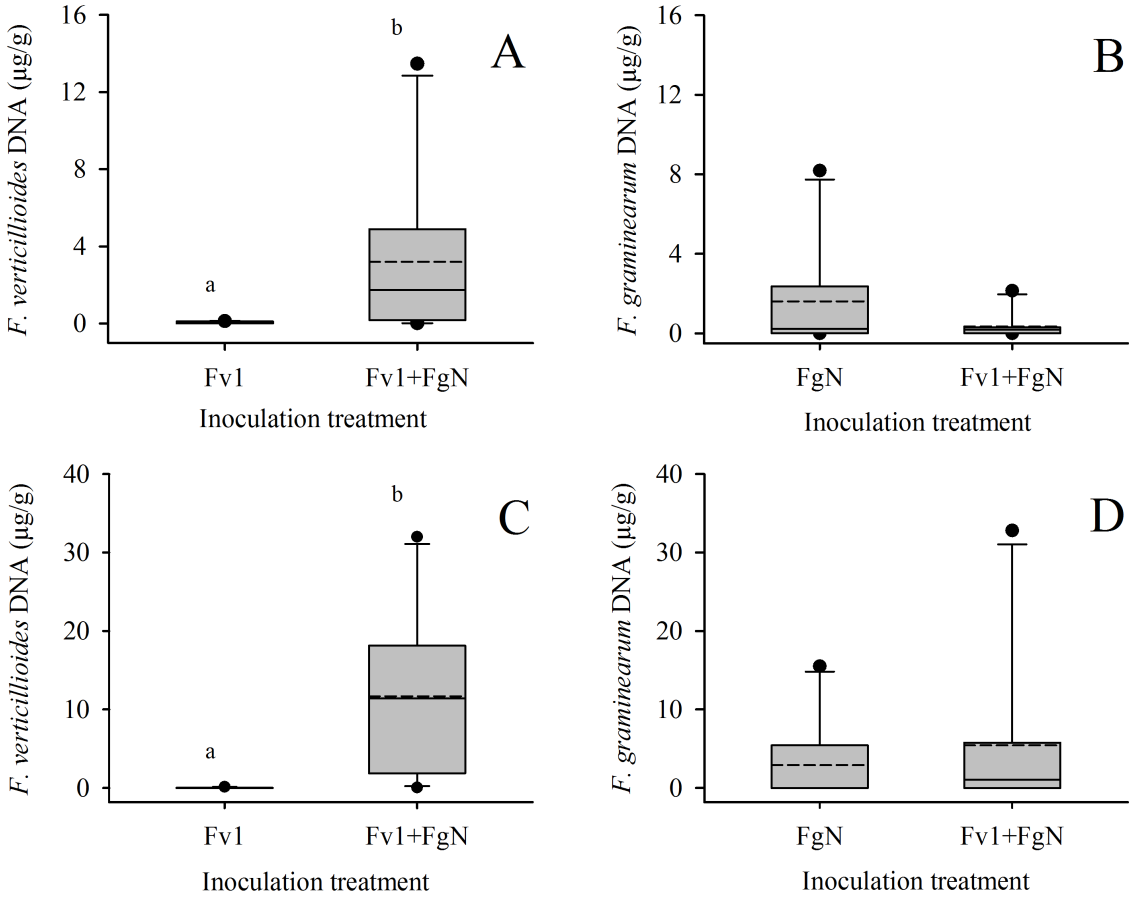

Figure 2. Amounts of DNA of *F. verticillioides* (A, C) and *F. graminearum* (B, D) in maize cobs of Amadeo (A, B) and Kabanas (C, D) in 2009, respectively. Fv1: single inoculation with *F. verticillioides* strain Fv1; FgN: single inoculation with *F. graminearum* strain FgN; Fv1+FgN: mixed inoculations with Fv1 and FgN. The black line (—) and the dotted line (---) in each boxplot represent the median and the mean value, respectively. Different letters represent significantly different values at $P<0.05$ (Mann Whitney Rank Sum test)

Mycotoxins in the field trial experiment of 2009

In Kabanas FB1 and ZEN were found in significantly higher frequencies in mixed inoculations than in the respective single inoculated treatments. These results were not found for NIV and with all three mycotoxins in Amadeo (Table 3).

Furthermore, samples of the single and mixed inoculated treatments were compared with

regard to their absolute content of mycotoxins.

The amounts of FB1 were significantly higher in mixed than in single inoculated treatments in both maize varieties (Fig. 3) The differences between treatments were consistent with the results obtained for an increase in infestation rate and biomass of *F. verticillioides*. In Kabanas, significantly higher amounts of ZEN (P=0.011) were detected in mixed inoculations with *F. verticillioides* than in single inoculations with *F. graminearum*. For NIV no differences between the treatments were determined. Normalization, that is, the toxin production per unit of fungal biomass, did not reveal significant differences between the treatments. For normalization only samples with fungal DNA above LOQ were used (S-Table 1 and 2).

Table 3. Evaluation of the differences between frequencies of mycotoxin-contaminated plants inoculated either with *F. verticillioides*, *F. graminearum* or a mixture of both fungal species. FB1 was obtained in the single inoculated treatment of *F. verticillioides*, and NIV and ZEN were obtained in the single inoculated treatment of *F. graminearum*. All mycotoxins were investigated in mixed treatments of both fungal species. * significant

		No. of plants containing the respective mycotoxins > LOQ (total No. of plants)		
Maize variety	Mycotoxins	Single inoculation	Mixed inoculation	Fisher exact test (P-value)
Amadeo	FB1	8 (10)	9 (10)	1.000
	NIV	7 (10)	7 (10)	1.000
	ZEN	8 (10)	9 (10)	1.000
Kabanas	FB1	2 (10)	10 (10)	<0.0001*
	NIV	4 (10)	9 (10)	0.057
	ZEN	5 (10)	10 (10)	0.033*

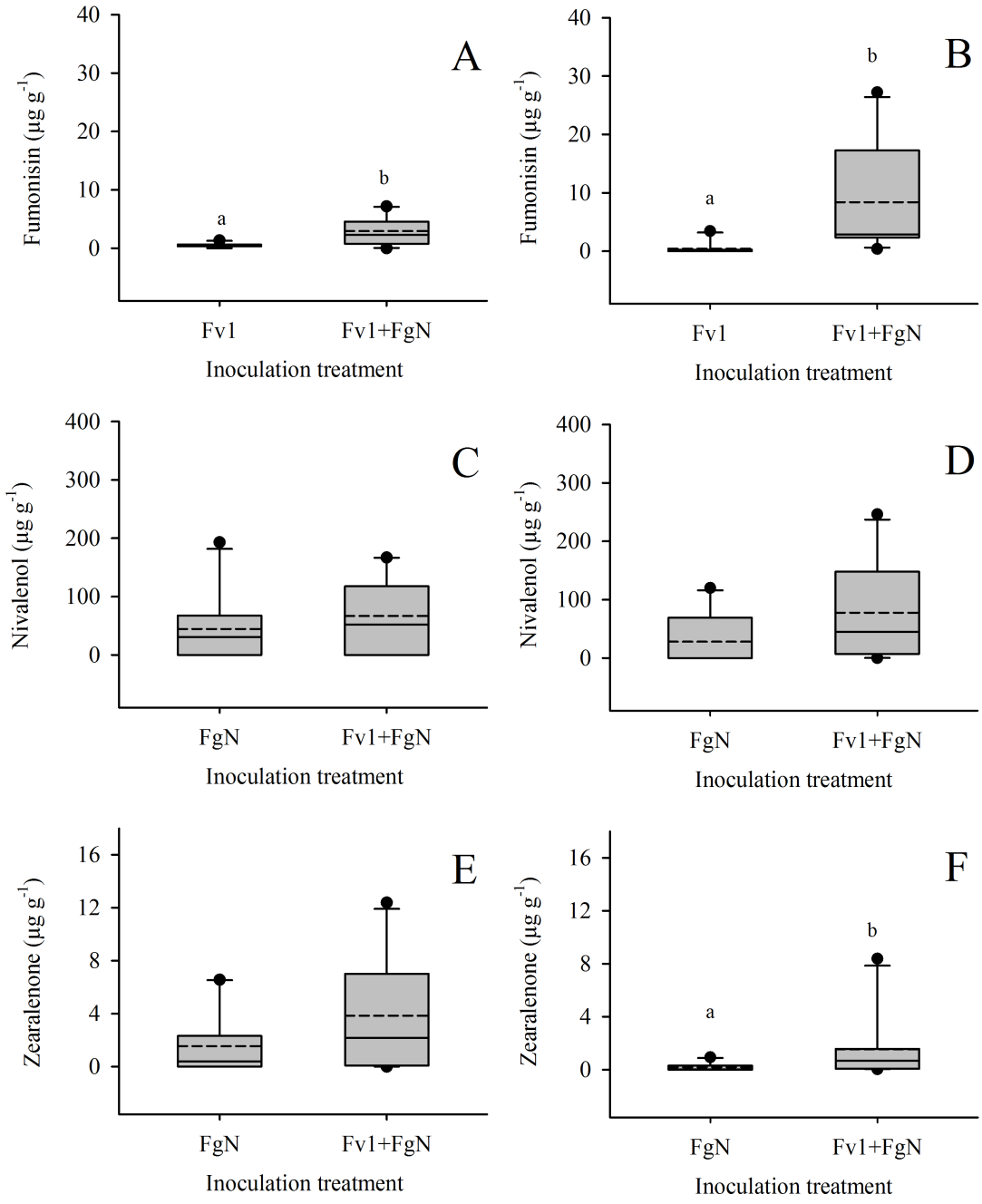

Figure 3. Absolute amounts of fumonisin B1 (A, B), nivalenol (C, D) and zearalenone (E, F) in maize cobs of Amadeo and Kabanas, respectively, in 2009. Fv1: single inoculation with *F. verticillioides*; FgN: single inoculation with *F. graminearum* FgN*;* Fv1+FgN: mixed inoculations with Fv1 and FgN. The black line (—) and the dotted line (---) in each boxplot represent the median and the mean value, respectively. Different letters represent significantly different values at $P<0.05$ (Mann-Whitney Rank Sum Test)

Field trial experiment 2010

Disease symptoms in the field trial experiment of 2010

In 2010, maize cobs were harvested at 21 dpi, 35 dpi and at maturity stage at 49 dpi. No differences in the visual disease severity between *F. verticillioides* and the mixed inoculation treatments were observed. Only inoculation with FgN partially led to significantly higher visual disease severity rates than the other treatments (Fig. 4).

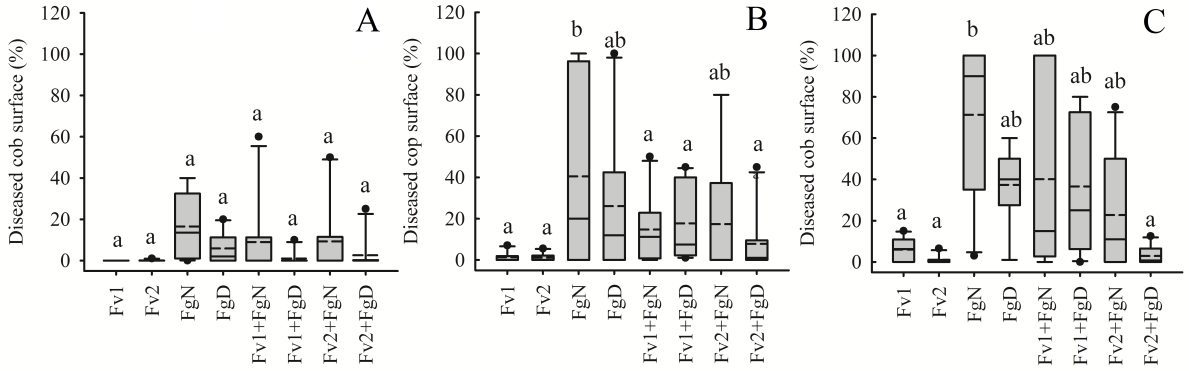

Figure 4. Visual disease symptoms estimated as percentage infected cob surface per cob at (A) 21 dpi, (B) 35 dpi and (C) 49 dpi in 2010. Fv1: single inoculation with Fv1; Fv2: single inoculation with Fv2; FgN: single inoculation with FgN; FgD: single inoculation with FgD; Fv1+FgN: mixed inoculations with Fv1 and FgN; Fv2+FgN: mixed inoculations with Fv2 and FgN; Fv1+FgD: mixed inoculations with Fv1 and FgD; Fv2+FgD: mixed inoculations with Fv2 and FgD. The black line (—) and the dotted line (---) in each boxplot represent the median and the mean value, respectively. Different letters represent significantly different values at $P<0.05$ (Kruskal-Wallis One Way Analysis of Variance on Ranks followed by multiple comparison with Tukey test)

DNA in the field trial experiment of 2010

The number of samples containing fungal DNA increased from one harvest time point to the next. However, the frequency of samples containing fungal DNA was relatively low for both fungal species. Single and mixed inoculations were compared with regard to the proportions of samples containing fungal DNA of both species. For *F. verticillioides,* no differences between the treatments were detected. In mixed inoculations with both *F. verticillioides* strains, *F. graminearum* FgD was detected in lower frequencies than in single inoculations at the third harvest time point. No further differences in frequencies of the fungal species were

detected between the treatments (Table 4).

Due to the low number of samples which tested positive for fungal DNA at the first two harvest time points, only samples of the third harvest time point were tested for differences in their amounts of DNA between the treatments. The amounts of *F. graminearum* DNA were significant lower in mixed inoculations of both strains of *F. graminearum* with *F. verticillioides* Fv2 than in single inoculated treatments (Fig. 5). No differences between treatments were determined concerning amounts of *F. verticillioides* DNA. By comparing only the fungal biomass of samples with amounts of DNA above LOQ, no significant differences between the treatments were determined (S-Table 3-5). Natural contamination of both fungal species occurred rarely and in amounts lower than LOQ in the control plants. Additionally, natural contamination within the treatments also only revealed amounts of fungal DNA close to or below LOQ (S-Table 3-5).

Table 4. Evaluation of the differences between frequencies of samples containing species-specific DNA inoculated either with *F. verticillioides*, *F. graminearum* or with a mixture of both fungal species. *significant

Days post inoculation	[Single]-[Mix treatment]	Species- specific DNA	No. of plants containing fungal DNA > LOQ (total No. of plants)		Fisher exact test (P-value)
			Single inoculation	Mixed inoculation	
21 dpi	[Fv1]-[Fv1+FgN]	*F. verticillioides*	0 (10)	1 (10)	1.000
	[FgN]-[Fv1+FgN]	*F. graminearum*	4 (10)	1 (10)	0.303
	[Fv1]-[Fv1+FgD]	*F. verticillioides*	0 (10)	1 (10)	1.000
	[FgD]-[Fv1+FgD]	*F. graminearum*	0 (10)	1 (10)	1.000
	[Fv2]-[Fv2+FgN]	*F. verticillioides*	0 (10)	2 (10)	0.474
	[FgN]-[Fv2+FgN]	*F. graminearum*	4 (10)	2 (10)	0.628
	[Fv2]-[Fv2+FgD]	*F. verticillioides*	0 (10)	0 (10)	1.000
	[FgD]-[Fv2+FgD]	*F. graminearum*	0 (10)	0 (10)	1.000
35 dpi	[Fv1]-[Fv1+FgN]	*F. verticillioides*	2 (10)	5 (10)	0.350
	[FgN]-[Fv1+FgN]	*F. graminearum*	2 (10)	2 (10)	1.000
	[Fv1]-[Fv1+FgD]	*F. verticillioides*	2 (10)	6 (10)	0.170
	[FgD]-[Fv1+FgD]	*F. graminearum*	2 (10)	2 (10)	1.000
	[Fv2]-[Fv2+FgN]	*F. verticillioides*	0 (10)	2 (9)	0.211
	[FgN]-[Fv2+FgN]	*F. graminearum*	2 (10)	2 (9)	1.000
	[Fv2]-[Fv2+FgD]	*F. verticillioides*	0 (10)	3 (10)	0.211
	[FgD]-[Fv2+FgD]	*F. graminearum*	2 (10)	1 (10)	1.000
49 dpi	[Fv1]-[Fv1+FgN]	*F. verticillioides*	6 (9)	6 (10)	1.000
	[FgN]-[Fv1+FgN]	*F. graminearum*	8 (10)	4 (10)	0.170
	[Fv1]-[Fv1+FgD]	*F. verticillioides*	6 (9)	6 (10)	1.000
	[FgD]-[Fv1+FgD]	*F. graminearum*	7 (9)	3 (10)	0.070*
	[Fv2]-[Fv2+FgN]	*F. verticillioides*	3 (6)	3 (10)	0.607
	[FgN]-[Fv2+FgN]	*F. graminearum*	8 (10)	6 (10)	0.628
	[Fv2]-[Fv2+FgD]	*F. verticillioides*	3 (6)	6 (10)	1.000
	[FgD]-[Fv2+FgD]	*F. graminearum*	7 (9)	0 (10)	<0.001*

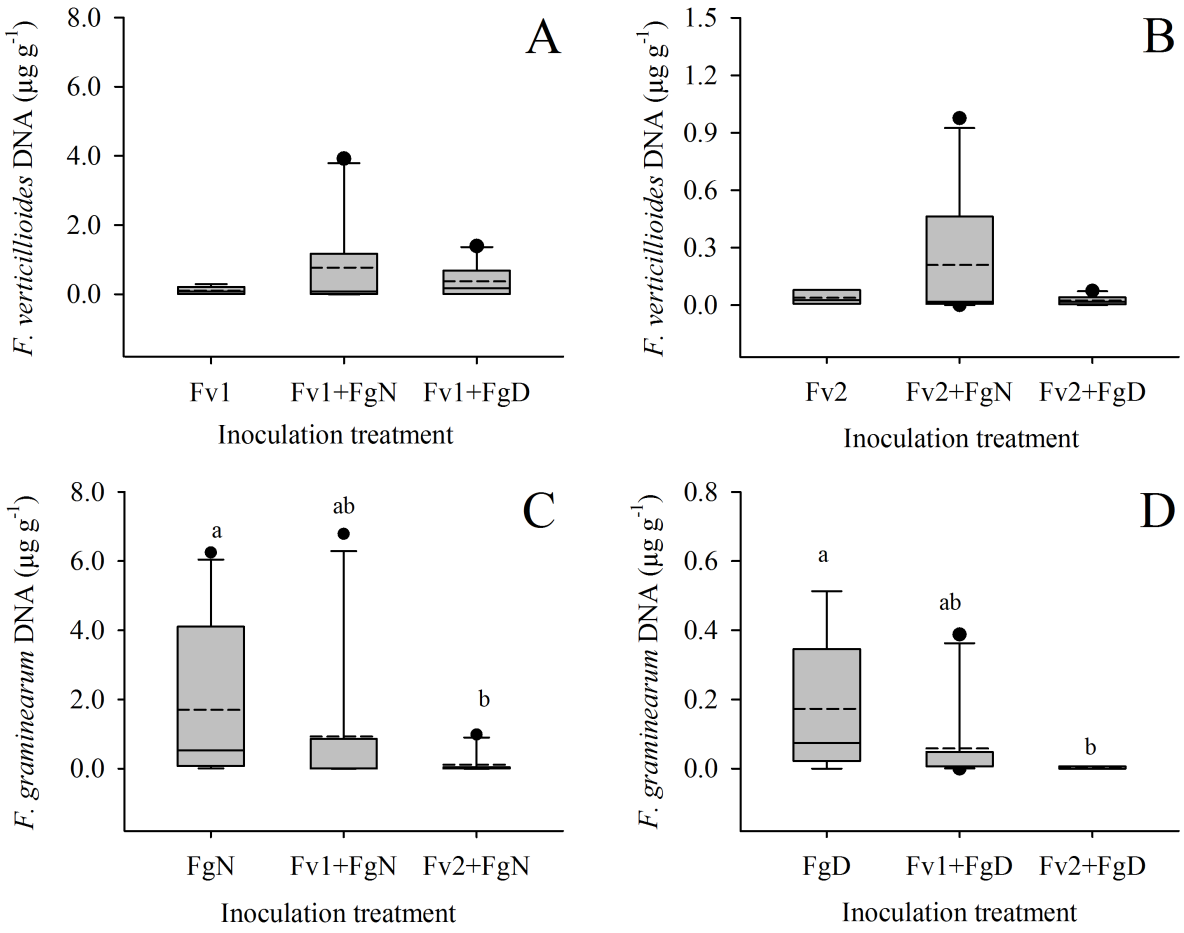

Figure 5. Amounts of DNA of *F. verticillioides* Fv1 (A) and Fv2 (B) and *F. graminearum* FgN (C) and FgD (D) in single and mixed inoculated treatments 49 dpi in 2010. Fv1 and Fv2: single inoculations with *F. verticillioides* strain Fv1 or Fv2; FgN and FgD: single inoculation with *F. graminearum* strain FgN or FgD; Fv1+FgN: mixed inoculations with Fv1 and FgN; Fv2+FgN: mixed inoculations with Fv2 and FgN; Fv1+FgD: mixed inoculations with Fv1 and FgD; Fv2+FgD: mixed inoculations with Fv2 and FgD. The black line (—) and the dotted line (---) in each boxplot represent the median and the mean value, respectively. Different letters represent significantly different values at $P<0.05$ (Kruskal-Wallis One Way Analysis of Variance on Ranks follwed by Tukey test (C) and Dunns`test (D)

Mycotoxins in field trial experiment of 2010

At the first harvest time point FB1 was only detected in mixed inoculated samples, but in low frequencies. In proportion analysis, FB1 was detected in significantly higher frequencies in mixed inoculations of *F. verticillioides* Fv1 with both *F. graminearum* strains than in the

single inoculations at the second harvest time point (Table 5). This result was not determined with *F. verticillioides* strain Fv2. At the last harvest time point no differences of the FB1 frequencies between the treatments were determined.

Due to the low infestation rates of the fungi NIV, DON and ZEN were only analyzed at the two later harvest time points. At the third harvest time point, the proportion analysis of DON revealed significantly lower frequencies (P=0.005) of DON in mixed inoculations of FgD with Fv2 than single inoculations which is consistent with the lower number of samples containing DNA of *F. graminearum*. Furthermore, a significant lower frequency of ZEN was found in mixed inoculations of FgN with Fv2 than in single inoculations. No further differences in the proportions of this mycotoxin was detected between the treatments.

Due to the low number of samples which tested positive for fungal DNA and mycotoxins at the first two harvest time points, only samples of the third harvest time point were tested for differences in their amounts of mycotoxins (Fig. 6). Significantly lower amounts of DON and NIV were determined in mixed inoculations of *F. graminearum* FgD and FgN with Fv2, respectively. For FB1 and ZEN no differences between the treatments were determined.

Normalization did not reveal significant differences between the treatments. For normalization only samples with fungal DNA above LOQ were used (S-Table 3-5).

Table 5. Differences between frequencies of mycotoxin-contaminated plants inoculated either with *F. verticillioides*, *F. graminearum* or a mixture of both fungal species. FB1 was obtained in the single inoculated treatment of *F. verticillioides;* NIV and ZEN were obtained in the inoculated treatments of *F. graminearum* FgN*;* and DON and ZEN were obtained in the inoculated treatments of *F. graminearum* FgD. Samples of the first harvest time point were only analyzed for FB1. *significant

			No. of plants containing mycotoxins > LOQ (total No. of plants)		
Days post inoculation	[Single]-[Mix treatment]	Mycotoxins	Single inoculation	Mixed inoculation	Fisher exact test (P-value)
21 dpi	[Fv1]-[Fv1+FgN]	FB1	0 (10)	4 (10)	0.087
	[Fv1]-[Fv1+FgD]	FB1	0 (10)	1 (10)	1.000
	[Fv2]-[Fv2+FgN]	FB1	0 (10)	3 (10)	0.211
	[Fv2]-[Fv2+FgD]	FB1	0 (10)	0 (10)	1.000
35 dpi	[Fv1]-[Fv1+FgN]	FB1	1 (10)	7 (10)	0.020*
	[FgN]-[Fv1+FgN]	NIV	4 (10)	9 (10)	0.057
	[FgN]-[Fv1+FgN]	ZEN	0 (10)	0 (10)	1.000
	[Fv1]-[Fv1+FgD]	FB1	1 (10)	9 (10)	0.001*
	[FgD]-[Fv1+FgD]	DON	0 (10)	4 (10)	0.087
	[FgD]-[Fv1+FgD]	ZEN	0 (10)	1 (10)	1.000
	[Fv2]-[Fv2+FgN]	FB1	2 (10)	1 (9)	1.000
	[FgN]-[Fv2+FgN]	NIV	4 (10)	2 (9)	0.628
	[FgN]-[Fv2+FgN]	ZEN	0 (10)	1 (9)	0.474
	[Fv2]-[Fv2+FgD]	FB1	2 (10)	4 (10)	0.628
	[FgD]-[Fv2+FgD]	DON	0 (10)	3 (10)	0.211
	[FgD]-[Fv2+FgD]	ZEN	0 (10)	0 (10)	1.000
49	[Fv1]-[Fv1+FgN]	FB1	8 (9)	8 (10)	1.000
	[FgN]-[Fv1+FgN]	NIV	10 (10)	8 (10)	0.474
	[FgN]-[Fv1+FgN]	ZEN	8 (10)	4 (10)	0.170
	[Fv1]-[Fv1+FgD]	FB1	8 (9)	7 (10)	0.582
	[FgD]-[Fv1+FgD]	DON	8 (10)	7 (10)	1.000
	[FgD]-[Fv1+FgD]	ZEN	3 (9)	4 (10)	1.000
	[Fv2]-[Fv2+FgN]	FB1	3 (6)	4 (10)	1.000
	[FgN]-[Fv2+FgN]	NIV	10 (10)	6 (10)	0.087
	[FgN]-[Fv2+FgN]	ZEN	8 (10)	2 (10)	0.023*
	[Fv2]-[Fv2+FgD]	FB1	3 (6)	7 (10)	0.607
	[FgD]-[Fv2+FgD]	DON	8 (10)	1 (10)	0.005*
	[FgD]-[Fv2+FgD]	ZEN	3 (9)	0 (10)	0.087

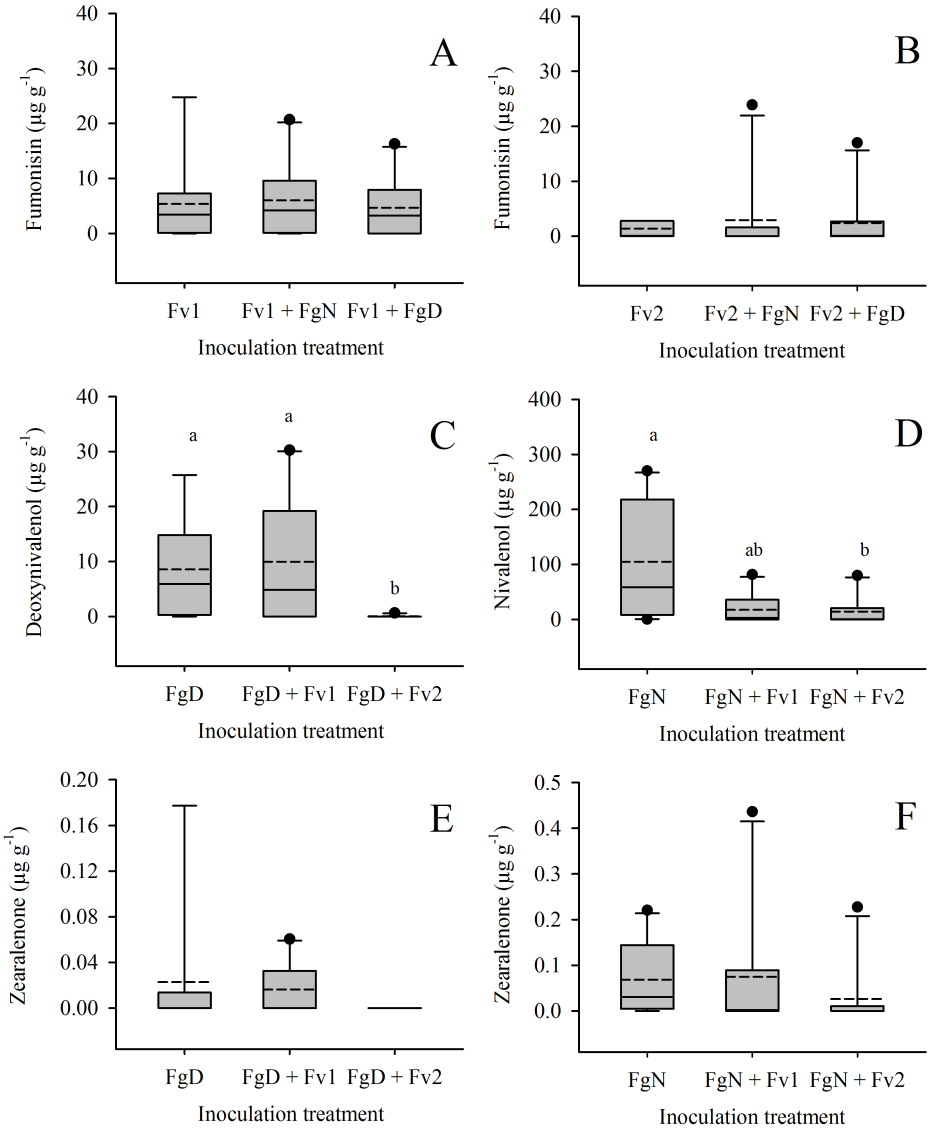

Figure 6. Amounts of mycotoxins of *F. verticillioides* Fv1 (A) and Fv2 (B) and *F. graminearum* FgD (C, E) and FgN (D, F) in single and mixed inoculated treatments 49 dpi in 2010. Fv1 and Fv2: single inoculations with *F. verticillioides* strain Fv1 or Fv2; FgN and FgD: single inoculation with *F. graminearum* strain FgN or FgD; Fv1+FgN: mixed inoculations with Fv1 and FgN; Fv2+FgN: mixed inoculations with Fv2 and FgN; Fv1+FgD: mixed inoculations with Fv1 and FgD; Fv2+FgD: mixed inoculations with Fv2 and FgD. The black line (—) and the dotted line (---) represent the median and the mean value, respectively. Different letters represent significantly different values at $P<0.05$ (Kruskal-Wallis One Way Analysis of Variance on Ranks follwed by Tukey (D) and Dunns` (C) test

Climatic conditions in 2009 and 2010

The minimum and maximum temperatures during the growing period in 2009 in Dassel close to Relliehausen and 2010 in Göttingen were relatively similar. The total precipitation during the growing period was higher in 2010 with 331 mm (one week before inoculation till harvest) than in 2009 with 142 mm (one week before inoculation till harvest). In both years the week before inoculation was characterized by large amounts of precipitation (Fig. 7).

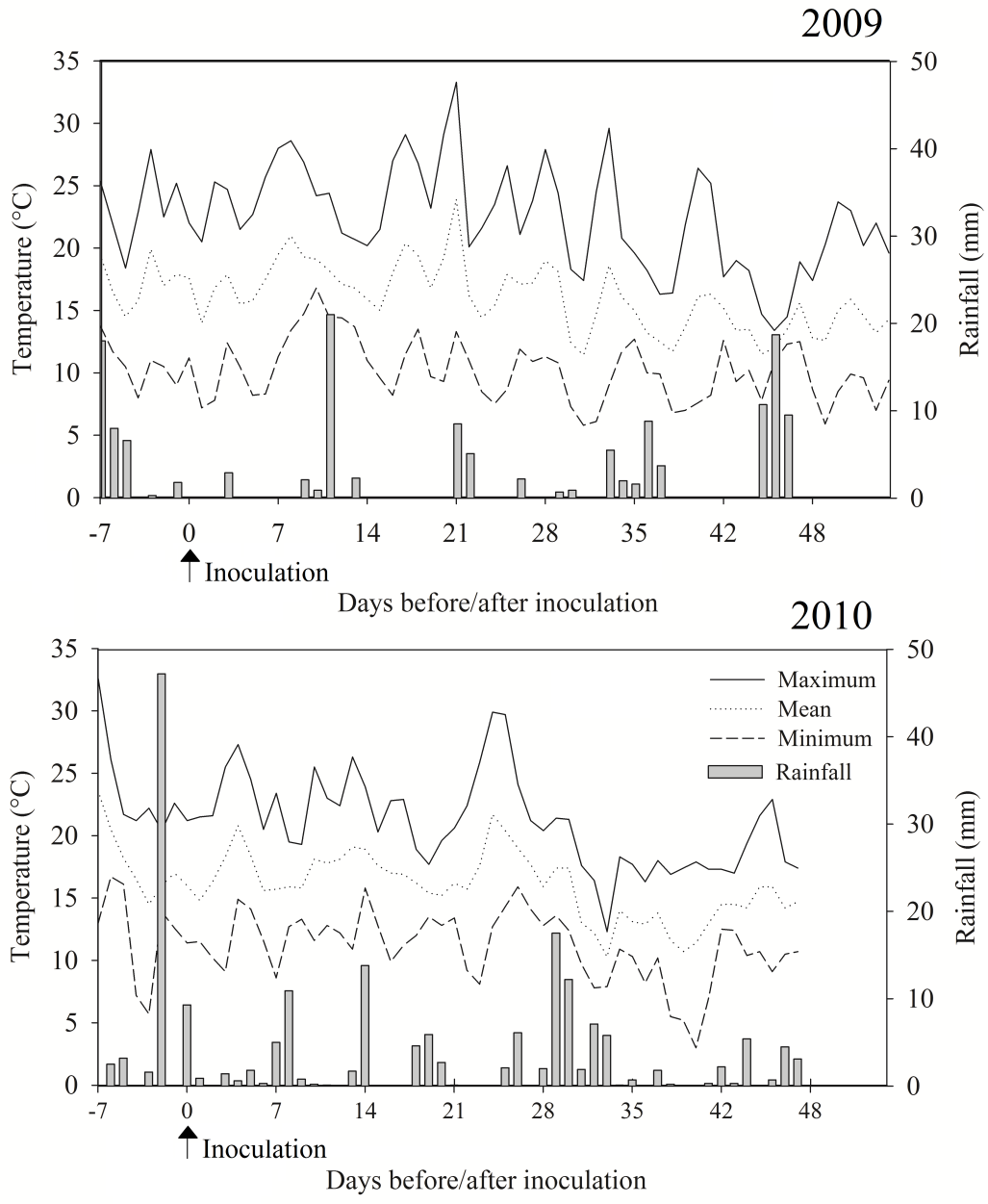

Figure 7. Temperature and precipitation in the trial periods of field experiments in 2009 and 2010

Climate chamber experiment

The influence of temperature and mixed inoculations with *F. graminearum* on the growth and mycotoxin production of *F. verticillioides* was investigated by way of inoculation experiments on the maize variety Gaspe Flint under controlled climate conditions (Fig. 8).

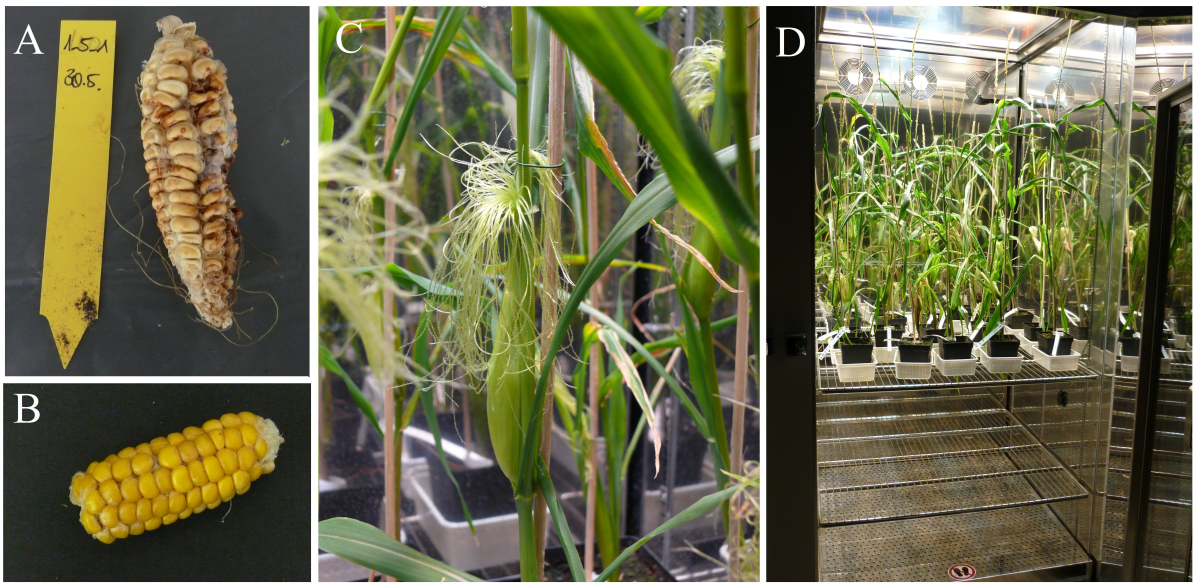

Figure 8. Cobs and plants of mini maize variety Gaspe Flint in the climate chamber experiment. Maize ear infected with Fv1 and FgN (A), an uninfected maize ear (B), a maize plant five days after silk emergence (C) and maize plants in climate chamber (D)

DNA in climate chamber experiment

The influence of temperature on the incidence of fungal DNA in kernels and cobs, defined as samples containing amounts of DNA above the LOQ, was determined using Pearson correlation test and the Fisher exact test.

Correlation analyses between percentage of samples containing amounts of DNA above the LOQ and temperature were carried out. For *F. verticillioides* the infestation rate was positively correlated with temperature in mixed inoculations with FgD in kernels (r=0.912, P=0.0311), in single inoculations in cobs (r=0.933, P=0.0207) and in mixed inoculations with FgN (r=0.954, P=0.0116) in cobs (Fig. 9). Neither *F. graminearum* strains showed correlations between temperature and their infestation rates.

Significant differences in the incidence of *F. verticillioides* between the temperatures were seen in the mixed treatment with FgD (Fig. 9, S-Table 9). The observed incidence rate of *F. verticillioides* in kernels was less at the lowest temperature T1 in comparison to all other temperatures ($P<0.001$). Similar results were observed in the cobs, with the lowest climate chamber T1 leading to lower incidence of *F. verticillioides* than at T3 ($P=0.025$) and T5 ($P=0.003$). Additionally, incidence at T5 was higher than at T1 but also than at T2 ($P=0.009$). Overall, the highest and the lowest temperature scenario led to significant differences in the incidence of *F. verticillioides* DNA compared to the other temperatures in mixed inoculations with FgD.

In kernels mixed inoculations of Fv1 and FgD led to significant lower incidence rates of *F. graminearum* in T5 in comparison to T4 ($P=0.02$) and T3 ($P=0.01$). No differences in the incidence of *F. graminearum* DNA inoculated with FgN were observed (Fig. 9, S-Table 7-10). Furthermore, statistical analysis of positive associations in the occurrence of both species in mixed inoculated treatments was carried out using the Fisher exact test. The different climate scenarios in mixed inoculations were analyzed but no significant relationship between *F. verticillioides* DNA and *F. graminearum* DNA was determined in kernels or cobs (S-Table 9, 10).

Furthermore, correlation analysis between amount of biomass and temperature was carried out by using samples containing fungal biomass and removing values below LOQ from the data set. This allowed to analyze the effects of temperature on biomass of the two fungal species. Moderate positive correlations between temperature and amounts of *F. verticillioides* DNA were obtained in single inoculations ($r=0.463$, $P=0.015$) and in mixed inoculations with FgD ($r=0.417$, $P=0.027$) in cobs (Fig. 10). Furthermore, the temperature was not determined to have had an effect in the kernels or in the treatment with FgN. Neither *F. graminearum* strains showed effects of temperatures in their amounts of DNA (Fig. 11, 12).

In about 40% of the control plants as well as 15% and 35% of the plants inoculated with FgD and FgN, respectively, DNA of *F. verticillioides* was found in amounts above LOQ (S-Table 7, 8, 11).

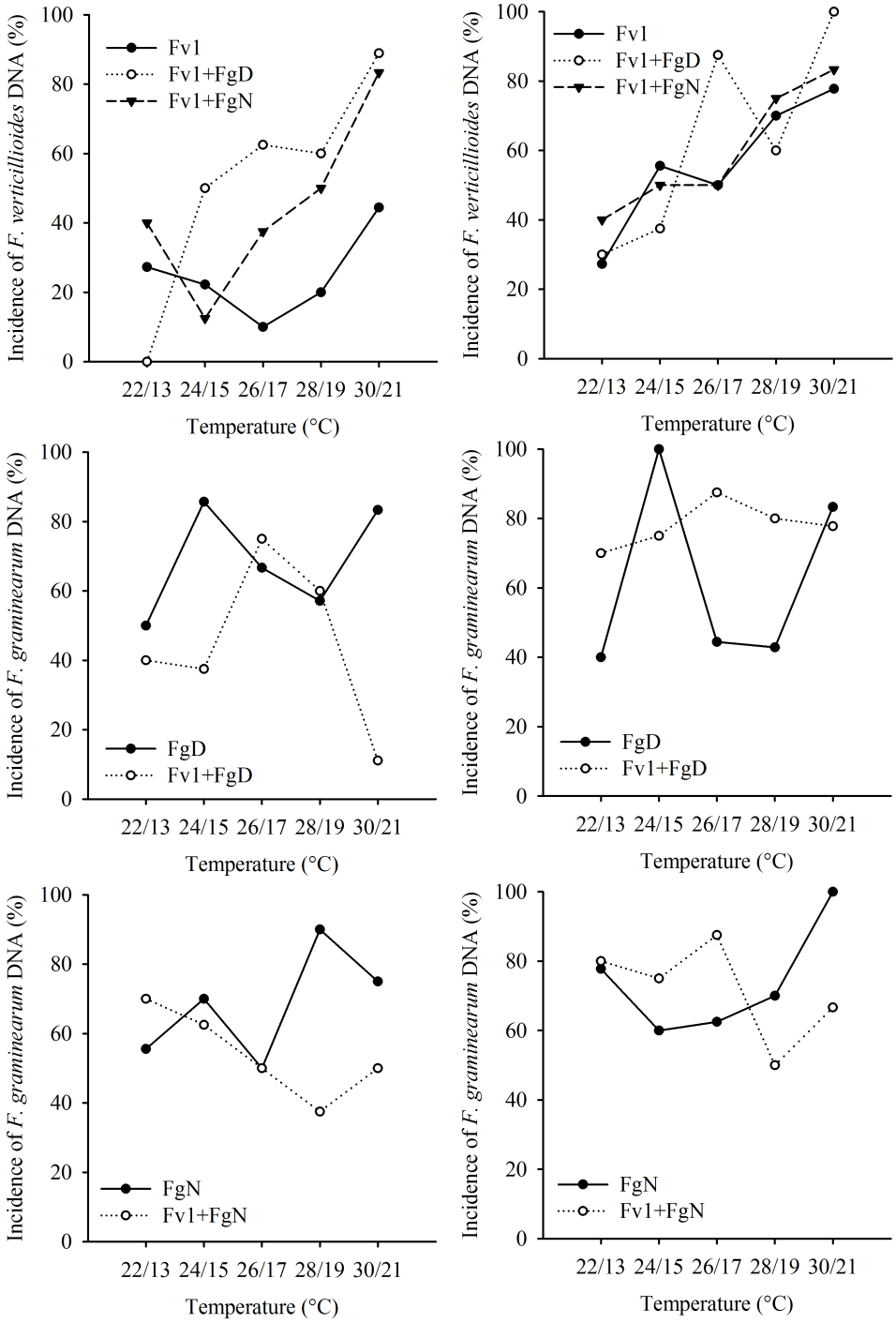

Figure 9. Percentages of samples containing fungal DNA of *F. verticillioides* and *F. graminearum* in amounts above LOQ in kernels (left) and cobs (right) at different temperature scenarios. Fv1: single inoculations with *F. verticillioides* strain Fv1; FgN and FgD: single inoculation with *F. graminearum* strain FgN or FgD; Fv1+FgN: mixed inoculations with Fv1 and FgN; Fv1+FgD: mixed inoculations with Fv1 and FgD

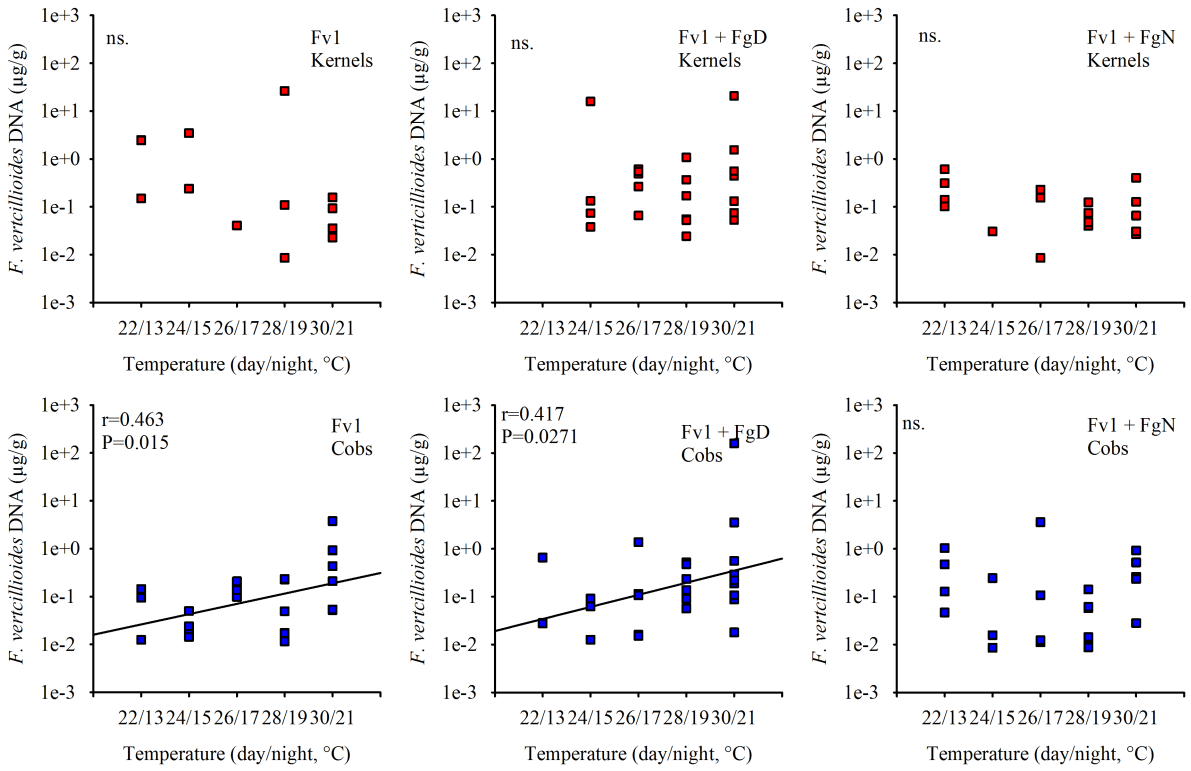

Figure 10. Amount of *F. verticillioides* DNA in kernels (red) and cobs (blue) grown under controlled conditions in single and mixed inoculation treatments at different temperatures. Samples without quantifiable amounts of biomass were excluded. Fv1: single inoculations with Fv1; Fv1+FgD: mixed inoculations with Fv1 and FgD, Fv1+FgN: mixed inoculations with Fv1 and FgN (Spearman correlation on Ranks, $P < 0.05$; ns. = not significant)

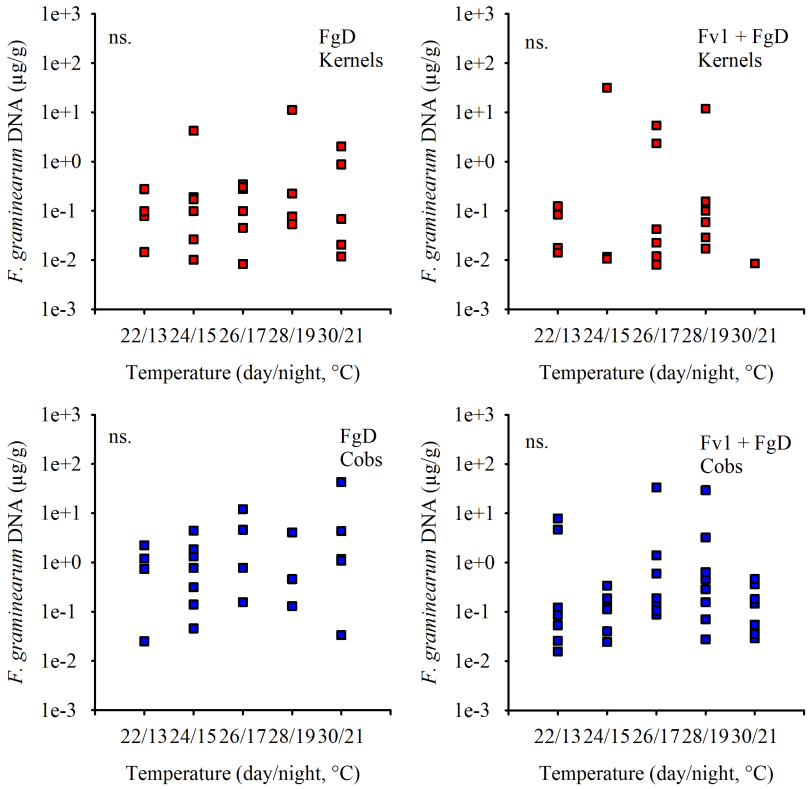

Figure 11. Amount of *F. graminearum* DNA strain FgD in kernels (red) and cobs (blue) grown under controlled conditions in single and mixed inoculation treatments at different temperatures. Samples without quantifiable amounts of biomass were excluded. FgD: single inoculation with FgD; Fv1+FgD: mixed inoculations with Fv1 and FgD (Spearman correlation on Ranks, $P < 0.05$; ns. = not significant)

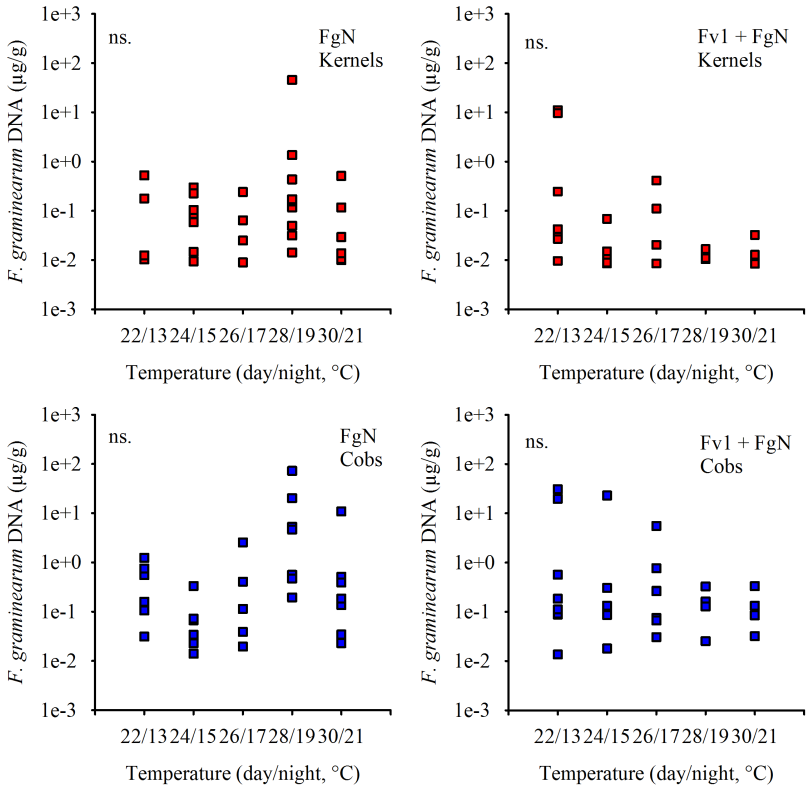

Figure 12. Amount of *F. graminearum* DNA strain FgN in kernels (red) and cobs (blue) grown under controlled conditions in single and mixed inoculation treatments at different temperatures. Samples without quantifiable amounts of biomass were excluded. FgN: single inoculation with FgN; Fv1+FgN: mixed inoculations with Fv1 and FgN (Spearman correlation on Ranks, $P < 0.05$; ns. = not significant)

Mycotoxins in climate chamber experiment

Using Pearson correlation test the relationship between temperature and incidence of mycotoxins was tested to support an integrated vision of the risk of mycotoxins due to changing temperature, seen as the consequence of frequency in the treatments. NIV and DON were not analyzed in cobs due to high inhibition effects in analysis, whereas FB1 was analyzed in kernels and cobs.

Positive correlations were determined between temperature and FB1 in kernels of mixed inoculations with FgD ($r=0.991$, $P=0.001$) and cobs of single inoculations with Fv1 ($r=0.983$, $P=0.026$) and mixed inoculations of Fv1 and FgD ($r=0.900$, $P=0.037$). For NIV a negative correlation was detected in mixed inoculations of Fv1 and FgN ($r=-0.926$, $P=0.024$) since NIV was not detected at 28 and 30 °C. In single inoculations of FgN, no correlation was

found. Correlations between DON and temperature were not detected in neither single nor mixed inoculations (Fig 13).

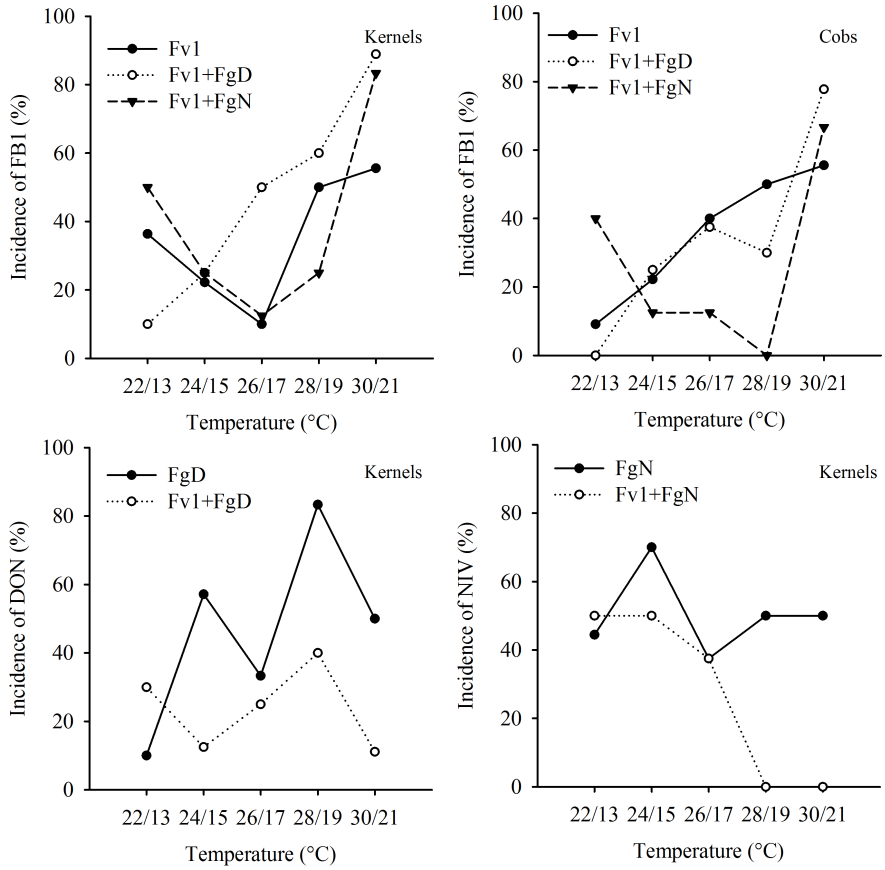

Figure 13. Percentages of samples containing mycotoxins in amounts above LOQ in kernels and cobs at different temperature scenarios. Fv1: single inoculations with *F. verticillioides* strain Fv1; FgN and FgD: single inoculation with *F. graminearum* strain FgN or FgD; Fv1+FgN: mixed inoculations with Fv1 and FgN; Fv1+FgD: mixed inoculations with Fv1 and FgD

Discussion

The biomass and mycotoxin production of *F. verticillioides* and both chemotypes of *F. graminearum* in response to single and mixed inoculations under controlled conditions in climate chambers at different temperatures as well as in field trials were elucidated.

In the field trials, the frequency of *F. verticillioides* detected was equal or higher in mixed inoculations than in single inoculations, but never lower. Especially in 2009 mixed inoculations lead to enhanced infection rates in both maize varieties. The consequence of

higher disease rates of *F. verticillioides* is the increase of contamination of samples with FB1. In contrast, the frequency of *F. graminearum* DNA was either not affected or reduced in mixed inoculations.

The observations of Picot *et al.* were similar and they formulated the hypothesis that *F. verticillioides* had a competitive advantage over *F. graminearum*. Furthermore, the authors suggest that *F. graminearum* may facilitate contamination by *F. verticillioides* in maize ears by acting as a breach for infection [28]. Two ways of positive co-occurrence may be possible: on the one hand, the infection by one fungus which increases the vulnerability of the plant and facilitates infection by another fungus, and, on the other hand, the existence of similar host requirements for both fungi and a lack of competitive exclusion [44]. The increase in the occurrence of *F. verticillioides* in mixed inoculations indicates a factor that facilitates the infection of maize ears by the fungus. In several studies, the role of mycotoxins in the plant pathogenesis of both fungal species has been evaluated. NIV was found to act as a moderate virulence factor of *F. graminearum* in maize and interacts with resistance to spread [45, 46]. Furthermore, Harris *et al.* found DON to be a putative virulence factor in maize cob colonization [47]. In wheat, DON plays an important role as virulence factor in the spread from one spikelet to another, while the mycotoxin does not seem not be necessary for initial infection by the fungus [48]. Instead, fumonisins were shown not to be necessary for *F. verticillioides* for infection or disease development [20, 49]. The production of NIV and also DON may have facilitated the infection of *F. verticillioides* of maize cobs. Undamaged maize kernels represent a barrier to fungal pathogens that infest kernel tissues. It has been proven that kernel damage caused by several insects facilitates infection with *F. verticillioides* [2]. Infection with *F. graminearum* may defeat the barriers in a similar facilitative way and may play a role in infection across the maize cob.

In addition to the higher occurrence of the fungus in mixed treatments, the amount of *F. verticillioides* also revealed an increase in the biomass in 2009. Nevertheless, in 2010 similar frequencies and amounts of *F. verticillioides* biomass were determined among the treatments; which show that *F. verticillioides* colonized the ear as successfully as in mixed treatments until the latest harvest time point. The reason for the differences in interaction are unknown, but environmental conditions may have influenced the infection and growth of both fungal species. Interestingly, in 2009 the statistical proportion analysis led partly to differences in results between DNA and mycotoxins. *F. verticillioides* DNA was enhanced in

mixed inoculations in both maize varieties while *F. graminearum* was not. In contrast, Fb1 and ZEN revealed a higher frequency in Kabanas in mixed treatments, but not in Amadeo. In all single inoculations, the frequency of mycotoxins was lower in Kabanas than in Amadeo. This may indicate different levels of resistance among the maize varieties towards *Fusarium* infection. However, this needs further, more detailed investigations. Lines resistant to *Fusarium* may present a possibility of reducing maize ear rot by *Fusarium verticillioides* or *F. graminearum* [50, 51]. It is assumed that modern field maize cultivars are almost universally infected with symptomless endophytic colonization by *F. verticillioides*, but disease symptoms are rarely exhibited unless stressed [52]. In the field and also in the climate chamber experiment, the ubiquitous fungus *F. verticillioides* was also found as a natural contaminant in controls and treatments of *F. graminearum*. The occurrence of natural contamination by the fungus in experiments *in planta* have also been described several times [28, 53]. Picot *et al.* even found an increase in natural contamination due to inoculation with *F. graminearum*, which may have facilitated *F. verticillioides* infection [28].

In contrast to *F. verticillioides,* the frequency of *F. graminearum* detected was not or negatively effected by mixed inoculation when compared to single inoculations. In 2010, the frequency of DNA of *F. graminearum* strain FgD was reduced in mixed inoculated samples with Fv1 and also Fv2 when compared to single inoculations. Often DON or NIV were found, but there was no DNA of *F. graminearum* in quantifiable amounts. This may indicate that either a further fungal species produced the mycotoxins, but natural contamination occurred only rarely and in low amounts, or that *F. graminearum* occurred only in amounts not quantifiable for real-time PCR, especially at the early harvest time points. Reduction at the later harvest time points may be the result of growth suppression by *F. verticillioides*.

Picot *et al.* found reduced biomass of *F. graminearum* in mixed inoculations and formulated the hypothesis that this may probably be attributed to a better growth rate of *F. verticillioides* over a wide range of temperatures [28]. Furthermore, Reid *et al.* revealed that *F. verticillioides* had a greater growth rate in mixed inoculations than did *F. graminearum* [53]. Faster colonization of the substrate by one fungus may lead to reduced growth of the other fungus as a result of indirect interaction between the two fungal species. On the other hand, *F. verticillioides* may directly influence *F. graminearum* by the production of secondary metabolites.

It is supposed that the production of secondary metabolites gives the producing organism an

advantage which increases fitness and assertiveness in the specific ecological niche [54]. However, no changes in the levels of FB1 in response to fungal competition were observed, indicating that the mycotoxin tested did not play a role as a factor in a putative competitive reaction of the fungus. Nevertheless, it cannot be excluded that further compounds may negatively effect the growth of *F. graminearum*.

Differences in the frequencies and growth of both fungal species may be partly explained by climatic conditions in the two years. Climate may be the most important factor influencing the co-existence and interactions of several filamentous fungi in the same geographical regions. Environmental conditions are the most likely candidate to account for the difference in mycotoxin content among maize grain produced in different regions. Weather affects the accumulation of mycotoxins in grain in three ways: temperature and humidity control the efficiency of infection, affect the ability of host plants to counteract the infection by defense responses, and influence fungal growth and mycotoxin production within plant tissue.

The low levels of fumonisins in maize grown in Northern Europe can be accounted by the absence of major fumonisin producers or by the suppression (or lack of induction) of fumonisin synthesis under local conditions. Published data on fumonisin content and the biomass of fumonisin producers in naturally contaminated maize grain in Northern Europe are limited. In a 2-year survey of 84 maize field samples in Germany, high contamination rates with *Fusarium verticillioides*, *F. graminearum*, and *F. proliferatum* were found in 2006, whereas in 2007 *F. graminearum*, *F. cerealis* and *F. subglutinans* were found more frequently. The authors explain this fact with the differences in climate between the two years. In 2006, the growing season was characterized by high temperature and low rainfall during anthesis and early grain filling, whereas in 2007 only moderate temperatures and frequent rainfall occurred during the growing season [55].

Reid *et al.* formulated the hypothesis that daytime temperatures are mainly responsible for conditions favoring the dominance of *F. verticillioides* over *F. graminearum* due to the former's ability to grow in a wide range of temperatures [27]. However, the temperature was quite similar between the two years and both years were characterized by rainfall before and after inoculation. In 2010, the amount of rain was even higher. Disease development caused by *F. graminearum* is generally associated with frequent rainfall and moderate temperatures during the summer, whereas *F. verticillioides* is usually favored by dry and hot climatic conditions, especially during pollination [1]. In many studies the optimal temperatures for

growth of *F. graminearum* and *F. verticillioides* were evaluated and it was shown that the optimal temperature ranges of the two fungal species differ but overlap. In *in vitro* experiments, the growth of *F. verticillioides* increased steadily across the temperature range with optimal conditions between 25 and <35 °C [56], while at 15 °C growth rate was very low [57]. Optimal temperatures for the growth of *F. graminearum* have been reported to be between 24 and 28 °C [34, 53, 58]. Temperatures during growing season were moderate and similar in both years. The lower frequency and amounts of precipitation in 2009 could theoretically have favored *F. verticillioides* infection and growth. High frequencies of natural infection also demonstrate the favorable conditions for infection in this year. In 2010, climatic conditions differed with regard to the frequency and amount of precipitation, which could lead to climate conditions not favorable to *F. verticillioides* growth. Nevertheless, mixed inoculations with Fv2 led to reduced biomass of both strains of *F. graminearum*, indicating that *F. verticillioides* may have dominated *F. graminearum* in this year. However, this result was not confirmed in mixed inoculations with Fv1, indicating differences in strain behavior.

Due to the complex relationship between climate and fungal disease development, investigations on the effects of temperature were carried out *in planta* under controlled conditions. Usually examinations on temperature are carried out *in vitro* at constant temperatures, which differs from temperature variation under field conditions. Garcia *et al.* demonstrated that growth rates differ in account of constant and cycling temperatures and that extrapolation from constant conditions to real field conditions is problematic [59]. Most of the studies are carried out in culture medium, only a few of them use plant material and no studies are published evaluating the effects of temperature on the growth of both fungi *in planta*.

In the current study the influence of five temperatures scenarios (22/13-30/21°C) in climate chambers on incidence and amounts of fungal DNA was determined. The incidence of *F. verticillioides* was positively effected by temperature, while *F. graminearum* showed reduced incidence only in mixed inoculations of Fv1 and FgD at the highest temperature. However, this effect of temperature was only seen in this treatment and, therefore, the result should not be overrated.

Correlation analysis revealed either no effect or a positive correlation between temperature and amounts of *F. verticillioides* biomass. Co-inoculation with *F. graminearum* never affected the growth negatively. Additionally, the increase in temperature also led to similar or higher contamination rates with FB1. Due to the fact that these results were obtained from one

experiment, further investigations are needed. Overall, either no effects or positive effects on the frequency and biomass of *F. verticillioides* were obtained. Although positive correlations between the biomass and incidence of *F. verticillioides* with temperature were found, it has to be taken into consideration that the samples were obtained after two weeks of growing and that the effect may increase with a longer growing period in the field.

The temperature of the field trials may correspond to the two lower temperature scenarios tested. Assuming global warming, it has to be taken into account that the severity of *Fusarium* ear rot caused by *F. verticillioides* may increase in moderate temperature areas. The results show interactions between *F. graminearum* and *F. verticillioides*, which lead to an increase in contamination by *F. verticillioides*. These aspect may additionally facilitate disease severity. The consequences may be enhanced contamination of maize and maize products with the carcinogenic mycotoxin FB1.

Further work is needed to evaluate the complex interactions between plant variety and both fungal strains. The interaction of *F. verticillioides* and *F. graminearum* during germination, the invasion as well as the early colonization of maize cobs all require further studies. Understanding of early events in infection seems to be critical for the mechanism behind the direct or indirect interactions leading to the facilitation of *F. verticillioides* infections.

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Supplementary part

S-Table 1. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1, nivalenol and zearalenone in maize kernels of Amadeo in 2009. <LOQ: below the limit of quantification; <LOD: below the limit of detection; -:not analyzed

No.	Treatment	Visual symptoms (% of cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	ZEN (µg/g)
1	Fv1	7.0	<LOD	0.11	0.56	<LOD	0.05
2	Fv1	0.5	<LOD	<LOQ	<LOD	<LOD	<LOD
3	Fv1	1.5	<LOD	0.13	0.58	<LOD	0.08
4	Fv1	1.5	<LOQ	<LOQ	0.45	<LOD	<LOD
5	Fv1	2.5	<LOD	0.02	1.34	<LOD	<LOD
6	Fv1	3.0	0.04	0.12	0.60	<LOD	0.04
7	Fv1	9.0	<LOQ	<LOQ	0.53	<LOD	0.04
8	Fv1	2.5	<LOQ	<LOQ	0.45	<LOD	0.02
9	Fv1	7.5	<LOD	<LOQ	0.71	<LOD	<LOD
10	Fv1	0.0	<LOD	<LOQ	<LOD	<LOD	<LOQ
1	FgN	95.0	8.19	0.05	1.01	39.83	0.83
2	FgN	50.0	1.79	0.08	0.43	22.24	6.22
3	FgN	100.0	0.25	3.15	0.54	193.20	0.36
4	FgN	100.0	0.19	1.86	1.06	78.79	6.57
5	FgN	0.5	<LOQ	<LOD	<LOD	<LOD	0.07
6	FgN	0.0	<LOD	<LOD	<LOD	<LOD	<LOD
7	FgN	40.0	0.11	0.93	3.10	4.70	<LOD
8	FgN	25.0	<LOQ	0.02	<LOD	<LOD	0.02
9	FgN	100.0	1.93	0.06	<LOD	63.86	1.02
10	FgN	100.0	3.65	0.01	<LOD	42.02	0.45
1	Fv1 + FgN	100.0	0.26	7.28	4.01	102.38	0.78
2	Fv1 + FgN	100.0	0.29	4.11	2.35	166.97	6.81
3	Fv1 + FgN	100.0	0.35	1.86	0.83	95.20	6.69
4	Fv1 + FgN	100.0	0.13	2.26	6.29	51.76	12.39
5	Fv1 + FgN	100.0	0.25	1.16	7.18	163.67	0.40
6	Fv1 + FgN	1.5	<LOD	0.05	0.62	<LOD	<LOQ
7	Fv1 + FgN	100	2.14	13.47	1.84	52.59	7.65
8	Fv1 + FgN	9.5	<LOQ	0.23	4.01	<LOD	0.11
9	Fv1 + FgN	100.0	<LOQ	1.13	2.19	38.50	3.57

S-Table 1. continued

No.	Treatment	Visual symptoms (% of cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	ZEN (µg/g)
10	Fv1 + FgN	0.0	<LOQ	0.02	<LOD	<LOD	0.04
1	Control	<LOQ	0.03	-	-	-	-

S-Table 2. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1, nivalenol and zearalenone in maize kernels of Kabanas in 2009. <LOQ: below the limit of quantification; <LOD: below the limit of detection; -:not analyzed

No.	Treatment	Visual symptoms (% of cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	ZEN (µg/g)
1	Fv1	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD
2	Fv1	2.5	<LOD	0.02	1.07	<LOD	0.03
3	Fv1	0.0	<LOD	0.05	<LOD	<LOD	0.05
4	Fv1	0.0	0.03	<LOD	<LOD	<LOD	<LOD
5	Fv1	0.0	<LOD	<LOQ	<LOD	<LOD	<LOD
6	Fv1	0.0	<LOD	<LOQ	<LOD	<LOD	0.31
7	Fv1	0.0	0.02	<LOQ	<LOD	<LOD	0.03
8	Fv1	0.5	<LOD	0.14	3.45	<LOD	<LOD
9	Fv1	0.0	<LOD	<LOD	<LOD	<LOD	<LOQ
10	Fv1	0.0	<LOD	<LOD	<LOD	<LOD	<LOD
1	FgN	0.0	<LOD	<LOD	<LOD	<LOD	<LOD
2	FgN	60.0	4.40	0.08	<LOD	65.66	0.29
3	FgN	0.0	<LOD	<LOQ	0.45	<LOD	<LOD
4	FgN	30.0	<LOD	0.02	<LOD	<LOD	0.03
5	FgN	4.0	<LOQ	0.10	0.45	<LOD	<LOD
6	FgN	0.0	<LOQ	0.04	0.43	<LOD	<LOQ
7	FgN	0.0	<LOD	<LOQ	<LOD	<LOD	<LOD
8	FgN	25.0	0.73	0.06	<LOD	14.76	0.21
9	FgN	90.0	8.59	0.71	1.58	78.92	0.37
10	FgN	100.0	15.52	0.13	<LOD	120.11	0.94
1	Fv1 + FgN	0.0	<LOD	0.05	0.42	<LOD	0.04
2	Fv1 + FgN	40.0	0.06	1.88	2.31	8.60	3.07
3	Fv1 + FgN	30.0	<LOD	1.86	3.23	2.16	0.07
4	Fv1 + FgN	50.0	0.69	3.68	2.35	32.14	0.65
5	Fv1 + FgN	95.0	<LOD	9.60	7.77	32.42	0.31
6	Fv1 + FgN	100.0	1.53	32.00	19.03	246.38	0.08

S-Table 2. continued

No.	Treatment	Visual symptoms (% of cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	ZEN (µg/g)
7	Fv1 + FgN	100.0	2.62	13.28	27.22	57.14	0.71
8	Fv1 + FgN	95.0	1.41	15.09	2.56	98.03	s1.02
9	Fv1 + FgN	100.0	32.80	16.53	2.39	155.37	8.38
10	Fv1 + FgN	100.0	15.25	22.93	16.71	145.48	1.08
1	Control	<LOD	<LOQ	-	-	-	-

S-Table 3. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1 in maize kernels of Kabanas in 2010 at the first harvest time point 21 dpi.. Deoxynivalenol, nivalenol and zearalenone were not analyzed due to the low infestation rates of the fungi. <LOQ: below the limit of quantification; <LOD: below the limit of detection; -:not analyzed

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
1	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
2	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
3	Fv1	0.0	<LOQ	<LOQ	<LOD	-	-	-
4	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
5	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
6	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
7	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
8	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
9	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
10	Fv1	0.0	<LOQ	<LOD	<LOD	-	-	-
1	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
2	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
3	Fv2	1.0	<LOQ	<LOD	<LOD	-	-	-
4	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
5	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
6	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
7	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
8	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
9	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
10	Fv2	0.0	<LOQ	<LOD	<LOD	-	-	-
1	FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
2	FgN	1.0	<LOQ	<LOD	<LOD	-	-	-

S-Table 3. continued

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
3	FgN	1.0	<LOQ	<LOD	<LOD	-	-	-
4	FgN	1.0	<LOQ	<LOD	<LOD	-	-	-
5	FgN	7.0	<LOQ	<LOD	<LOD	-	-	-
6	FgN	20.0	<LOQ	<LOD	<LOD	-	-	-
7	FgN	40.0	0.05	<LOD	<LOD	-	-	-
8	FgN	30.0	0.03	<LOD	<LOD	-	-	-
9	FgN	40.0	0.04	<LOD	<LOD	-	-	-
10	FgN	25.0	0.01	<LOD	<LOD	-	-	-
1	FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
2	FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
3	FgD	2.0	<LOQ	<LOQ	<LOD	-	-	-
4	FgD	15.0	<LOQ	<LOD	<LOD	-	-	-
5	FgD	10.0	<LOQ	<LOD	<LOD	-	-	-
6	FgD	10.0	<LOQ	<LOD	<LOD	-	-	-
7	FgD	2.0	<LOQ	<LOD	<LOD	-	-	-
8	FgD	20.0	<LOQ	<LOD	<LOD	-	-	-
9	FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
10	FgD	0.0	<LOQ	<LOQ	<LOD	-	-	-
1	Fv1 + FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
2	Fv1 + FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
3	Fv1 + FgN	5.0	<LOQ	<LOQ	0.04	-	-	-
4	Fv1 + FgN	10.0	<LOQ	<LOQ	0.05	-	-	-
5	Fv1 + FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
6	Fv1 + FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
7	Fv1 + FgN	15.0	<LOQ	<LOQ	0.07	-	-	-
8	Fv1 + FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
9	Fv1 + FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
10	Fv1 + FgN	60.0	0.07	0.09	0.05	-	-	-
1	Fv1 + FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
2	Fv1+ FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
3	Fv1+ FgD	0.0	<LOQ	<LOQ	<LOD	-	-	-
4	Fv1+ FgD	10.0	0.02	0.04	0.15	-	-	-
5	Fv1+ FgD	0.0	<LOQ	<LOQ	<LOD	-	-	-
6	Fv1+ FgD	0.0	<LOQ	<LOQ	<LOD	-	-	-
7	Fv1+ FgD	0.0	<LOQ	<LOQ	<LOD	-	-	-

S-Table 3. continued

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
8	Fv1+ FgD	0.0	<LOQ	<LOQ	<LOD	-	-	-
9	Fv1+ FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
10	Fv1+ FgD	0.0	<LOQ	<LOD	<LOD	-	-	-
1	Fv2+ FgN	50.0	0.02	0.24	0.18	-	-	-
2	Fv2+ FgN	2.0	<LOQ	<LOD	<LOD	-	-	-
3	Fv2+ FgN	1.0	<LOQ	<LOD	<LOD	-	-	-
4	Fv2+ FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
5	Fv2+ FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
6	Fv2+ FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
7	Fv2+ FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
8	Fv2+ FgN	40.0	0.01	0.04	0.07	-	-	-
9	Fv2+ FgN	0.0	<LOQ	<LOQ	0.01	-	-	-
10	Fv2+ FgN	0.0	<LOQ	<LOD	<LOD	-	-	-
1	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
2	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
3	Fv2+ FgD	1.0	<LOQ	<LOD	<LOQ	-	-	-
4	Fv2+ FgD	25.0	<LOQ	<LOQ	<LOQ	-	-	-
5	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
6	Fv2+ FgD	0.0	<LOQ	<LOQ	<LOQ	-	-	-
7	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
8	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
9	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
10	Fv2+ FgD	0.0	<LOQ	<LOD	<LOQ	-	-	-
1	Control	0.0	<LOQ	<LOD	-	-	-	-
2	Control	0.0	<LOQ	<LOD	-	-	-	-
3	Control	0.0	<LOQ	<LOD	-	-	-	-
4	Control	0.0	<LOQ	<LOD	-	-	-	-
5	Control	0.0	<LOQ	<LOD	-	-	-	-
6	Control	0.0	<LOQ	<LOD	-	-	-	-
7	Control	0.0	<LOQ	<LOD	-	-	-	-
8	Control	0.0	<LOQ	<LOD	-	-	-	-
9	Control	0.0	<LOQ	<LOD	-	-	-	-
10	Control	0.0	<LOQ	<LOD	-	-	-	-

S-Table 4. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1, deoxynivalenol, nivalenol and zearalenone in maize kernels of Kabanas in 2010 at the second harvest time point 35 dpi. <LOQ: below the limit of quantification; <LOD: below the limit of detection; -:not analyzed

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
1	Fv1	3.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
2	Fv1	1.5	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
3	Fv1	0.0	<LOQ	0.02	<LOD	<LOD	<LOD	<LOD
4	Fv1	1.5	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	Fv1	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
6	Fv1	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
7	Fv1	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
8	Fv1	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
9	Fv1	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
10	Fv1	7.0	<LOQ	0.01	1.31	<LOD	<LOD	<LOD
1	Fv2	1.5	<LOQ	<LOQ	<LOD	-	-	-
2	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
3	Fv2	5.5	<LOQ	<LOQ	1.20	-	-	-
4	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
5	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
6	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
7	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
8	Fv2	4.0	<LOQ	<LOQ	0.18	-	-	-
9	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
10	Fv2	0.0	<LOQ	<LOQ	<LOD	-	-	-
1	FgN	95.0	<LOQ	<LOQ	<LOD	19.24	<LOD	<LOD
2	FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
3	FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
4	FgN	70.0	<LOQ	<LOQ	<LOD	2.19	<LOD	<LOD
5	FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
6	FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
7	FgN	100.0	0.22	<LOQ	<LOD	22.61	<LOD	<LOD
8	FgN	40.0	0.29	<LOQ	<LOD	<LOD	<LOD	<LOD
9	FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
10	FgN	100.0	<LOQ	<LOQ	<LOD	41.67	<LOD	<LOD
1	FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD

S-Table 4. continued

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
2	FgD	4.0	<LOQ	<LOQ	<LOD	17.71	<LOD	<LOD
3	FgD	0.0	<LOQ	<LOQ	<LOD	5.84	<LOD	<LOD
4	FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	FgD	20.0	<LOQ	<LOQ	<LOD	0.46	<LOD	<LOD
6	FgD	30.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
7	FgD	100.0	0.22	<LOQ	<LOD	0.57	<LOD	<LOD
8	FgD	80.0	0.29	<LOQ	<LOD	<LOD	<LOD	<LOD
9	FgD	26.5	<LOQ	<LOQ	<LOD	0.85	<LOD	<LOD
10	FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
1	Fv1 + FgN	3.5	<LOQ	0.07	0.36	44.82	<LOD	<LOD
2	Fv1 + FgN	30.0	0.04	0.17	0.91	0.52	<LOD	<LOD
3	Fv1 + FgN	50.0	<LOQ	0.14	1.77	0.40	<LOD	<LOD
4	Fv1 + FgN	6.5	<LOQ	<LOQ	1.16	13.64	<LOD	<LOD
5	Fv1 + FgN	20.0	<LOQ	<LOQ	0.02	0.63	<LOD	<LOD
6	Fv1 + FgN	0.0	<LOQ	<LOQ	<LOD	0.12	<LOD	<LOD
7	Fv1 + FgN	16.0	<LOQ	0.03	<LOD	66.78	<LOD	<LOD
8	Fv1 + FgN	0.0	<LOQ	<LOQ	<LOD	3.05	<LOD	<LOD
9	Fv1 + FgN	20.5	0.01	0.02	0.09	<LOD	<LOD	<LOD
10	Fv1 + FgN	1.0	<LOQ	<LOQ	1.31	121.84	<LOD	<LOD
1	Fv1 + FgD	30.0	<LOQ	0.12	0.13	<LOD	4.39	<LOD
2	Fv1 + FgD	45.0	0.03	0.35	0.61	<LOD	16.60	0.03
3	Fv1 + FgD	1.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
4	Fv1 + FgD	12.0	<LOQ	0.04	0.33	0.26	<LOD	<LOD
5	Fv1 + FgD	40.0	0.07	0.22	0.09	<LOD	10.22	<LOD
6	Fv1 + FgD	2.5	<LOQ	0.02	0.24	<LOD	<LOD	<LOD
7	Fv1 + FgD	2.5	<LOQ	<LOQ	0.34	<LOD	<LOD	<LOD
8	Fv1 + FgD	1.5	<LOQ	<LOQ	1.67	<LOD	<LOD	<LOD
9	Fv1 + FgD	3.0	<LOQ	<LOQ	0.06	0.84	<LOD	<LOD
10	Fv1 + FgD	40.0	<LOQ	0.03	0.13	<LOD	15.15	<LOD
1	Fv2 + FgN	4.5	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
2	Fv2 + FgN	80.0	0.37	0.07	0.18	134.65	<LOD	0.03
3	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
4	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
6	Fv2 + FgN	2.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD

S-Table 4. continued

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
7	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
8	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
9	Fv2 + FgN	70.0	0.25	0.02	<LOD	55.54	<LOD	<LOD
1	Fv2 + FgD	20.0	<LOQ	<LOQ	0.26	<LOD	2.65	<LOD
2	Fv2 + FgD	6.0	<LOQ	0.04	0.22	<LOD	<LOD	<LOD
3	Fv2 + FgD	2.0	<LOQ	0.02	1.99	0.14	<LOD	<LOD
4	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	0.19	<LOD	<LOD
6	Fv2 + FgD	5.5	<LOQ	0.06	1.02	<LOD	1.00	<LOD
7	Fv2 + FgD	45.0	0.08	<LOQ	<LOD	<LOD	22.60	<LOD
8	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
9	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
10	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
1	Control	0.0	<LOQ	<LOQ	-	-	-	-
2	Control	0.0	<LOQ	<LOQ	-	-	-	-
3	Control	0.0	<LOQ	<LOQ	-	-	-	-
4	Control	0.0	<LOQ	<LOQ	-	-	-	-
5	Control	0.0	<LOQ	<LOQ	-	-	-	-
6	Control	0.0	<LOQ	<LOQ	-	-	-	-
7	Control	0.0	<LOQ	<LOQ	-	-	-	-
8	Control	0.0	<LOQ	<LOQ	-	-	-	-
9	Control	0.0	<LOQ	<LOQ	-	-	-	-
10	Control	0.0	<LOQ	<LOQ	-	-	-	-

S-Table 5. Absolute amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1, deoxynivalenol, nivalenol and zearalenone in maize kernels of Kabanas in 2010 at the third harvest time point 49 dpi. <LOQ: below the limit of quantification; <LOD: below the limit of detection; -:not analyzed

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
1	Fv1	7.0	<LOQ	0.04	8.53	<LOD	<LOD	<LOD
2	Fv1	10.5	<LOQ	0.12	3.72	<LOD	<LOD	<LOD
3	Fv1	5.5	<LOQ	0.08	3.40	<LOD	<LOD	<LOD
4	Fv1	0.0	<LOQ	<LOQ	0.17	<LOD	<LOD	<LOD
5	Fv1	12.0	<LOQ	0.29	24.76	<LOD	<LOD	<LOD
6	Fv1	15.0	<LOQ	0.29	1.49	<LOD	<LOD	<LOD
7	Fv1	9.0	<LOQ	0.13	6.08	<LOD	<LOD	<LOD
8	Fv1	1.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
9	Fv1	0.0	<LOQ	<LOQ	0.07	<LOD	<LOD	<LOD
1	Fv2	1.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
2	Fv2	0.0	<LOQ	0.08	<LOD	<LOD	<LOD	<LOD
3	Fv2	1.0	<LOQ	<LOQ	0.16	<LOD	<LOD	<LOD
4	Fv2	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	Fv2	2.5	<LOQ	0.09	1.42	<LOD	<LOD	<LOD
6	Fv2	6.5	<LOQ	0.05	6.91	-	-	-
1	FgN	100.0	0.48	<LOQ	<LOD	79.33	<LOD	0.01
2	FgN	3.0	<LOQ	<LOQ	<LOD	0.38	<LOD	<LOD
3	FgN	100.0	0.59	0.04	<LOD	239.45	<LOD	0.14
4	FgN	70.0	0.88	<LOQ	<LOD	37.23	<LOD	0.04
5	FgN	80.0	0.42	<LOQ	<LOD	37.66	<LOD	0.02
6	FgN	100.0	4.06	0.02	<LOD	210.55	<LOD	0.08
7	FgN	100.0	6.25	<LOQ	<LOD	163.67	<LOD	0.22
8	FgN	100.0	4.23	<LOQ	<LOD	270.11	<LOD	0.15
9	FgN	20.0	<LOQ	<LOQ	<LOD	11.54	<LOD	<LOD
10	FgN	40.0	0.10	<LOQ	<LOD	10.48	<LOD	0.02
1	FgD	40.0	0.04	<LOQ	<LOD	0.71	5.93	0.02
2	FgD	1.0	<LOQ	0.01	<LOD	0.69	0.33	<LOD
3	FgD	40.0	0.16	<LOQ	0.50	1.74	<LOD	<LOD
4	FgD	50.0	0.07	<LOQ	<LOD	0.80	10.09	<LOD
5	FgD	40.0	0.07	<LOQ	<LOD	<LOD	5.27	<LOD
6	FgD	25.0	<LOQ	<LOQ	<LOD	<LOD	0.28	<LOD

S-Table 5. continued

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
7	FgD	50.0	0.36	<LOQ	<LOD	0.74	19.50	0.01
8	FgD	60.0	0.33	0.02	<LOD	<LOD	10.00	0.18
9	FgD	30.0	0.51	-	<LOD	0.16	25.76	<LOD
1	Fv1 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
2	Fv1 + FgN	20.0	<LOQ	0.26	2.42	1.58	<LOD	<LOD
3	Fv1 + FgN	100.0	0.13	0.69	7.12	4.08	<LOD	0.04
4	Fv1 + FgN	100.0	6.79	3.92	5.94	11.23	<LOD	0.44
5	Fv1 + FgN	60.0	0.54	<LOQ	<LOD	38.09	<LOD	<LOD
6	Fv1 + FgN	7.5	<LOQ	0.12	15.40	0.17	<LOD	<LOD
7	Fv1 + FgN	100.0	1.81	2.62	20.71	81.70	<LOD	0.22
8	Fv1 + FgN	0.0	<LOQ	<LOQ	7.65	35.28	<LOD	0.04
9	Fv1 + FgN	3.5	<LOQ	<LOQ	0.14	<LOD	<LOD	<LOD
10	Fv1 + FgN	10.0	<LOQ	0.05	0.81	0.11	<LOD	<LOD
1	Fv1 + FgD	25.0	<LOQ	<LOQ	<LOD	<LOD	8.28	<LOD
2	Fv1 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
3	Fv1 + FgD	80.0	0.14	1.10	10.61	<LOD	27.86	0.03
4	Fv1 + FgD	25.0	<LOQ	<LOQ	0.16	<LOD	1.30	<LOD
5	Fv1 + FgD	50.0	0.02	<LOQ	<LOD	<LOD	16.29	0.03
6	Fv1 + FgD	70.0	<LOQ	0.55	6.04	<LOD	14.14	0.05
7	Fv1 + FgD	4.0	<LOQ	0.12	1.37	0.13	<LOD	<LOD
8	Fv1 + FgD	7.0	<LOQ	0.24	7.04	0.28	<LOD	<LOD
9	Fv1 + FgD	25.0	<LOQ	0.34	5.08	<LOD	1.45	<LOD
10	Fv1 + FgD	80.0	0.39	1.39	16.29	<LOD	30.27	0.06
1	Fv2 + FgN	2.0	<LOQ	<LOQ	0.55	<LOD	<LOD	<LOD
2	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
3	Fv2 + FgN	75.0	<LOQ	0.12	<LOD	44.45	<LOD	0.03
4	Fv2 + FgN	0.0	0.02	<LOQ	<LOD	0.15	<LOD	<LOD
5	Fv2 + FgN	50.0	0.02	<LOQ	<LOD	12.52	<LOD	<LOD
6	Fv2 + FgN	0.0	0.47	0.99	4.75	79.89	<LOD	0.23
7	Fv2 + FgN	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
8	Fv2 + FgN	50.0	0.14	0.02	0.18	5.30	<LOD	<LOD
9	Fv2 + FgN	20.0	0.46	<LOQ	23.90	0.39	<LOD	<LOD
10	Fv2 + FgN	30.0	0.98	<LOQ	<LOD	<LOD	11.18	<LOD
1	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
2	Fv2 + FgD	6.5	<LOQ	0.08	2.50	<LOD	<LOD	<LOD

S-Table 5. continued

No.	Treatment	Visual symptoms (% cob surface)	*F. graminearum* DNA (µg/g)	*F. verticillioides* DNA (µg/g)	FB1 (µg/g)	NIV (µg/g)	DON (µg/g)	ZEN (µg/g)
3	Fv2 + FgD	6.5	<LOQ	0.05	3.30	0.17	<LOD	<LOD
4	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	Fv2 + FgD	0.0	<LOQ	0.02	0.03	<LOD	<LOD	<LOD
6	Fv2 + FgD	0.0	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
7	Fv2 + FgD	2.0	<LOQ	0.02	0.05	<LOD	<LOD	<LOD
8	Fv2 + FgD	12.5	<LOQ	0.04	18.00	<LOD	0.67	<LOD
9	Fv2 + FgD	1.5	<LOQ	0.03	0.83	<LOD	<LOD	<LOD
10	Fv2 + FgD	0.0	<LOQ	<LOQ	0.11	<LOD	<LOD	<LOD
1	Control	0.0	<LOQ	<LOQ	-	-	-	-
2	Control	0.0	<LOQ	<LOQ	-	-	-	-
3	Control	0.0	<LOQ	<LOQ	-	-	-	-
4	Control	0.0	<LOQ	<LOQ	-	-	-	-
5	Control	0.0	<LOQ	<LOQ	-	-	-	-
6	Control	0.0	<LOQ	<LOQ	-	-	-	-
7	Control	0.0	<LOQ	<LOQ	-	-	-	-
8	Control	1.0	<LOQ	<LOQ	-	-	-	-
9	Control	1.0	<LOQ	<LOQ	-	-	-	-
10	Control	0.0	<LOQ	<LOQ	-	-	-	-

S-Table 6. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxin fumonisin B1 in maize kernels and cobs of maize cobs inoculated with Fv1 and grown under five temperature scenarios. <LOQ: below the limit of quantification; <LOD: below the limit of detection

		F. verticillioides DNA		*F. graminearum* DNA		FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 1 (22/13 °C)	1	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	2	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	3	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD
	4	<LOD	0.09	<LOQ	<LOQ	<LOD	<LOD
	5	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	6	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	7	<LOQ	<LOQ	<LOQ	<LOQ	0.04	<LOD
	8	2.49	0.01	<LOQ	<LOQ	110.30	<LOD
	9	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	10	0.15	<LOQ	<LOQ	<LOQ	0.41	<LOD
	11	2.44	0.14	<LOQ	<LOQ	93.73	7.83
Scenario 2 (24/15 °C)	1	<LOD	0.02	<LOQ	<LOQ	<LOD	0.28
	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	3	<LOD	0.05	<LOQ	<LOQ	<LOD	0.62
	4	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	5	<LOQ	0.02	<LOQ	<LOQ	<LOD	<LOD
	6	<LOD	<LOD	<LOQ	<LOQ	0.12	<LOD
	7	0.24	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	8	<LOQ	0.01	<LOQ	<LOQ	<LOD	<LOD
	9	3.46	0.02	<LOQ	<LOQ	29.29	<LOD
Scenario 3 (26/17 °C)	1	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD
	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	4	<LOQ	0.18	<LOQ	<LOQ	<LOD	<LOD
	5	<LOD	0.16	<LOQ	<LOQ	<LOD	0.60
	6	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD
	7	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	8	0.04	0.10	<LOQ	<LOQ	0.03	1.48
	9	<LOD	0.14	<LOQ	<LOQ	<LOD	0.77
	10	<LOQ	0.21	<LOQ	<LOQ	<LOD	3.73

S-Table 6. continued

		F. verticillioides DNA		*F. graminearum* DNA		FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 4 (28/19 °C)	1	0.01	0.01	<LOQ	<LOQ	0.04	<LOD
	2	<LOQ	0.02	<LOQ	<LOQ	<LOD	0.56
	3	<LOQ	0.02	<LOQ	<LOQ	<LOD	<LOD
	4	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	5	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD
	6	0.11	0.23	<LOQ	<LOQ	0.39	2.24
	7	<LOQ	<LOQ	<LOQ	<LOQ	0.21	<LOD
	8	<LOQ	0.23	<LOQ	<LOQ	0.04	2.93
	9	26.30	0.01	<LOQ	<LOQ	455.19	0.20
	10	<LOD	0.05	<LOQ	<LOQ	<LOD	0.51
Scenario 5 (30/21 °C)	1	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD
	2	<LOQ	0.92	<LOQ	<LOQ	<LOD	<LOD
	3	<LOQ	0.05	<LOQ	<LOQ	<LOD	1.12
	4	0.09	0.21	<LOQ	<LOQ	0.20	0.14
	5	0.04	3.77	<LOQ	<LOQ	0.03	0.26
	6	0.16	0.43	<LOQ	<LOQ	0.85	0.35
	7	<LOQ	<LOD	<LOQ	<LOQ	0.04	<LOD
	8	<LOQ	0.93	<LOQ	<LOQ	<LOD	3.86
	9	0.02	0.05	<LOQ	<LOQ	0.09	<LOD

S-Table 7. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1 and deoxynivalenol in maize kernels and cobs of maize cobs inoculated with FgD and grown under five temperature scenarios. Deoxynivalenol was not analyzed in cobs due to strong inhibition effects. <LOQ: below the limit of quantification; <LOD: below the limit of detection

		F. verticillioides DNA		*F. graminearum* DNA		DON	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 1 (22/13 °C)	1	<LOD	<LOQ	0.01	<LOD	<LOD	<LOD	<LOD
	2	<LOQ	<LOD	0.09	<LOD	30.74	<LOD	<LOD
	3	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
	4	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
	5	<LOQ	<LOQ	0.28	1.20	<LOD	<LOD	<LOD
	6	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD
	7	<LOD	<LOQ	<LOD	0.03	<LOD	<LOD	<LOD
	8	<LOQ	<LOQ	0.08	0.74	<LOD	<LOD	<LOD
	9	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
	10	<LOD	0.02	0.10	2.22	<LOD	<LOD	<LOD
Scenario 2 (24/15 °C)	1	<LOD	<LOD	<LOD	0.14	<LOD	<LOD	<LOD
	2	<LOD	<LOD	0.10	0.77	3.72	<LOD	<LOD
	3	<LOQ	0.05	0.01	0.31	<LOD	<LOD	<LOD
	4	<LOQ	<LOD	0.03	0.05	1.17	<LOD	<LOD
	5	0.07	<LOQ	4.22	4.45	92.81	<LOD	<LOD
	6	<LOQ	0.02	0.19	1.33	<LOD	<LOD	<LOD
	7	<LOD	<LOD	0.17	1.85	1.54	<LOD	<LOD
Scenario 3 (26/17 °C)	1	<LOD	<LOD	0.28	<LOD	24.75	<LOD	<LOD
	2	<LOD	<LOD	<LOD	<LOD	301.41	<LOD	<LOD
	3	0.19	<LOQ	<LOQ	<LOD	<LOD	<LOQ	0.31
	4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	5	<LOQ	<LOQ	0.35	0.78	<LOD	<LOD	<LOD
	6	<LOD	<LOQ	0.110	<LOD	<LOD	<LOD	<LOD
	7	<LOD	<LOQ	0.05	12.06	<LOD	<LOD	<LOD
	8	<LOD	<LOQ	0.01	0.16	<LOD	<LOD	<LOD
	9	<LOQ	0.12	0.30	4.61	6.64	<LOD	<LOD

S-Table 7. continued

		F. verticillioides DNA		*F. graminearum* DNA		DON	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 4 (28/19 °C)	1	0.14	<LOQ	0.08	0.46	5.17	0.01	<LOD
	2	<LOQ	<LOD	11.25	<LOD	162.11	<LOD	<LOD
	3	<LOD	<LOD	<LOD	<LOD	606.17	<LOD	<LOD
	4	0.42	0.19	0.05	4.08	16.95	0.01	<LOD
	5	<LOQ	<LOD	<LOQ	0.13	<LOD	<LOD	<LOD
	6	<LOQ	23.68	0.22	<LOD	<LOD	<LOD	0.55
	7	<LOQ	0.04	<LOD	<LOD	<LOD	<LOD	<LOD
Scenario 5 (30/21 °C)	1	<LOD	<LOD	0.07	1.17	100.87	<LOQ	<LOD
	2	<LOD	<LOD	0.02	0.03	<LOD	<LOD	<LOD
	3	5.85	<LOD	2.03	4.33	55.56	1.18	<LOD
	4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	5	<LOQ	0.01	0.01	1.08	<LOD	<LOD	<LOD
	6	0.97	0.28	0.88	43.12	32.01	<LOD	<LOD

S-Table 8. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1 and nivalenol in maize kernels and cobs of maize cobs inoculated with FgN and grown under five temperature scenarios. Nivalenol was not analyzed in cobs due to strong inhibition effects.

		F. verticillioides DNA		*F. graminearum* DNA		NIV	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 1 (22/13 °C)	1	0.07	<LOQ	0.01	0.13	<LOD	0.57	2.02
	2	0.13	<LOD	0.53	<LOD	14.15	<LOD	<LOD
	3	<LOD	<LOD	<LOD	0.11	<LOD	<LOD	<LOD
	4	0.02	0.01	0.01	0.56	3.84	0.39	<LOD
	5	<LOD	<LOD	0.01	0.75	0.26	<LOD	<LOD
	6	<LOD	<LOD	<LOD	0.03	<LOD	<LOD	<LOD
	7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	8	<LOD	<LOD	<LOQ	0.16	<LOD	<LOD	<LOD
	9	<LOQ	<LOD	0.18	1.24	0.92	<LOD	<LOD
Scenario 2 (24/15 °C)	1	0.07	<LOQ	0.09	0.33	0.58	<LOD	<LOD
	2	<LOD	<LOD	0.3	<LOD	9.82	<LOD	<LOD
	3	<LOD	<LOD	<LOD	<LOD	2.90	0.56	7.24
	4	<LOD	<LOD	0.23	<LOD	4.40	<LOD	<LOD
	5	0.03	<LOQ	0.01	0.03	<LOD	<LOD	<LOD
	6	<LOD	<LOD	0.10	0.07	0.13	<LOD	<LOD
	7	<LOD	<LOQ	0.01	0.02	<LOD	<LOD	<LOD
	8	<LOQ	<LOD	0.06	<LOD	0.03	<LOD	<LOD
	9	<LOD	<LOD	<LOQ	0.01	<LOD	<LOD	<LOD
	10	0.01	<LOQ	<LOQ	0.07	24.38	<LOD	<LOD
Scenario 3 (26/17 °C)	1	<LOD	<LOD	<LOD	<LOD	5.47	<LOD	<LOD
	2	0.04	<LOQ	0.01	0.11	164.01	<LOD	<LOD
	3	<LOQ	0.02	<LOD	0.02	<LOD	<LOD	<LOD
	4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	5	<LOD	<LOQ	<LOD	0.04	<LOD	<LOD	<LOD
	6	33.75	0.86	0.02	<LOD	<LOD	213.97	5.06
	7	<LOD	<LOQ	0.06	0.41	0.16	<LOD	<LOD
	8	<LOD	<LOQ	0.24	2.55	<LOD	<LOD	<LOD

S-Table 8. continued

		F. verticillioides DNA		*F. graminearum* DNA		NIV	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 4 (28/19 °C)	1	<LOQ	<LOQ	0.12	0.56	<LOD	<LOD	<LOD
	2	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
	3	<LOQ	0.01	0.16	<LOD	<LOD	<LOD	<LOD
	4	0.02	0.02	0.01	0.19	0.64	<LOD	<LOD
	5	1.40	<LOD	45.38	<LOD	2.04	<LOD	<LOD
	6	<LOD	0.04	0.43	5.31	<LOD	<LOD	<LOD
	7	<LOD	0.02	0.17	20.23	5.90	<LOD	<LOD
	8	<LOQ	<LOQ	1.35	4.68	3.73	<LOD	<LOD
	9	<LOQ	<LOD	0.05	0.47	125.83	<LOD	<LOD
	10	0.16	0.02	0.03	72.38	<LOD	<LOD	<LOD
Scenario 5 (30/21 °C)	1	<LOD	<LOD	0.01	0.03	0.40	<LOD	<LOD
	2	<LOD	<LOD	0.03	0.51	<LOD	<LOD	<LOD
	3	0.04	0.10	0.01	0.39	0.10	<LOD	<LOD
	4	3.00	2.86	<LOD	0.03	<LOD	1.63	3.29
	5	6.35	0.20	0.12	0.14	5.08	17.53	2.49
	6	0.06	0.43	0.01	0.02	<LOD	0.20	1.66
	7	0.30	2.42	0.51	10.84	<LOD	0.28	<LOD
	8	0.13	0.23	<LOD	0.18	0.86	<LOD	<LOD

S-Table 9. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1 and deoxynivalenol in maize kernels and cobs of maize cobs inoculated with *F. verticillioides* strain Fv1 and *F. graminearum* FgD and grown under five temperature scenarios. Deoxynivalenol was not analyzed in cobs due to strong inhibition effects. <LOQ: below the limit of quantification; <LOD: below the limit of detection

		F. verticillioides DNA		*F. graminearum* DNA		DON	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 1 (22/13 °C)	1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	2	<LOD	<LOD	0.02	<LOD	72.44	0.08	<LOD
	3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	4	<LOD	<LOD	0.01	0.12	<LOD	<LOD	<LOD
	5	<LOD	<LOD	<LOD	0.09	<LOD	<LOD	<LOD
	6	<LOQ	0.03	<LOD	0.03	<LOD	<LOD	<LOD
	7	<LOD	<LOQ	<LOD	0.05	<LOD	<LOD	<LOD
	8	<LOD	0.66	0.12	4.71	0.24	<LOD	<LOD
	9	<LOQ	0.03	0.08	7.86	1.85	<LOD	<LOD
	10	<LOD	<LOD	<LOD	0.02	<LOD	<LOD	<LOD
Scenario 2 (24/15 °C)	1	<LOD	<LOD	<LOQ	0.02	<LOD	<LOD	<LOD
	2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	3	<LOQ	0.01	<LOQ	0.12	<LOD	0.17	<LOD
	4	<LOD	<LOD	0.01	0.34	<LOD	<LOD	<LOD
	5	0.07	<LOQ	31.63	<LOD	3.04	0.03	<LOD
	6	0.04	<LOQ	0.01	0.19	<LOD	<LOD	<LOD
	7	0.13	0.06	<LOD	0.04	<LOD	<LOD	0.34
	8	15.84	0.09	<LOD	0.11	<LOD	33.35	2.29
Scenario 3 (26/17 °C)	1	0.07	0.11	<LOD	0.09	<LOD	<LOD	0.21
	2	0.61	0.02	0.02	<LOD	<LOD	0.10	<LOD
	3	0.26	0.11	0.04	0.60	<LOD	0.35	0.42
	4	<LOQ	0.02	<LOD	0.19	<LOD	<LOD	<LOD
	5	0.49	1.38	5.42	33.38	189.22	1.04	<LOD
	6	<LOQ	0.02	0.01	0.13	<LOD	<LOD	<LOD
	7	<LOQ	<LOD	0.01	0.11	<LOD	<LOD	<LOD
	8	0.55	0.11	2.35	1.40	51.86	0.37	0.20

S-Table 9. continued

		F. verticillioides DNA		*F. graminearum* DNA		DON	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 4 (28/19 °C)	1	0.06	0.06	0.02	0.29	2.95	5.60	0.74
	2	0.17	0.52	<LOD	0.03	<LOD	0.03	1.96
	3	0.37	<LOD	0.06	0.46	11.35	5.79	5.01
	4	0.02	0.09	<LOD	<LOD	<LOD	<LOD	<LOD
	5	<LOD	0.48	0.03	0.64	<LOD	<LOD	<LOD
	6	<LOQ	<LOD	<LOD	0.16	<LOD	0.05	<LOD
	7	<LOQ	<LOQ	0.01	0.07	<LOD	<LOD	<LOD
	8	0.05	0.23	0.16	3.22	2.56	0.16	<LOD
	9	1.07	0.14	11.84	29.57	667.89	0.24	<LOD
	10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Scenario 5 (30/21 °C)	1	0.07	0.09	<LOD	0.03	<LOD	0.29	0.63
	2	0.13	0.11	<LOD	<LOD	<LOD	6.82	7.02
	3	<LOD	0.29	<LOD	0.06	<LOD	0.02	<LOD
	4	0.44	3.55	0.01	0.15	0.75	0.30	0.25
	5	0.56	0.19	<LOD	<LOD	<LOD	0.42	0.65
	6	20.72	159.02	<LOD	0.36	<LOD	147.19	102.22
	7	0.05	0.02	<LOD	0.04	<LOD	<LOD	<LOD
	8	0.08	0.22	<LOD	0.18	<LOD	0.61	0.37
	9	1.54	0.56	<LOD	0.46	<LOD	0.79	0.61

S-Table 10. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1 and nivalenol in maize kernels and cobs of maize cobs inoculated with *F. verticillioides* strain Fv1 and *F. graminearum* FgN and grown under five temperature scenarios. Nivalenol was not analyzed in cobs due to strong inhibition effects.<LOQ: below the limit of quantification; <LOD: below the limit of detection

		F. verticillioides DNA		*F. graminearum* DNA		NIV	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 1	1	0.14	0.05	0.25	0.57	7.36	0.56	0.74
(22/13 °C)	2	0.31	0.47	11.10	30.82	83.70	<LOD	<LOD
	3	<LOD	<LOQ	<LOQ	0.09	<LOD	<LOD	<LOD
	4	<LOQ	<LOD	0.03	<LOQ	1.02	2.62	2.00
	5	0.10	0.13	0.04	0.09	0.81	0.18	0.88
	6	0.61	1.03	9.67	19.65	132.75	2.03	2.69
	7	<LOD	<LOQ	0.04	0.18	<LOD	0.02	<LOD
	8	<LOQ	<LOQ	0.01	0.11	<LOD	<LOD	<LOD
	9	<LOD	<LOQ	<LOQ	0.01	<LOD	<LOD	<LOD
	10	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD
Scenario 2	1	0.03	<LOQ	0.01	<LOQ	2.27	1.24	1.29
(24/15 °C)	2	<LOQ	0.02	<LOQ	0.11	<LOD	<LOD	<LOD
	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD
	4	<LOQ	<LOQ	0.07	0.09	2.00	0.18	<LOD
	5	<LOQ	0.24	0.01	23.05	6.81	<LOD	<LOD
	6	<LOQ	0.01	<LOQ	0.30	<LOD	<LOD	<LOD
	7	<LOQ	0.01	0.01	0.02	0.08	<LOD	<LOD
	8	<LOQ	<LOQ	0.02	0.13	<LOD	<LOD	<LOD
Scenario 3	1	0.23	<LOD	0.11	0.03	<LOD	<LOD	<LOD
(26/17 °C)	2	<LOD	0.01	<LOQ	5.54	9.61	<LOD	<LOD
	3	0.01	3.60	<LOQ	0.07	<LOD	<LOD	<LOD
	4	<LOQ	<LOQ	0.41	0.76	0.10	0.17	0.37
	5	<LOD	<LOD	<LOQ	0.27	0.08	<LOD	<LOD
	6	0.16	0.01	0.02	<LOQ	<LOD	<LOD	<LOD
	7	<LOQ	0.11	0.01	0.26	<LOD	<LOD	<LOD
	8	<LOD	<LOD	<LOQ	0.07	<LOD	<LOD	<LOD

S-Table 10. continued

		F. verticillioides DNA		*F. graminearum* DNA		NIV	FB1	
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	cobs (µg/g)
Scenario 4 (28/19 °C)	1	0.04	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD
	2	0.12	0.06	0.01	<LOQ	<LOD	<LOD	<LOD
	3	0.07	0.14	<LOQ	0.03	<LOD	0.01	<LOD
	4	<LOQ	0.01	<LOQ	0.16	<LOD	<LOD	<LOD
	5	<LOD	0.06	0.02	<LOQ	<LOD	0.07	<LOD
	6	0.05	0.01	<LOQ	0.13	<LOD	<LOD	<LOD
	7	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD
	8	<LOQ	0.01	0.01	0.33	<LOD	<LOD	<LOD
Scenario 5 (30/21 °C)	1	0.07	0.52	0.03	0.08	<LOD	0.10	0.71
	2	<LOQ	<LOQ	0.01	0.33	<LOD	<LOD	<LOD
	3	0.40	0.26	0.01	<LOQ	<LOD	0.96	0.96
	4	0.03	0.03	<LOQ	<LOQ	<LOD	0.05	<LOD
	5	0.03	0.23	<LOQ	0.03	<LOD	0.01	0.45
	6	0.13	0.92	<LOQ	0.13	<LOD	0.61	0.98

S-Table 11. Amounts of species-specific DNA of *F. verticillioides* and *F. graminearum* as well as the mycotoxins fumonisin B1, deoxynivalenol and nivalenol in maize kernels and cobs of non-inoculated control maize cobs. Nivalenol and Deoxynivalenol were not analyzed in cobs due to strong inhibition effects. <LOQ: below the limit of quantification; <LOD: below the limit of detection; -: not analyzed

		F. verticillioides DNA		*F. graminearum* DNA		FB1	DON	NIV
	No.	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	cobs (µg/g)	kernels (µg/g)	kernels (µg/g)	kernels (µg/g)
Scenario 1	1	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
(22/13 °C)	2	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
	3	<LOQ	-	<LOD	-	<LOD	<LOD	<LOD
Scenario 2	1	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
(24/15 °C)	2	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
	3	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
Scenario 3	1	0.05	-	<LOD	-	0.02	<LOD	<LOD
(26/17 °C)	2	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
	3	0.29	-	<LOD	-	0.31	<LOD	<LOD
Scenario 4	1	0.01	-	<LOD	-	<LOD	<LOD	<LOD
(28/19 °C)	2	<LOD	-	<LOD	-	<LOD	<LOD	<LOD
	3	0.14	-	<LOD	-	<LOD	<LOD	<LOD
Scenario 5	1	0.01	-	<LOD	-	0.87	<LOD	<LOD
	2	<LOQ	-	<LOD	-	<LOD	<LOD	<LOD
(30/21 °C)	3	2.19	-	<LOD	-	2.25	<LOD	<LOD
	4	6.47	-	<LOD	-	19.46	<LOD	<LOD

Chapter 5: Detection of the chemical response of *Aspergillus nidulans* against the fungivorous springtail *Folsomia candida*

This chapter describes the work of Katharina Döll carried out as part of a project in collaboration with Marko Rohlfs at the J.F. Blumenbach Institute of Zoology and Anthropology, University of Göttingen, Germany.

The data are part of the following publication:

Döll K, Chatterjee S, Scheu S, Karlovsky P, Rohlfs M (2013): Fungal metabolic plasticity and sexual development mediate induced resistance to arthropod fungivory. Proceedings of the Royal Society B 280:20131219.

Abstract

Drastic losses of fungal biomass can occur after the feeding of fungivorous soil arthropods on filamentous fungi. The induced chemical defense reactions of fungi expressed in the synthesis of toxic metabolites to fungivory was conjectured. In this study grazing of collembolan *Folsomia candida* on *Aspergillus nidulans* induced significant up-regulation of the highly toxic sterigmatocystin, the two meroterpenoids austinol and dehydroaustinol as well as the cyclic nonribosomal depsipeptides emericellamides C, D, E and F. Our study suggests that these secondary metabolites are involved in the defense of *A. nidulans* to fungivores and that the chemical response is much more complex than expected.

Introduction

The soil mould ascomycete *A. nidulans,* teleomorph *Emericella nidulans,* produces numerous secondary metabolites with unknown ecological functions. Although several secondary metabolites produced by *A. nidulans* have already been identified, including sterigmatocystin, austinol, dehydroaustinol and emericellamides, it is predicted that the majority of *A. nidulans* secondary metabolites is still unknown [1]. Due to its high toxicity and similarity to

aflatoxins, sterigmatocystin is one of the most studied metabolites produced by *A. nidulans* [2, 3]. Sterigmatocystin shows toxicological, mutagenic and carcinogenic effects in animals and is classified as a 2B carcinogen for humans by the International Agency for Research on Cancer [4].

There has been very little research on the influence of secondary metabolites in biotic interactions. Many invertebrates use saprophytic fungi in soil as an important food source. It has been suggested that fungal secondary metabolites of saprophytic fungi in soil act as putative resistance mechanisms against invertebrate fungivores [5]. It is predicted that fungivores feeding on fungal hyphae lead to a chemical defense reaction of the fungi by increasing synthesis of secondary metabolites to fungivores [6].

Collembola are highly abundant fungivores [7] which can drastically reduce the fungal biomass. In interactions between *A. nidulans* and the springtail *Folsomia candida* secondary metabolites seem to enhance fungal competitiveness by playing a key role in the protection of the fungus against grazing of the fungivores [8]. In food choice experiments colonies of *A. nidulans* with an interrupted pathway of secondary metabolites (deletion of *LaeA* gene), including sterigmatocystin, penicillin and terrequinone A, were more attractive to *F. candida* than the wild type [6]. Colonies producing increased amounts of secondary metabolites, including sterigmatocystin, decreased in attractiveness to the fungivores as a food source [9].

In the present study, the chemical response of *A. nidulans* to attacks of the fungivore *F. candida* was investigated. With the use of mass spectrometry mycelia of *A. nidulans* colonies treated and not treated with *F. candida* were analyzed for the content of secondary metabolites. An non-targeted metabolic profiling approach was first used to identify metabolites which were induced in colonies of *A. nidulans* after *F. candida* had fed on them. Upregulated metabolites were then identified using tandem mass spectrometry.

Material and methods

Chemicals

Methanol (HPLC-grade), isopropyl alcohol (p.a. grade), n-hexane (for synthesis) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Acetonitrile, methanol,

both LC-MS grade, were supplied by Th.Geyer GmbH & Co. KG (Renningen, Germany) and acetic acid (LC-MS grade) was purchased from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany).

Analytical mycotoxin standards

Sterigmatocystin purchased from Sigma-Aldrich, Steinheim, Germany was dissolved in acetonitrile, resulting in a concentration of 1 mg mL^{-1}. Out of this stock solution a dilution series of 10 concentrations from 1 ng mL^{-1} to 10 µg mL^{-1} in methanol/water (1:1) was prepared.

Sample preparation

A. nidulans was incubated on KOH-treated, sterile cellophane placed on malt extract agar. The fungi were treated with 25 fungivores *Folsomia candida* ("Berlin" strain) for seven days. Fungal tissue was frozen in liquid nitrogen and lyophilized. Five colonies were pooled to generate one biological sample. This experiment was carried out by Dr. Marko Rohlfs.

Freeze dried mycelia (100 mg) of 7-day old colonies of *Aspergillus nidulans* (strain RDIT2.3; veA1), treated (n=6) and not treated (n=6) with the fungivores, were prepared for targeted and non-targeted metabolite analysis by adding 1 mL acetonitrile/water (84:16). The samples were shaken over night and centrifuged at 4800 x g for 10 min. Afterwards 400 µL supernatant was evaporated to dryness in a speed vacuum concentrator at 40 °C and redissolved in the same amount of methanol/water (1:1). Cyclohexane (400 µL) was added for defatting and the samples were thoroughly mixed and centrifuged for 10 min at 14000 x g. The lower phase was taken and stored at -20 °C for analysis.

Analysis of sterigmatocystin

HPLC-MS/MS analysis was conducted using a system consisting of a binary pump system (ProStar 210, Varian, Darmstadt, Germany), a degasser, a column oven, a prostar 410 autosampler (Varian, Darmstadt, Germany) and a triple quadrupole mass spectrometer 1200L

coupled with electrospray ion source (ESI) (Varian, Darmstadt, Germany). Separation was carried out by HPLC at 40 °C on a reverse phase column Kinetex C_{18} (50.0 x 2.1 mm, particle 2.6 µm) coupled with a C_{18} security guard cartridge (4 mm × 2 mm i.d., both from Phenomenex (Aschaffenburg, Germany). Solvent A consisted of water with 5% acetonitrile and solvent B of methanol, both containing 7 mM acetic acid. The binary gradient started at 50% B and increased linearly to 98% within 5 minutes at a flow rate of 0.2 ml min^{1}. After washing for 3 minutes the column was re-equilibrated using starting conditions for 7.7 minutes. The sample volume was 10 µL. Electrospray ionization was performed in positive mode. The needle voltage and the shield voltage were set to 5 kV and 600 V, respectively. Air served as a nebulizing gas (50 psi) and nitrogen as a drying gas (19 psi, 250 °C). Argon was used as a collision gas in quadrupol 2. LC-MS/MS operating in multiple reaction mode with specific mass transitions of *m/z* 325>281 and 325>310 with collision energies of 36 V and 24 V, respectively, were used for the detection of sterigmatocystin. Analytes were analyzed with a dwell time of 500 ms at a detector voltage of 1300 V. The software Varian MS workstation 6.9.1 was used for system control, data acquisition and evaluation. The concentration of the samples was calculated on the basis of a linear calibration curve constructed with pure external standards.

Tandem mass spectrometry of secondary metabolites

Metabolic profiling (conducted by Dr. Subhankar Chatterjee) was carried out to obtain differences in mass signal intensities between treated samples and controls.

Masses with enhanced signal intensities in treated samples were analyzed with tandem mass spectrometry. Analysis was performed using a system consisting of a binary pump system (ProStar 210, Varian, Darmstadt, Germany), a degasser, a column oven, a prostar 410 autosampler (Varian, Darmstadt, Germany) and a ion trap mass spectrometer 500 MS coupled with electrospray ion source (ESI) (Varian, Darmstadt, Germany). Separation of the metabolites was carried out at 40 °C on a reverse phase column Polaris C_{18}-Ether, 100 x 2 mm, 3 µm particle size; Agilent, Darmstadt, Germany). Solvent A was water with 5% acetonitrile and solvent B was methanol, both solvents contained 7 mM acetic acid. The solvent system and the gradient were identical to those used in the metabolic profiling. The

binary gradient started at 10% B for 5 minutes and increased to 98% within 25 minutes, followed by a washing step for 8 minutes. Afterwards, the column was re-equilibrated using starting conditions for 20 minutes. The sample volume was 10 µL and the flow rate was set to 0.2 mL min^{1}. The precursor ions of *m/z* 624; 596; 459 and 457 were fragmented using CID excitation voltages of 2.46 V, 2.36 V, 1.84 V and 1.83 V, respectively. Electrospray ionization was conducted in positive mode. For all analytes the capillary voltage, the needle and shield voltage were set to 40 V, 5 kV and 600 V, respectively. RF loading was set to 100%. Air served as a nebulizing gas at 50 psi and nitrogen as a drying gas at 25 psi and 250 °C. Helium was used as a collision gas. Daughter ions were analyzed in standard mode with 15000 u sec^{-1} and 2.35 or 2.71 seconds per scan depending on the analyte.

System control, data acquisition and evaluation was carried out with a Varian MS workstation 6.9.1. For the identification of the metabolites the specific mass spectra resulting from the fragmentation and the order of their elution in the gradient (retention time) were compared to published data.

Statistical analysis

Statistical analysis was carried out using the normalized data of sterigmatocystin (µg g^{-1}), calculated on the basis of a linear calibration curve, and the peak areas for all other secondary metabolites. Differences in the levels of secondary metabolites between treated and untreated mycelia of *A. nidulans* were carried out using a t-test. Data not following normality or homogeneity of variance were analyzed using the Mann-Whitney rank sum test. Statistical analyses were performed using the software SigmaPlot version 11.0 (Systat software Inc.).

Results

Production of sterigmatocystin

Mycelia of 7-day old *A. nidulans* colonies treated and not treated with *F. candida* were analyzed for the production of sterigmatocystin using tandem mass spectrometry. The amounts of sterigmatocystin in samples treated with the fungivores were significantly higher in comparison to untreated controls (Fig. 1).

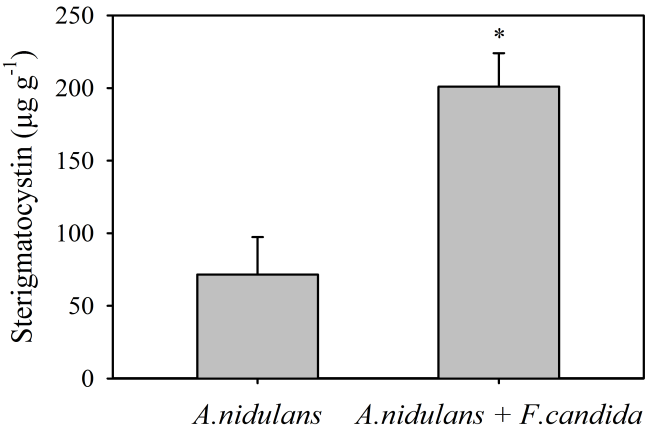

Figure 1. Production of sterigmatocystin by *Aspergillus nidulans* after predation by *Folsomia candida* in comparison to untreated controls. Data indicate means (n=6) and the standard deviation. Significant differences are illustrated by *, indicating $P \leq 0.001$ according to paired t-test

Identification of further enhanced secondary metabolites

For the detection of further metabolites the amount of which differ considerably in the two groups metabolic profiling was carried out (conducted by Subhankar Chatterjee). Signals (*m/z* 596, 624, 457, 459) which displayed enhancement in all biological replications of the treated colonies in comparison to the untreated controls were examined further with tandem mass spectrometry for accurate identification. The specific mass spectra as well as the retention time of all analytes tested were compared to published data.

Fragmentation with *m/z* of 596 and 624 via HPLC-MS/MS resulted in both cases in the

detection of two peaks (Fig. 2). The compounds were identified as emericellamides C, D, E and F comparing them with data published by Chiang *et al.* [10]. The cyclic nonribosomal depsipeptides are two separate pairs of isomers. The isomers differ in the position of their methyl group at C21 and C23 (Fig. 3) with a slight effect on their polarity. Each pair of isomer resulted in the same fragmentation pattern (Fig. 4). For emericellamides C and D the product ion spectrum revealed signals at *m/z* 295, 323, 436, 507, 525, 568, 578 and for emericellamides E and F at *m/z* 323, 351, 436, 464, 482, 535, 553 and 606. Between the isomers the fragmentation pattern was the same, but the intensity of the fragments differed.

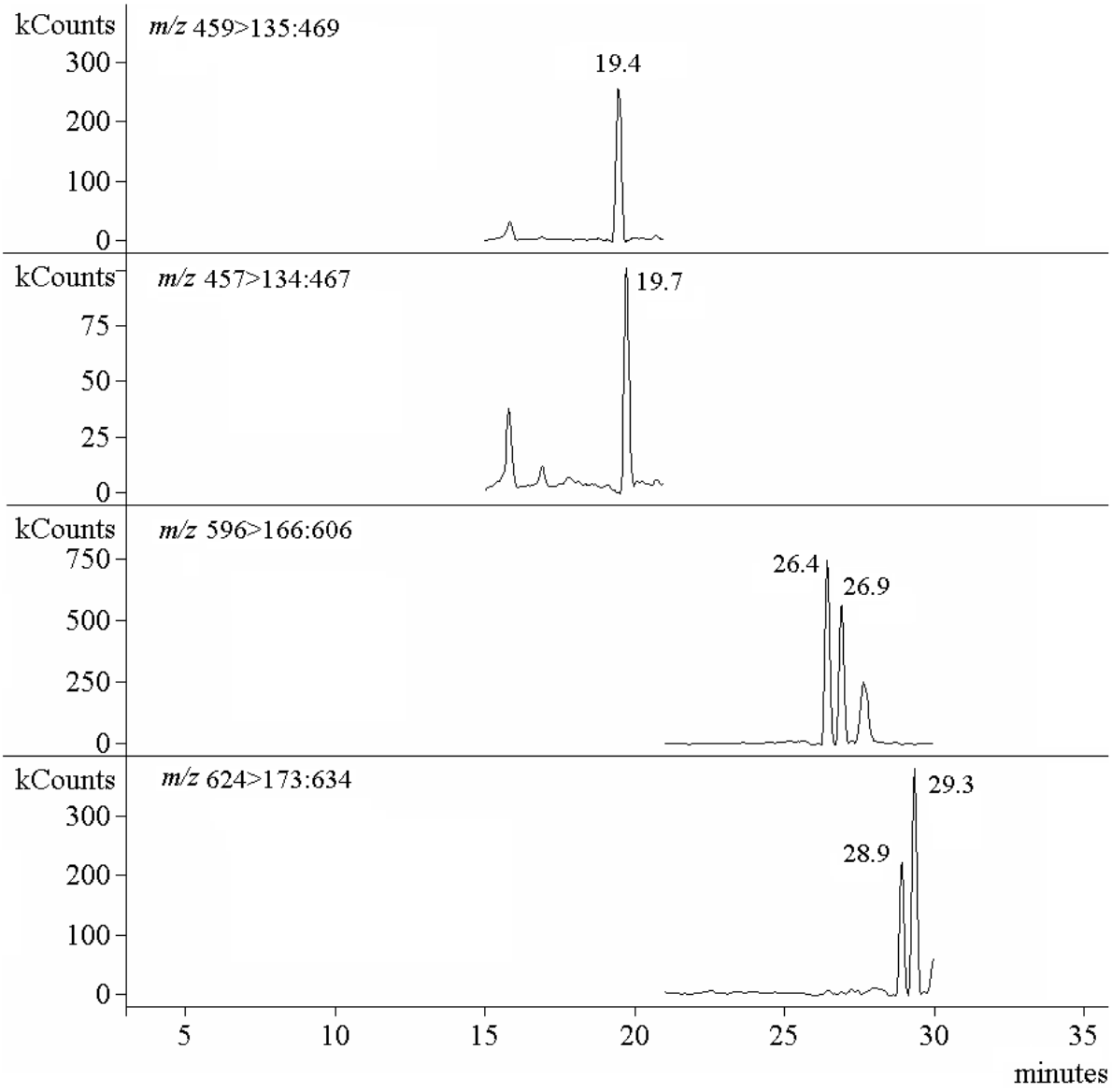

Figure 2. Total ion chromatograms of product ions received after fragmentation of the precursor ions

at *m/z* 459, 457, 596, 624 using tandem mass spectrometry

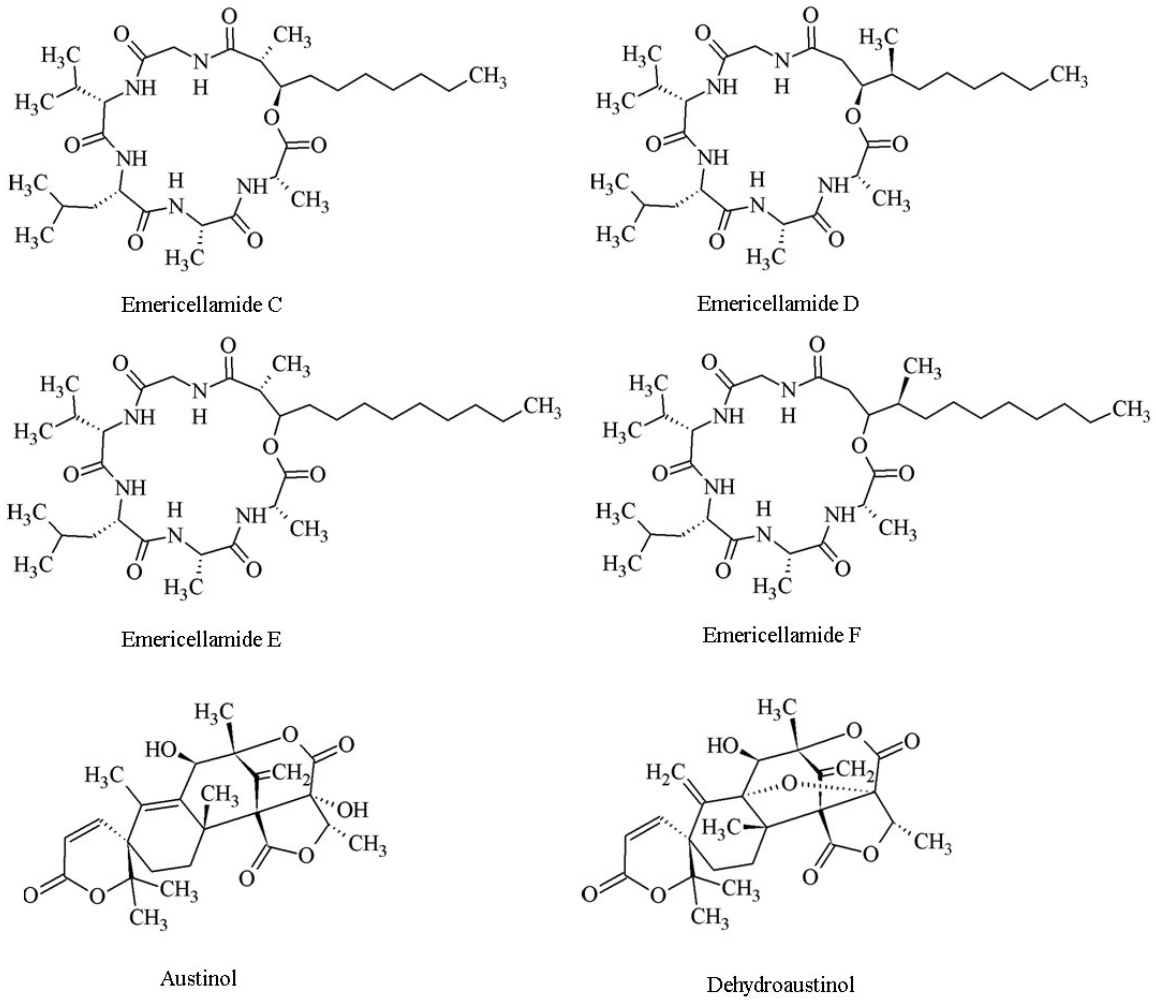

Figure 3. Chemical structures of secondary metabolites found in mycelium of *Aspergillus nidulans*

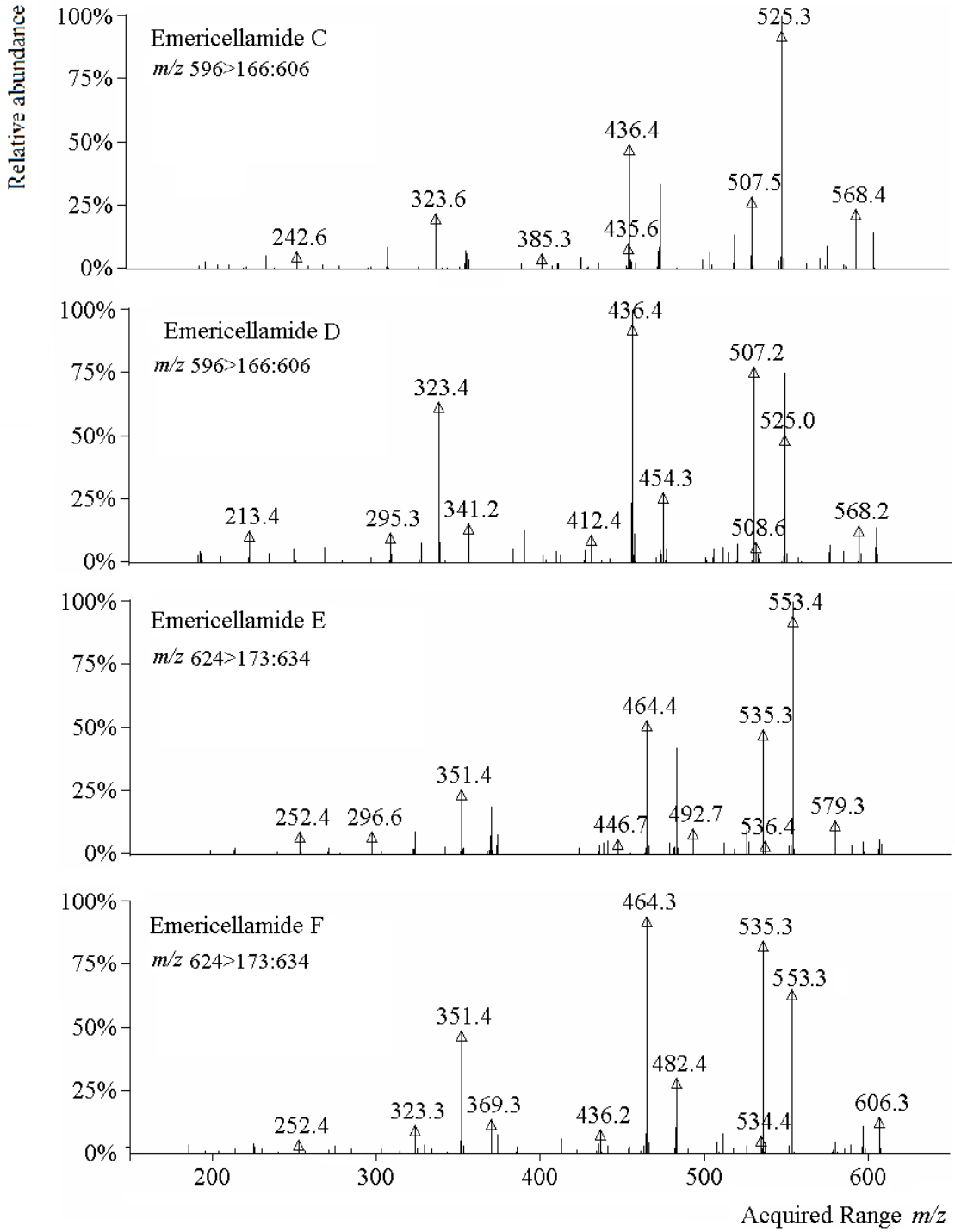

Figure 4. Product ion mass spectra of emericellamides C, D, E and F

Apart from the emericellamides two compounds with *m/z* of 459 and 457 were identified as the meroterpenoids austinol and dehydroaustinol. The product ion spectrum, obtained by tandem mass spectrometry, revealed signals at *m/z* 441, 423, 323, 223 and at *m/z* 439, 421,

359, 339, respectively (Fig. 5). Identification was confirmed by comparing the mass spectra with those kindly provided by Clay C. C. Wang. The signals of all six metabolites were significantly enhanced in mycelia treated with *F. candida* in comparison to those of the untreated controls (Fig. 6).

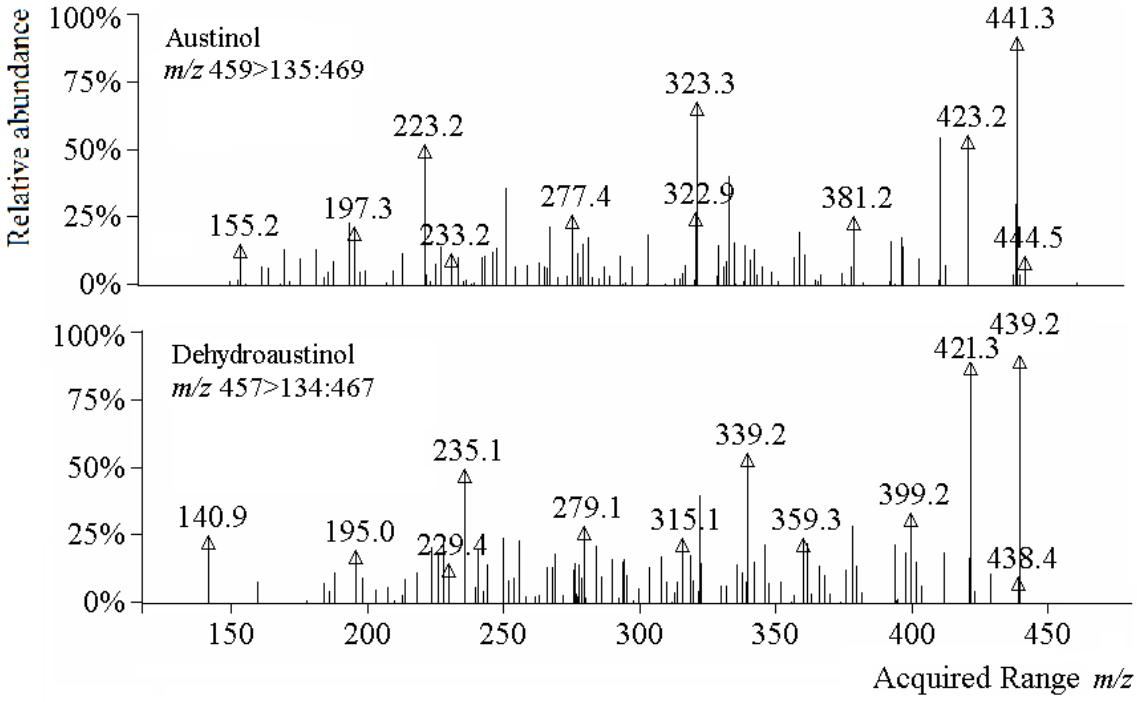

Figure 5. Product ion mass spectra of austinol and dehydroaustinol

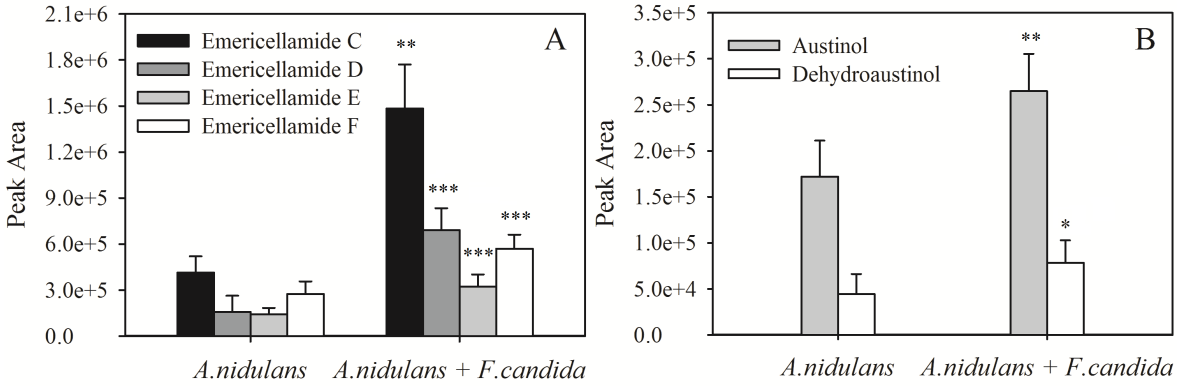

Figure 6. Production of emericellamides C, D, E and F (A) and austinol and dehydroaustinol (B) by *Aspergillus nidulans* after predation by *Folsomia candida* in comparison to untreated controls. Data indicate means (n=6) ± standard deviation. Significant differences in the production of secondary metabolites are illustrated by ***, ** and *, indicating P≤0.001, P≤0.01 and P≤0.05, respectively according to paired t-test, except for emericellamide C statistically analyzed with Mann-Whitney Rank Sum Test

Discussion

The biological role of many fungal secondary metabolites has not been demonstrated, but the hypothesis is formulated that some might be involved in the defense against predators [5]. In the present study enhanced amounts of sterigmatocystin, emericellamides C, D, E and F as well as austinol and dehydroaustinol in mycelia treated with the fungivore *F. candida* were found. This observation suggests that these metabolites might be involved in defense reaction towards fungivore arthropods. Earlier studies also indirectly support this hypothesis for sterigmatocystin.

F. candida avoided the mutant-producing enhanced amounts of sterigmatocystin and consumed almost exclusively the wild type in food choice experiments. The restorer gene of secondary metabolism RsmA, a putative YAP-like bZIP protein, was overexpressed and the authors believe that RsmA is responsible for the upregulation of sterigmatocystin and other secondary metabolites as a defensive response in stress situations [9]. Furthermore, the highly toxic effects of sterigmatocystin to insects were reported. Sterigmatocystin-producing colonies of *A. nidulans* led to 100% mortality of the insect *Drosophila melanogaster,* while mutants not producing the mycotoxin did not affect the insects [11].

Several secondary metabolites produced by *Aspergillus* spp. have been reported to be toxic to insects, including the structurally related aflatoxins [5]. Also a non-aflatoxigenic strain of *A. flavus* showed a substantial antifeedant activity against *Carpophilus hemipterus* (*Nitidulidae*). In this study, the most abundant active component found in sclerotia was dihydroxyaflavinine [12]. Furthermore, ochratoxin A isolated from the sclerotia of the fungus *A. carhonarius* was reported to cause feed reduction of the larvae of the detritivorous beetle *Carpophilus hemipterus* as well as weight gain and mortality of the maize ear worm *Helicoverpa zea [13]*. It is predicted that the predation by insects acts a selective force that has shaped the chemical defense systems of *Aspergillus* spp. [12].

Unlike in the case of sterigmatocystin, there has been little research on the biological function of emericellamides, austinol and dehydroaustinol. The production of emericellamides A, C, D, E and F by *A. nidulans* was first described by Chiang *et al.* [10]. The cyclic nonribosomal depsipeptides emericellamides are molecules of mixed polyketide/peptide origin [1]. An increase of approximately 100 times of emericellamide A and B, structurally closely related metabolites, was found in co-cultures of the marine fungus *Emericella* sp. strain CNL-878

(not further described) and the actinomycete *Salinispora arenicola* in comparison to pure cultures of the fungus. Emericellamide A and B are antibiotic agents with moderate effectiveness, with emericellamide A being slightly more effective than B [14]. Furthermore, the two meroterpenoids austinol and dehydroaustinol, molecules of mixed polyketide/terpenoid origin [15] showed toxic effects on insects [16]. Together with the increase in the production of emericellamides and austinols observed in our study after grazing by arthropods, this suggests that, in addition to sterigmatocystin both families of metabolites may act as protection against arthropods and bacteria.

In addition to insecticidal activities, dehydroaustinol was reported to be involved in regulatory processes in sterigmatocystin and conidia production [17]. The production of secondary metabolites and conidia are co-regulated in *A. nidulans* due to a common signal transduction pathway. [18]. Two genes, *fluG* and *flbA*, are known to regulate both conidia and sterigmatocystin production [19] whereas the *fluG*-dependent extra-cellular factor is required for the initiation of the developmental pathway [20]. Dehydroaustinol was found to be the initiation factor (called FluG factor), whereas diorcinol may act as an essential accompanying compound preventing the crystallization of dehydroaustinol on the hyphae surface by adduct formation. This supports its spread over the hyphae and, finally, contact with the putative receptor leading to production of asexual conidia and sterigmatocystin [17]. In addition to its function as a putative biocontrol agent, dehydroaustinol may be involved in regulatory processes leading to increased synthesis of sterigmatocystin. Future research is needed to improve our understanding of the biological role of the detected metabolites in biotic interactions.

The present study shows the induced chemical reaction of *A. nidulans* to grazing activities by *F. candida.* Our study suggests that these secondary metabolites are involved in the defense of *A. nidulans* against fungivores and that the chemical response is much more complex than expected. The exact role of these metabolites in the interaction and their effects on fungivores remain to be determined.

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Chapter 6: Mycotoxin production of *Fusarium oxysporum* f. sp. *strigae* in interaction with *Striga hermonthica* and sorghum

This chapter describes the work of Katharina Döll as part of a project in collaboration with Dr. Beninweck Ndambi Endah at the laboratory of Dr. Annerose Heller at the Institute for Plant production and Agroecology in the Tropics and Subtropics (380a), University of Hohenheim, 70599 Stuttgart, Germany.

Abstract

The fungal isolate *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2 is highly host specific and aggressive towards the parasitic weed *Striga hermonthica*, but is not pathogenic towards sorghum. *Striga* shoots and mature sorghum grains were tested for their content of fumonisins, beauvericin, enniatins and moniliformin. The samples originate from *Striga* and sorghum plants whose seeds have been sown together in pots. Sorghum seeds were coated with Foxy 2. Furthermore, the production of these mycotoxins was also analyzed in rice cultures of the fungus. Among the tested mycotoxins beauvericin was found in *Striga* shoots as well as in rice cultures. The possibility that the fungus is able to produce fumonisins was excluded by proving the absence of the gene FUM1 which is an essential gene involved in fumonisin synthesis. Furthermore, no toxin was found in mature sorghum grains from plants grown from of Foxy 2-coated seeds and attached to *Striga* plants. The results indicate that the use of Foxy 2 as biocontrol agent of *Striga hermonthica* poses no risks to animals or humans as consumers of sorghum grains.

Introduction

Striga hermonthica is a parasitic weed leading to severe agricultural losses in several crops including sorghum, maize, millet, rice and even wheat, especially on the African continent [1, 2]. Eleven species of the genus *Striga* are known to parasitize plants, with *S. asiatica* and *S. hermonthica* on cereals, and *S. gesnerioides* on legumes leading to the most serious damage to agricultural plants. Economic losses due to *Striga* are estimated to be more than US $7 billion annually [3].

The fungal strain *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2, was isolated from diseased *Striga* plants in Ghana. ITS sequence analysis of Foxy 2 and the closely related isolate PSM197 showed that the ITS sequences differ from those of pathogenic *F. oxysporum* strains deposited in GenBank. It was concluded that the fungi, both highly specific towards *Striga,* belong to a new *forma specialis* which was called *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines [4]. The fungus Foxy 2 is highly host-specific and aggressive to all developmental stages of the parasitic weed, including seeds, but is not pathogenic towards sorghum. The emergence of *Striga* is drastically reduced by the fungus while the yield of sorghum plants is increased [5]. Foxy 2 is a potential, highly host-specific mycoherbicide for the control of *Striga* plants. The fungus infects *Striga* seeds leading to the inhibition of seed germination as well as infestation on host plant`s roots by the parasitic weeds. Furthermore, the fungus reduces the total numbers of seeds in the soil and prevents the production of new seeds [6, 7].

There has been hardly any research on the production of toxins by the fungus which also may play a role in the host-specific activities of the fungus. The only toxic metabolites known to be produced are fusaric acid and 9,10- dehydrofusaric acids [8]. No further toxins have been detected as yet, but the production of toxins may play an important role in the aggressiveness of the fungus. Before the use of the fungus as a biocontrol agent, it is necessary to investigate and exclude the possibility of contamination of agricultural products by mycotoxins in order to prevent health risks to animals and humans.

Mycotoxin production among subspecies of *F. oxysporum* is diverse, and the production of enniatins, fusaric acid, moniliformin, sambutoxin [9] and beauvericin [10] have been reported. Additionally, some strains belonging to the *F. oxysporum* species have also been found to produce fumonisins B and C [11–13]. Of these toxins, the production of fumonisins in

particular is of importance because the International Agency for Research on Cancer (IARC) has classified fumonisins as probably carcinogenic to humans (group 2B). Fumonisins have also been shown to cause equine leukoencephalomalacia, pulmonary oedema in pigs and hepatocarcinogenesis in rats [14]. Fumonisins are a class of mycotoxins which were first described by Gelderblom *et al.* in 1988 [15]. In total 28 fumonisin analogs have been characterized until today, and these can be classified as fumonisin A, B, C, and P series, with fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3) being the most abundant.
In the current study the fungal strain Foxy 2 was tested for its production of fumonisins, beauvericin, enniatins and moniliformin in *Striga* shoots. Furthermore, fungal DNA was screened for the presence of the gene FUM1, which is an essential gene involved in fumonisin synthesis. Additionally, mature sorghum grains which were grown on *S. hermonthica* parasitized sorghum plants from Foxy 2-coated seeds were analyzed for their mycotoxin contents.

Materials and methods

Chemicals

Methanol (HPLC-grade), was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Acetonitrile, methanol, both LC-MS grade, were supplied by Th. Geyer GmbH & Co. KG (Renningen, Germany) and acetic acid (LC-MS grade) was purchased from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany).

Analytical mycotoxin standards

Analytical standards of beauvericin, enniatin A, A1, B, B1 were obtained from Sigma Aldrich (Steinheim, Germany) in powder form (1 mg). Individual stock solutions were prepared by redissolving the powder in acetonitrile, which resulted in a concentration of 1 mg mL^{-1}. Moniliformin was obtained from alexis Deutschland GmbH (Grünberg, Germany). Certified mixed solutions of FB1, FB2 and FB3 in concentrations of 50 ng mL^{-1} per toxin were purchased from Sigma Aldrich (Steinheim, Germany). A standard mixture with equal

concentrations of each mycotoxin was prepared. Out of this stock solution a dilution series in methanol/water (1:1) was prepared for quantification.

Fungal strains

The fungal isolate *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2 was obtained from Dr. Annerose Heller, University of Hohenheim Stuttgart, Germany. The fungus was originally isolated from *S. hermonthica* plants in Northern Ghana by Abbasher *et al.* [7]. The strain has the accession number BBA- 67547-Ghana in the collection of the Federal Biological Research Center for Agriculture and Forestry, Berlin, Germany.
F. verticillioides strain VP2 [16], obtained from Francesca Cardinale, University of Turin, Italy, was used as positive control in FUM1 analysis. *F. oxysporum* 121 obtained from Evelyn Möller (University of Hohenheim, Stuttgart, Germany) was used as a positive control for the species-specific detection of *F. oxysporum*.

Plant material and greenhouse experiment

Greenhouse experiments were planned and carried out by Dr. Ndambi Beninweck Endah (University of Hohenheim, Stuttgart, Germany) [17]. Sorghum seeds were coated with a suspension of arabic gum (AG 40%) and dried Foxy 2 chlamydospores by Saat und Ernte-Technik GmbH (Eschwege, Germany) as described by Elzein *et al.* [18]. Three Foxy 2-coated *Sorghum bicolor* L. Moench seeds were planted together with 50 mg seeds of *S. hermonthica* and cultivated up to four months. Two cultivars of *Sorghum bicolor* L. Moench seeds were used: the *Striga*-susceptible cultivar “Cowbaula” (provided by the International Institute of Tropical Agriculture (IITA-Benin Station) and the *Striga*-tolerant cultivar “Wad Admed” (provided by the Agricultural Research Corporation (ARC), Sudan).
Additionally, two weeks after emergence of *Striga* shoots microconidia (500 µL of 2.45 x 10^9 conidia mL^{-1}) of Foxy 2 were injected directly into the *Striga* shoots.
In both treatments, coating and coating and injection *Striga* shoots were harvested three and six weeks after their emergence. Mature sorghum grains were harvested four months after being sown. Non-infected *S. hermonthica* shoots and mature sorghum grains grown from non-coated seeds, were used as controls. Finally, fine powder of freeze-dried *Striga* shoots and

dried grains of each sorghum cultivar were pooled together resulting in one sample per treatment. Grains from 9 and 6 seed non-coated and coated plants from variety Cowbaula as well as grains from 10 and 8 seed non-coated and coated plants from variety Wad Admed were pooled, respectively.

Mycotoxin extraction

Fungal cultures on polished rice (50 g) were prepared by adding 70 mL of demineralized water to 50 g of polished rice, autoclaving and after cooling down inoculating with small agar blocks overgrown with mycelium of Foxy 2. The cultures were incubated for four weeks at 25 °C.

Meal of *Striga* shoots, sorghum grains and rice cultures (4 g) were extracted with 40 mL of methanol/water (75:25) and shaken overnight at room temperature. The samples were centrifuged at 4500 x g for 10 min and the supernatant was stored at -20 °C until analysis.

For fumonisin analysis plant extracts were purified using strong anion exchange (SAX) solid phase cartridges (Varian, Darmstadt, Germany) following Shephard *et al.* [19] with modifications. The SAX columns were preconditioned with 8 mL of methanol (HPLC grade) followed by methanol/water (75:25). Sample extract (10 mL) was applied to the cartridge, which was washed thoroughly with 8 mL methanol/water (75:25) followed by 4 mL methanol. Finally, 10 mL of 5% acetic acid in methanol were used for elution. The flow rate was always lower than 1 mL min^{-1}. The elute was evaporated to dryness at 40 °C and redissolved in 500 µL of methanol/water (50:50).

Mycotoxin analysis

Striga shoots, sorghum grains and rice cultures were analyzed for their content of beauvericin, enniatins A, A1, B and B1, FB1, FB2, FB3, fumonisins of the C and P series and moniliformin.

Chromatographic separation was performed by high performance liquid chromatography (HPLC) on a reverse phase Kinetex C18 column, 50.0 x 2.1 mm, particle 2.6 µm, equipped with C_{18} security guard cartridge, 4 mm × 2 mm i.d., both from Phenomenex (Aschaffenburg, Germany) with the temperature maintained at 40 °C. The mobile phase consisted of (A) water

with 5% acetonitrile and (B) methanol, both containing 7 mM acetic acid. The flow rate was set at 0.2 mL min^{-1}. The analytical system was equipped with an autosampler (ProStar 410, Varian, Darmstadt, Germany), a binary pump system (ProStar 210, Varian, Darmstadt, Germany), a degasser (Degassit, MetaChem Technologies) and a column oven (Jetstream 2 plus, Techlab, Germany). HPLC was coupled to an electrospray ionization source (ESI) attached to which was a 500MS ion trap or 1200L Triple Quadrupol mass spectrometer (both Varian, Darmstadt, Germany). An aliquot of 10 µL of the sample was used for injection.

In ion trap ESI was operated in positive mode for all analytes with the following settings: Spray chamber temperature 50 °C, nebulizing gas (nitrogen) 50 psi, drying gas (nitrogen) 25 psi at 350 °C, shield voltage 600 V, needle voltage 5000 V. The trap damping gas was helium with 0.8 mL min^{-1}. The detector was operated in standard mode with 15.000 Da sec^{-1}. System control was carried out using Varian MS workstation 6.9.1.

Beauvericin, enniatins A, A1, B and B1 were analyzed by using the LC–MS/MS multi-toxin method as described in Chapter 2. No matrix effects were observed, therefore pure standards in methanol/water (1:1) were used for quantification. FB1 as well as FB2 were also analyzed in positive mode with *m/z* of 722 and 706 as precursor ions. Specific fragment ions for FB1 were *m/z* 686, 528 and 352 and for FB2 and FB3 *m/z* 670, 512 and 336. For the detection of fumonisins belonging to the C and P series, HPLC-MS in full-scan mode (*m/z* 600-850) as well as MS/MS were performed on the ion trap scanning for the specific masses described by Musser *et al*. [20] and Seo and Lee [21]. For fumonisin analysis, extracts were analyzed after cleanup on SAX cartridges, which leads to a 20-fold enrichment.

Only moniliformin separation was performed on a HILIC system [22] followed by analysis for the specific fragmentation of *m/z* 97 to 41 using the triple quadrupole mass spectrometer in negative mode.

The limits of quantification and detection were 10 µg kg^{-1} and 3 µg kg^{-1} for beauvericin, enniatin A, B and B1, 20 µg kg^{-1} and 7 µg kg^{-1} for enniatin A1, 5 µg kg^{-1} and 2 µg kg^{-1} for FB1, FB2 and FB3, and 300 µg kg^{-1} and 100 µg kg^{-1} for moniliformin, respectively.

Real-time PCR analysis

After incubation in 100 mL of potato dextrose broth for one week at 25 °C without shaking,

pure mycelium of the fungal strain Foxy 2 was harvested by filtration. 400 µL of ethanol (99%) was added to the mycelium in a 2 mL reaction tube, the mixture was vortexed and the sample was dried in a speed vacuum concentrator. DNA from dried fungal mycelium and freeze dried *S. hermonthica* meal was extracted using the CTAB method as described by Brandfass and Karlovsky [23].

Real-time PCR followed by melting curve analysis was used for the detection of species specific DNA of *F. oxysporum* in *S. hermonthica* shoots. The primer pair Clox1 (CAGCAAAGCATCAGACCACTATAACTC) and Clox2 (CTTGTCAGTAACTGGACGTT GGTACT) specific for *F. oxysporum* were used as described by Mulè *et al.* [24].

Furthermore, the presence of the gene FUM1 in Foxy 2 was examined by analyzing DNA from pure mycelium. For the detection of the FUM1 gene in the DNA of Foxy 2, two sets of primer were used. The first primer pair was rp32 (ACAAGTGTCCTTGGGGTCCAGG) and rp33 (GATGCTCTTGGAAGTGGCCTACG) described by Proctor *et al.* [12]. The second primer pair FUM1F7328 (ATGGAACTTGGAACCTGCAC) and FUM1R7664 (AGCTGGTACTCGGGATGATG) was derived from the FUM1 gene sequence (GenBank accession AF 155773). The size of the amplification product was 337 bp and the corresponding melting temperature was 88 °C .

PCR was carried out using PCR premix QPCR SYBR Green Mix (Abgene/Thermo Fisher, Schwerte, Germany) with 300 nM of each primer. Real-time PCR was performed in an iCycler thermocycler (BioRad, Hercules, CA, USA) using the thermocycle program described for species-specific PCR for *F. verticillioides* in Chapter 3 [25].

Results

F. oxysporum in Striga shoots

Striga shoot samples were analyzed for the presence of *F. oxysporum* DNA. *F. oxysporum* strain 121 was used as a positive control for the species-specific detection of *F. oxysporum*. The melting curve temperature of the amplification products of pure Foxy 2 DNA as well as of infected *Striga* shoot samples complied with those of the positive control (Table 1). DNA of *Striga* shoot samples grown with Foxy 2-coated sorghum seeds and harvested 3 weeks after

emergence were not analyzed because there was not enough material for extraction.

Table 1. Real-time PCR results for the detection of *F. oxysporum* DNA in *Striga* shoots infected with Foxy 2 as well as in pure mycelia of *F. oxysporum* strains. Coating: sorghum grains coated with Foxy 2; inoculation: additional inoculation of *Striga* shoots with spores of Foxy 2; not infected: no inoculation as well as planted with non-coated sorghum seeds; 3 and 6 weeks: harvest of *Striga* shoots 3 and 6 weeks after emergence; nd: not detected; Ct: treshold cycle

Sample	Ct- value	Melting temperature (°C)
F. oxysporum 121 (positive control)	19.64	86.50
Foxy 2	21.21	86.50
Striga shoots, not infected, 6 weeks	nd	nd
Striga shoots, coating, 6 weeks	27.23	86.50
Striga shoots, coating and inoculation, 3 weeks	30.61	86.50
Striga shoots, coating and inoculation, 6 weeks	28.85	86.50

Mycotoxin production of Foxy 2

Of the toxins for which the rice cultures of Foxy 2 were analyzed (beauvericin, FB1, FB2, FB3 as well as fumonisins of the C and P series, enniatins A, A1, B and B1, and moniliformin), only beauvericin was found (data not shown). In *Striga* shoots beauvericin was also the only mycotoxin detected (Fig. 1, Table 2), and this applies both to *Striga* shoots grown with Foxy 2 coated sorghum grains as well as to those with additional inoculation with the fungus. No mycotoxins were detected in the grains of either sorghum cultivar, coated and not-coated with Foxy 2 (Table 2).

Table 2. Content of mycotoxins in *Striga* shoots and sorghum grains. Coating: sorghum grains coated with Foxy 2; no coating: sorghum grains not coated with Foxy 2; inoculation: additional inoculation of *Striga* shoots with Foxy 2; not infected: no inoculation as well as planted with not-coated sorghum seeds; 3 and 6 weeks: harvest of *Striga* shoots 3 and 6 weeks after emergence

	Mycotoxin [µg kg^{-1} plant material]*								
Treatment	Bea	Enniatin				Fumonisin			Mon
		A	A1	B	B1	B1	B2	B3	
Rice culture									
Culture of Foxy 2	>20000	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Striga shoots									
Not infected, 6 weeks	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Coating, 3 weeks	60	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Coating, 6 weeks	760	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Coating, inoculation, 3 weeks	550	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Coating, inoculation, 6 weeks	930	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sorghum grains									
Cowbaula, no coating	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Cowbaula, coating	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Wad Admed, no coating	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Wad Admed, coating	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

*LOD values were 3 µg kg^{-1} for beauvericin (Bea), enniatin A, B and B1, 7 µg kg^{-1} for enniatin A1, 2 µg kg^{-1} for fumonisin B1, B2 and B3, and 100 µg kg^{-1} for moniliformin (Mon).

FUM1 analysis

In order to ensure that the strain is not able to produce fumonisins pure mycelium of the strain Foxy 2 was tested for the presence of the gene FUM1 with real-time PCR followed by melting curve analysis. No amplification products were obtained in Foxy 2 DNA with either primer pair. By contrast, *F. verticillioides* strain VP2, containing FUM1 gene and used as a positive control, obtained PCR products with the expected melting temperatures with both pairs of primers.

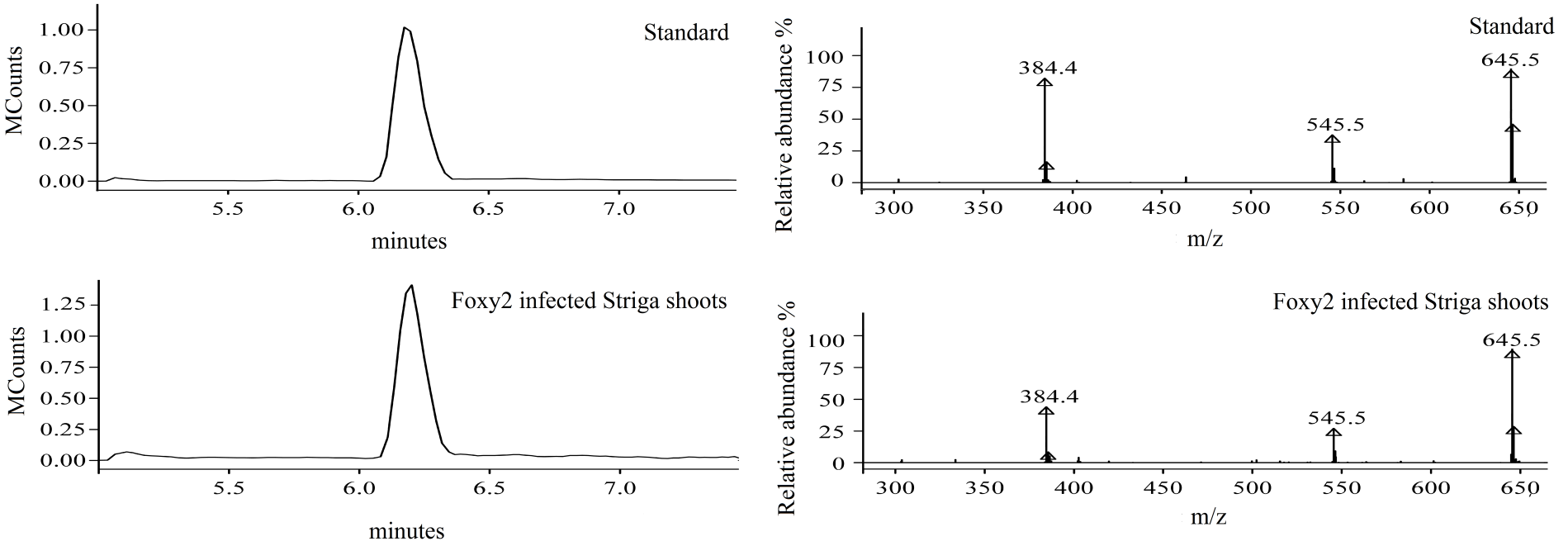

Figure 1. LC-ESI-MS/MS chromatograms (left) and MS/MS spectra (right) of beauvericin standard of 50 ng mL^{-1} and a sample of *Striga* shoots after infection with Foxy 2 coated sorghum seeds 6 weeks after emergence

Discussion

The hemiparasite *Striga hermonthica* is a major biotic constraint to sorghum and several other field plants, leading to severe agricultural losses in several crops, especially on the African continent [1, 2]. Since the damage to sorghum plants occurs primarly before the emergence of the parasite, controlling the parasitic weeds is difficult. Several conventional approaches have been tried in attempts to control *Striga*, but they were only partially successful [3]. Biological control may be an alternative approach for the specific control of the parasitic weed *Striga*.

The fungal strain *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2, is highly specific for its host plant *Striga hermonthica* and is discussed as a potential biocontrol agent.

However, before the release of a fungal plant pathogen as a biocontrol agent, host-specificity and risk assessment are required, including investigations on the production of mycotoxins by the pathogen which could endanger the health of animals and humans [3]. In previous studies, it has already been demonstrated that the strain Foxy 2 produces the toxin fusaric acid, a non-host specific phytotoxic compound putatively involved in the virulence of the fungus [8]. Zonno *et al.* showed that fusaric acid leads to dramatic reductions in seed germination of *Striga hermonthica* [26]. However, no further information concerning the mycotoxin production of this fungus is available.

In the current study the fungal strain Foxy 2, was tested for its production of the mycotoxins fumonisins, beauvericin, enniatins and moniliformin. Among these toxins the strain was shown to produce the mycotoxin beauvericin.

Beauvericin was first isolated from cultures of the entomopathogen *Brassica bassiana* [27] but it is mainly produced by several *Fusarium* species and occur on wide range of host plants [9]. The mycotoxin is a cyclic hexadepsipeptide and consists of D-*α*-hydroxy-isovaleryl acids alternating with three aromatic amino acid residues of N-methyl-phenylalanines linked by peptide and ester bonds (Fig. 2) [27].

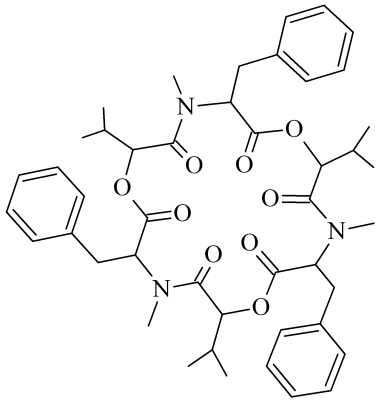

Figure 2. Chemical structure of beauvericin

Beauvericin is an ionophoric molecule that can form stable and lipophilic complexes with several cations or neutral molecules [28]. Its toxic effects are attributed to its activity as a membrane carrier, forming ionophoric lipophilic complexes [28]. As a consequence, insecticidal, antimicrobial [27] and antiviral effects as well as cytotoxic activities have been demonstrated [29]. Information on the toxicity of beauvericin against plants is limited. Beauvericin was reported to be toxic to tomato protoplasts and induced protoplast death caused by imbalance of the ascorbate system and subsequent oxidative stress [30]. Furthermore, in young maize leaves beauvericin significantly depolarized the membrane potential of leaf parenchymal cells, increased membrane permeability and K^+ leakage and led, finally, to the inhibition of respiration [31].

Following our discovery of the production of beauvericin by Foxy 2, effects of the mycotoxin were examined by applying the pure toxin to shoots. This was carried out by Ndambi Beninweck as part of this collaborative project. Beauvericin caused cell death in all types of tissues at concentrations of 10 µM and 50 µM and some membranes of the cells appeared to have been dissolved [17]. Furthermore, Zonno and Vurro tested 14 toxins for their effects on germination on *Striga hermonthica*. Among the toxins tested, beauvericin revealed moderate toxicity and led to a reduction in seed germination of about 30% [32]. These findings suggest that beauvericin may be involved in the suppression of seed germination as well as in disease development.

When using a plant pathogen as a biocontrol agent, it must be guaranteed that there is no risk of the agricultural product being contaminated with compounds toxic to humans or animals.

Only limited data concerning toxic effects of beauvericin to animals and humans are available, but currently no mycotoxicoses caused by consumption of beauvericin are known [28]. Additionally, fusaric acid has been proven to have only low to moderate toxicity to consumers [9]. The most toxic secondary metabolite produced by some strains of *F. oxysporum* are fumonisins. After no fumonisins were found in *Striga* shoots, sorghum grains or in rice cultures, the presence of the gene FUM1 in the genome of Foxy 2 was analyzed. The data confirmed that the strain is not able to produce fumonisins, lacking the essential gene in fumonisin synthesis. Overall, the toxicity of the mycotoxins produced by the fungus is low, but nevertheless it has to excluded that the harvest product is not contaminated by these mycotoxins.

Furthermore, the host-specificity of the fungus is of major importance. It must be taken into consideration that not only sorghum but also the other agricultural field crops in a crop rotation need to remain unaffected by the fungus. Elzein and Kroschel showed that Foxy 2 only infected *Striga asiatica* and *S. hermonthica*, which can be considered to be the host of the fungal strain Foxy 2, while 25 non-target plant species tested were immune and the fungus did not cause any effects on vegetative growth parameters [33] supporting the high specificity of the fungus towards *Striga*.

It was observed that the fungus grew on the surface of the sorghum roots and penetrated rhizodermal cells including root hairs, colonizing the intercellular space and the cells of the cortical parenchyma. However, the fungus was not penetrating the endodermal layer to invade the tissues of the central cylinder and the xylem vessels in sorghum shoots [34].

Although it was reported that fungus does not invade xylem vessels of sorghum, it is necessary to exclude the possibility that mycotoxins produced by the fungus are translocated to sorghum grains. Therefore, sorghum grains were analyzed for their content of mycotoxins. However, none of the mycotoxins for which the grains were tested were found, including beauvericin, indicating that the mycotoxin was not carried over to sorghum grains.

Overall, Foxy 2 was found to produce the mycotoxin beauvericin but without risks to animal or human consumers of sorghum grains. Investigation concerning phytotoxic activities of beauvericin as a potential virulence factor in plant disease development in *Striga* plants is needed.

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Chapter 7: General discussion-evolutionary aspects of secondary metabolites

Why do filamentous fungi produce secondary metabolites?

Various theories for the role of secondary metabolites in ecological systems have been discussed, ranging from considering them as less important useless "waste products" to compounds important for increasing the fitness of an organism in its environment. However, interest in research on secondary metabolites has increased in the last decades due to the acknowledgment of their importance in ecological systems [1]. The functions of many secondary metabolites are still unexplored and they are difficult to determine, but the involvement of several metabolites in the behavior of an organism has been shown. Fungi can be involved in numerous complex biotic inter- and intraspecific interactions, such as symbiosis, antagonism, mutualism and parasitism.

Understanding biotic interactions of filamentous fungi and the putative role of secondary metabolites in these interactions requires experimental studies based on specific and accurate analytical techniques. In the current study, complex interactions of toxic fungal species with plants, fungi, and insects were investigated in order to identify secondary metabolites which may play a role in biotic interactions. The bases of these investigations are the accurate determination and quantification of the secondary metabolites and the biomass of their producers in complex matrices. However, investigations have been difficult due to the lack of sufficient analytical techniques. Only in the last decade the analytical technologies have been developed which enable the quantitative analysis of both parameters in complex matrices. The development of accurate and sensitive methods is still in progress. Therefore, parts of this study address the development and assessment of analytical methods as bases for investigations of secondary metabolites in biotic interactions. The methods of choice nowadays are tandem mass spectrometry for the analysis of secondary metabolites as well as real-time PCR for the quantification of species-specific fungal biomass, and these were also chosen in the current study.

Costs and benefits of the production of secondary metabolites

The type of interaction can change over ecological and evolutionary time scales depending on cost/benefit ratios [2]. Simplified evolution theories assume that a compound with beneficial activities for the organism in interactions led to the favored conservation of this organism in contrast to those phenotypes that do not carry out such activities. The benefit of the biological activity of the compound to its producer must always be greater than the cost of its synthesis. However, in complex biotic interactions the excretion of metabolites by an organism exerts selection pressure also on the target. The population of phenotypes which react in favorable ways to the activities of the metabolite will be supported, and this results in adaptation to the situation. Biotic interactions are dynamic and are subject to a constant selection pressure exerted on all partners involved [3].

As part of this dissertation, enhanced amounts of sterigmatocystin and further secondary metabolites were detected in mycelium of *Aspergillus nidulans* after attack by the fungivore *Folsomia candida*. The results indicate the putative involvement of these metabolites in a defense reaction of the fungus. The synthesis of sterigmatocystin represents a physiological cost to the fungus, but the ability to produce and up-regulate this metabolite results in an indirect ecological benefit due to the successful defense of the fungal biomass. In addition to the indirect beneficial effects due to the defense of biomass, Wilkinson *et al.* found an increase in conidiation, with the progression in sterigmatocystin synthesis additionally leading to a direct beneficial effect which increases the fitness of the fungus [4]. Natural selection appears to have favored the ability of *A. nidulans* to respond to stress situations caused by insects with the excretion of toxic metabolites.

Apart from toxic metabolites produced as defense reaction, plant pathogenic fungi often produce phytotoxins, which are involved in the virulence or pathogenicity of the fungus. The production of these toxins is associated with costs for its synthesis. It is assumed that the virulence of a pathogen is positively correlated to growth and reproduction within the host, which are two important parameters describing the fitness of the pathogen [5]. The successful infection and, furthermore, growth within the plant enables the fungus to acquire nutrients, which is beneficial for further fungal growth and spore production. In the current study, both the non-host-specific *Fusarium* species *F. verticillioides* and *F. graminearum* as well as the host-specific strain *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2, were

investigated. It was shown that Foxy 2 produces the mycotoxin beauvericin and as part of the collaboration the toxic effects of beauvericin towards *Striga hermonthica* were investigated [6]. In previous studies the production and toxic effects of fusaric acid were also described. Both toxins caused toxic effects on *Striga hermonthica*, indicating that they may be involved in the specific plant pathogenicity, which enables the fungus to infect the host [6–8].

In a further study of this dissertation (Chapter 4) dealing with toxigenic plant pathogens, the results indicate that the infection as well as the spread of *F. verticillioides* on maize ears may be facilitated by the simultaneous infection with *F. graminearum*. Similar observations were determined by previous studies [9, 10]. Nivalenol has been reported to act as a moderate virulence factor of *F. graminearum* in maize. The function of deoxynivalenol in the infection of maize ears is not completely understood [11–13]. The results of this study in combination with previous observations indicate that *F. verticillioides* may indirectly benefit from infection with *F. graminearum* and perhaps also from the latter cost-intensive production of phytotoxins.

Dynamics of biotic interactions in time and space

Species distribution in ecosystems is dynamic and the prediction of the occurrence of species in geographical areas in the future requires a better understanding of how abiotic conditions effect dispersal processes. Changes in climate may result in shifts of the geographical distribution of organisms and have an impact on the alteration of fungal community structures. Wisz *et al.* described to what extent biotic interactions can affect the response of species to environmental conditions and underlined the importance of the incorporation of biotic interaction in the prediction of species distribution due to climate change [14]. As a consequence, changes in geographical dispersal and the alteration of community interactions may lead to existing interactions between species being dissolved or new ones being created [15].

Changes in environmental conditions and fungal communities may lead to strong natural selection pressures on traits important for fitness in the dynamic ecological system. Environmental conditions are the most likely factor to account for the difference in mycotoxin content among agricultural plants in different regions. Weather affects the accumulation of mycotoxins in three ways: temperature and humidity control the efficiency of infection, affect

the ability of host plants to counteract the infection by defense responses, and influence fungal growth and mycotoxin production within plant tissue.

Several plant pathogens, such as *Fusarium verticillioides* and *Fusarium graminearum* occur on many different host plants. It is assumed that the ability to infect several hosts strongly influences the evolution of virulence due to the fact that a generalist has more opportunities for transmission and survival [5]. The lipase FGL1 is a general virulence factor of *F. graminearum* and is involved in colonization of wheat, barley and maize. The mycotoxin deoxynivalenol is also an important virulence factor in wheat, while nivalenol is assumed to be a virulence factor in maize [11]. In contrast to these two non-host-specific fungal species , *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2 was demonstrated to be highly host-specific to *Striga hermonthica*. Although non-host-specific species have more opportunities for infection, it is predicted that evolution will favor specialism, because different hosts lead to different environments exerting selection pressure. The co-evolution of the host and the pathogen could result in functional trade-offs that would limit the generalist's fitness in any one host [5]. The production of the detected mycotoxin beauvericin in the species-specific host-pathogen interaction between Foxy 2 and *Striga* needs to be elucidated.

In addition to host-pathogen interactions, inter- and intraspecific interactions also influence the distribution of fungal species. Environmental conditions, host resistance or susceptibility all influence growth, survival, dissemination and fungal community composition [16, 17]. The dominance of fungal species colonizing the same host plant is influenced by abiotic conditions like temperature and humidity, and the predominant spectrum of fungal species and their mycotoxins appears to be specific to geographical areas. In Lower Saxony, Germany, an increase of about +1.3 °C in the mean temperature occurred between 1951 and 2005 [18]. Depending on the emissions scenario, further temperature increases of about +2 °C by 2050 and up to +4 °C by 2100 are predicted in Germany (1961-1990 base period) [19]. Similar values are also estimated for global warming, ranging from 1.1-3.5 °C by 2100, depending on the emissions scenario (1980–1999 base period) [20].

We examined the possibility that global warming will cause an increase in fumonisin content due to infection with *F. verticillioides* of maize grain produced in northern parts of Germany.

As part of this thesis, the biotic interaction between the two main maize pathogens *F. verticillioides* and *F. graminearum* was investigated. The experiment setup was based on temperatures which represent an area with a cooler climate in Lower Saxony. Temperature

increase led either to no or positive correlations in biomass and fumonisin contents in corn and cobs. It must be taken into consideration that the samples were harvested two weeks after inoculation, and growing periods in fields are much longer. The effect may even intensify significantly under field conditions. Interactions with *F. graminearum* never led to negative effects in infection, biomass or mycotoxin production by *F. verticillioides*. In contrast, positive effects in infection and biomass production were observed in interaction with *F. graminearum*, which lead to the conclusion that the interaction may even favor infection and disease development of *F. verticillioides.*

Juroszek and von Tiedemann summarized published data on the potential effects of climate change on wheat pathogens. As with many different genera, an increase of *F. graminearum* and further *Fusarium* species causing *Fusarium* head blight is predicted to occur in Germany and other European countries [21]. In the current study no increase of disease severity with temperature was observed by *F. graminearum* in maize. An increase in the occurrence of *F. graminearum* may additionally facilitate the incidence and disease severity of *Fusarium* ear rot caused by *F. verticillioides*. For *F. verticillioides* either no effects or slightly positive effects on the frequency of infection, biomass or mycotoxin content were obtained with increasing temperature. Temperature increase due to climate change may increase the risk of infection by *F. verticillioides* and contamination of food by fumonisins in Germany in the future.

A paradox: toxic fungi as biocontrol agents

Worldwide cereal grains are colonized by fungi which pose enormous problems in feed and food security due to their production of harmful mycotoxins. To protect consumers from contaminated food, special regulations with detailed guidelines regarding several mycotoxins in food have been established in many countries worldwide. Due to an increasing recognition of the significant risk for human and animal health, the number of countries with specific mycotoxin regulations in many food and feed products increased continuously from 33 in 1981 to 56 in 1987, 77 in 1995, and 100 in 2003 [22]. The Food and Agriculture Organization of the United Nations (FAO) estimates that about 25% of the world's food crops are contaminated with mycotoxins [23] and that about 1000 million tonnes of food are lost due to mycotoxin contamination every year [24].

Toxic fungi are severe contaminants of food products, but, on the other hand, fungi producing secondary metabolites provide new research perspectives. The discovery of the first broad-spectrum antibiotic penicillin in 1929 by Alexander Fleming [25] aroused great interest and widespread attention was given to the study of new secondary metabolites. So far, pharmaceutical research has discovered thousands of compounds that inhibit the growth of bacteria, fungi, protozoa, parasites, insects, viruses and even human tumor cells in extensive research programmes [26]. Fungal secondary metabolites offers an enormous reservoir for new potential drugs.

However, the enormous potential of fungi with regard to the production of secondary metabolites also offered new opportunities in other areas, such as plant disease, weed and pest control. The possibility of using fungal biocontrol agents results from the variety of fungal interactions with other organisms. In table 1 genera or species of important fungal biocontrol agents and their metabolites are listed.

Table 1. Secondary metabolites of important fungal biocontrol agents according to Vey *et al.* [23]

Fungal biocontrol agent	Main target organism	Secondary metabolites
Beauveria bassiana	insects	bassianin, beauvericin, bassianolide, beauverolides, tenellin
Beauveria bronniartii	insects	oosporein
Colletotrichum	weeds	colletotrichin
Fusarium spp.	fungi, insects, weeds	trichotheens, beauvericin, naphtazarins, fusaric acid
Gliocladium spp.	fungi, insects, weeds	viridin, gliovirin, glisoprenins, hepelidic acid
Hirsutella thompsonii	insects and mites	hirsutellin A, and B, phomalactone
Metharizium anizoplae	insects	destruxins, swainsinone, cytochalasin C
Paecilomyces fumosoroseus	insects	beauvericin, beauverolides, pyridine-2,6-dicarboxylic acid
Tolypocladium spp.	insects	cyclosporin, efrapeptins
Trichoderma spp.	fungi, insects, weeds	harzianic acid, alamethicins, tricholin, peptaibols, massoilactone
Verticillium lecanii	insects	dipcolonic acid, hydroxycarboxylic acid, cyclosporin

Biocontrol agents are often less effective and more labour-intensive than chemical pesticides and, therefore, investment in this area of research is limited. However, the use of natural resources as biocontrol agents offers alternatives to chemical pesticides and they generally

have a reduced environmental impact due to high target specificity compared to chemical pesticides. Before using fungi as biocontrol agents, it must be assessed extensively whether their metabolites enter the food chain and whether they pose a risk to human and animal health [27].

The parasitic weed *Striga hermonthica* is responsible for severe agricultural losses of yield in several crops, including sorghum, maize, millet and rice, and it occurs in most regions south of the Sahara [28–30]. The weed plant acts as a parasite to the roots in order to obtain water, nutrients and carbohydrates [30]. Some isolates have been proposed for use as biocontrol agents against parasitic weeds. It was found that the fungal isolate *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, Foxy 2, is highly host-specific and aggressive towards all developmental stages of the parasitic weed, including its seeds, but is not pathogenic towards sorghum [31]. Therefore, the use of Foxy 2 as a biocontrol agent against *Striga hermonthica* was proposed. However, hardly any investigation of the production of mycotoxins by the fungus as well as the risk of their contamination of sorghum grains has been carried out. Until now, only the ability to produce fusaric acid, a phytotoxin putatively involved in the virulence of the fungus, has been detected [8]. In the current study, the production of fumonisins, beauvericin, enniatins and moniliformin by Foxy 2 was investigated by analyzing rice cultures with HPLC-MS. It was found that Foxy 2 produces the mycotoxin beauvericin.

Toxic fungi as biocontrol agents against plant pathogens or pests have to be used with care because of the risk of contamination of harvest products with mycotoxins. The analysis of mature sorghum grains revealed that no mycotoxin was found in collected sorghum grains, indicating that beauvericin is not carried over to sorghum grains. Overall, the use of Foxy 2 as biocontrol agent against the parasite *Striga* causes no risks for animal or human consumers of sorghum grains.

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Summary

Filamentous fungi are producers of a broad range of secondary metabolites with a high diversity of biological activities. It is generally assumed that the production of these metabolites leads to benefits which allow the fungi to survive in their ecological niche. The role of most secondary metabolites is still unknown, but many of them may be involved in biotic interactions. Understanding the function of secondary metabolites in biotic interactions requires experimental studies for which sensitive and accurate analytical techniques for the quantification of fungal biomass and secondary metabolites are necessary. The aim of this study was the development and validation of analytical detection methods based on real-time PCR and HPLC-MS/MS as well as the identification and investigation of toxic secondary metabolites involved in diverse fungal interactions.

First of all a sensitive method for the simultaneous quantification of the six hexadepsipeptides beauvericin, enniatin A, A1, B and B1 and destruxin A in various matrices like asparagus, potato, maize, tomato, rice and wheat were developed and validated. Fragmentation of sodium adducts of the analytes and determination of their specific mass spectra with LC-ESI-MS/MS using an ion trap allowed the specific and sensitive identification of the mycotoxins. Furthermore, sample preparation steps, including choice of suitable organic solvents for extraction and defatting, were thoroughly investigated. A new solvent combination acetonitrile/isopropyl alcohol/water (70:15:15) lead to high efficiency rates and low matrix effects. The limits of quantification and limits of detection ranged from 1-12 ng g^{-1} and 0.3-4 ng g^{-1} respectively across all mycotoxins and matrices.

Furthermore real-time PCR is a common method for species-specific quantification of fungal biomass in epidemiological studies. However, no methods for the determination of the two performance characteristic parameters in real time PCR assays, that is, the limit of quantification and the limit of detection, exist as they do in common chemical analytical techniques. In this study a method based on receiver operating characteristic (ROC) curve analysis in combination with Youden index was established to determine both performance characteristic parameters. The concept was applied to two species-specific real-time PCR assays which had been developed in earlier studies and which served the quantification of *F. verticillioides* and *F. proliferatum*.

Apart from the establishment of analytical methods, studies on biotic interactions of fungi with a focus on the production of secondary metabolites were carried out.

Interactions of two major causal agents of maize ear rot *F. verticillioides* and *F. graminearum* were investigated by inoculating maize ears with a spore suspension of *F. verticillioides*, *F. graminearum* and a mixture of both in field trials. Maize kernels were analyzed for their content of fungal biomass and mycotoxins. Amounts of *F. verticillioides* and fumonisin B1 either increased or were not affected by mixed inoculations. By contrast, the incidence of *F. graminearum* were either not affected or decreased in mixed inoculations. However, the amounts of mycotoxins relative to biomass revealed no differences between single and mixed inoculated treatments. Additionally, the effect of different temperature scenarios on fungal disease severity and interactions were investigated under controlled conditions. Overall, incidence and amounts of *F. verticillioides* and fumonisin B1 were either not affected or correlated positively with increased temperature in single and mixed inoculations. The results indicate that an increase of temperature due to climate warming may favor maize ear rot caused by *F. verticillioides*. The interaction with *F. graminearum* may additionally facilitate infestation by *F. verticillioides.* This may result in increased risk of fumonisin B1 contamination of maize in moderate climate areas.

Another part of this dissertation deals with the production of secondary metabolites produced by *Aspergillus nidulans* as a putative chemical defense reaction against the fungivore *Folsomia candida*. Grazing of *F. candida* on *A. nidulans* induced significant up-regulation of the highly toxic mycotoxin sterigmatocystin. Furthermore, the enhanced production of two meroterpenoids austinol and dehydroaustinol as well as the cyclic nonribosomal depsipeptides emericellamides C, D, E and F was identified. These findings may indicate the involvement of these secondary metabolites in the defense reaction of *A. nidulan*s to fungivores.

Finally, the fungal strain *Fusarium oxysporum* f. sp. *strigae* Elzein et Thines, was analyzed for its production of the mycotoxins fumonisins, beauvericin, enniatins and moniliformin. It was found that the strain in question produced the mycotoxin beauvericin. Furthermore, the ability of the fungus to produce cancerogenic fumonisins was additionally excluded by genetic analysis. The fungal strain is highly host specific and aggressive towards the parasitic weed *Striga hermonthica* but is nonpathogenic towards sorghum. Beauvericin was obtained in *Striga* shoots but not in sorghum grains. The host-specific pathogenicity of the fungus towards *S. hermonthica* in combination with unaffected sorghum grains indicates that the fungus

acting as biocontrol agent poses no risks for animal or human consumers of sorghum grains. The reported studies revealed the involvement of several secondary metabolites in diverse types of complex biotic interactions demonstrating the high diversity and biological activities of fungal secondary metabolites.

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Publications

Journal publications

Ammar GA, Tryono R, Döll K, Karlovsky K, Deising HB, Wirsel SGR (2013): Identification of ABC transporter genes of *Fusarium graminearum* with roles in azole tolerance and/or virulence. PLoS ONE 8:e79042

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Presentations at national and international conferences

Döll, K and Karlovsky P. Mykotoxinbelastung an Mais unter Einfluss des Klimas. Oral presentation on 58. Deutsche Pflanzenschutztagung, 10-14 September 2012, Braunschweig, Germany

Döll, K and Karlovsky P. Sample preparation for the quantification of beauvericin and enniatins in vegetables and cereals. Oral presentation on 34. Mykotoxin-Workshop der Gesellschaft für Mykotoxin Forschung, 14-16 Mai 2012, Braunschweig, Germany

Alkhayat D M, Döll K, Karlovsky P, Vidal S. Entomopathogens as endophytes. Poster presentation on 34. Mykotoxin-Workshop of the Society for Mycotoxin Research, 14-16 Mai 2012, Braunschweig, Germany

Arici S E, Döll K, Karlovsky P. Comparison of virulence of *Fusarium avenaceum* isolates in wheat with emphasis on the role of enniatins in plant colonization. Poster presentation on 34. Mykotoxin-Workshop of the Society for Mycotoxin Research, 14-16 Mai 2012, Braunschweig, Germany

Amato B, Prodi A, Döll K, Karlovsky P, Nipoti P, Pisi A. Interaction field trials between mycotoxin producers *F. graminearum* and *F. poae* strains involved in *Fusarium* Head Blight of durum wheat. Poster presentation on 32. Mykotoxin-Workshop of the Society for Mycotoxin Research, 14. - 16. Juni 2010, Lyngby, Dänemark

Döll K and Karlovsky P. Effect of stress factors and carbonhydrates on the production of fumonisins by *Fusarium verticillioides*. Poster presentation on 31. Mykotoxin-Workshop of the Society for Mycotoxin Research, 15-17 June 2009, Münster, Germany

Döll K, Nutz S, and Karlovsky P. A species-specific real-time PCR assay for quantification of *Fusarium proliferatum*. Poster presentation on 30. Mykotoxin-Workshop of the Society for Mycotoxin Research, 27-30 April 2008, Utrecht, Netherlands

Döll K and Karlovsky P. Control of fumonisin synthesis in *Fusarium verticillioides* and *F. proliferatum*. Poster presentation on 30. Mykotoxin-Workshop of the Society for Mycotoxin Research, 27-30 April 2008, Utrecht, Netherlands

Nutz S, Döll K, and Karlovsky P. Real-time PCR assays for *Fusarium verticillioides* and *F. proliferatum*. Poster presentation on 10th International *Fusarium* Workshop and *Fusarium* Genomics Workshop, 30.August-02.September 2008, Alghero, Sardinia, Italy

Eidesstattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, im März 2013