Occurrence and significance of *Fusarium* and *Trichoderma* ear rot in maize

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Annette Pfordt
born in Heilbad Heiligenstadt (Thuringia)

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1. Name of referee: Prof. Dr. Andreas von Tiedemann
2. Name of co-referee: Prof. Dr. Petr Karlovsky

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Chapter I: Introduction

1.1 Maize cultivation

Maize (*Zea mays* L.), along with wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), belongs to the most important crops worldwide grown on approx. 194 M hectares [1]. Maize is not only one of the most important food crop, but also one of the world's most important animal feeds [2]. In Germany, maize is the second most important crop grown on 2.6 M hectares with a total harvest in 2019 of around 90.4 M tons, which is only exceeded by wheat [3].

The introduction of modern silage preparation techniques in the first half of the 20th century, can be considered as a milestone, which led to the initial cultivation increase in the late 1960s. Further increase in the economic importance of maize is primarily caused by breeding success, i.e. better adapted varieties, which allowed cultivation in cooler climatic regions in Germany, improved sowing and harvest techniques as well as advances in chemical control of weeds [4].

The main types of use in Germany are silage maize and grain maize, as well as corn-cob mix (CCM). In 2019, approximately 2.2 M hectares were harvested as silage maize and 416,000 hectares as grain maize and corn-cob mix [3]. Grain maize and CCM-maize is mainly produced in the southern regions of Germany, especially Bavaria and Baden-Wuerttemberg as well as in the Rhine valley for animal feed production (swine and poultry). In contrast, silage maize is mainly produced in central to northern regions of Germany used as feed for ruminants and as substrate for biogas production. In the last decades, silage maize cultivation increased in particular due to the Renewable Energy Law (EEG) in Germany to promote the use of renewable resources for energy and biogas production [5]. Especially after the adoption of the guideline in 2004, the production area increased by about 70% between 2000 and 2014 due to federal subsidies.

In grain maize production, only the grains are harvested, while corn-cob-mix (CCM) contains the grounded grains and spindles. Grain maize is harvested at a dry matter content of 60-65%. To produce silage maize, the whole plant is chopped and harvested and serves as basis for maize silage to be fed to ruminants. In contrast to grain maize, the optimal silage

maize ripeness is around 30-35% DM. A good silage maize variety is also characterized by a high biomass yield and a high energy density in the dry matter content [4].

Due to the increasing maize cultivation, the subsequent increase of this crop within the crop rotation and the spread of insects that promote infestation such as the European corn borer (*Ostrinia nubilalis*) [6], the infestation pressure and the importance of *Fusarium* infections on maize have increased in Germany [7,8].

1.2 Ear rot diseases in maize

Ear rots, caused by fungi, are among the most important maize diseases worldwide with high potential yield losses and a reduction of grain quality [9]. The largest damage is caused by their ability to produce toxicogenic secondary metabolites, so called mycotoxins [10]. The most important diseases in maize due to their mycotoxin production are *Aspergillus* ear rot mainly caused by *Aspergillus flavus*, *Penicillium* ear rot and *Fusarium* ear rot caused by various species of the genus *Penicillium* and *Fusarium* [9,11]. Several other fungi are known to cause ear rots in maize, however, usually less harmful with minor incidence and severity, such as *Cladosporium* ear rot (*Cladosporium* spp.), *Diplodia* ear rot (*Stenocarpella maydis* and *S. macrospora)*, *Nigrospora* ear rot (*Nigrosspora oryzae*), *Trichoderma* ear rot (*Trichoderma* spp.) and corn smut (*Ustilago maydis*) [8,12]. Fungal toxins of most concern are produced by species within the genera of *Aspergillus*, *Fusarium* and *Penicillium*. Among these mycotoxins, aflatoxin B1 (AFB1), fumonisin B1 (FB1), and ochratoxin A (OTA) (Figure 1) are the most toxic to mammals, causing a variety of toxic effects including hepatotoxicity, teratogenicity, and mutagenicity, resulting in diseases such as edema, immunosuppression, hepatic carcinoma, esophageal cancer, and kidney failure [10,13,14]. Aflatoxin B1 has been classified as a class I human carcinogen, while fumonisin B1 and ochratoxin A have been classified as class 2B carcinogens by the international agency for research on cancer (IARC) (2002) [15].

1.2.1 *Fusarium* ear rot

The fungi of the genus *Fusarium* are widespread pathogens causing economically important diseases, ranging from root and stem rot to ear rot on maize in temperate and semi-tropical areas [16]. Several toxigenic *Fusarium* species are known to cause yield losses and reduction of grain quality, thus endangering the safety of both animal feed and human food products

[15,17]. Among the most important *Fusarium* species in pre- and post-harvest ear rots of maize are *F. graminearum* and *F. verticillioides* [4–6], but also other species, such as *F. poae* [7,8], *F. proliferatum* [1], *F. subglutinans* [9] and *F. temperatum* [10], are frequently reported. Infections are typically characterized by the growth of fungal white or reddish mycelium with rotting symptoms on the cob and on stored grains. They are associated with the production of numerous, chemically diverse mycotoxins such as deoxynivalenol, nivalenol, zearalenon and fumonisin [18,19].

Fusarium infected ears develop a white, salmon to cinnamon-colored or pink-purple colored mycelium, which covers the cob and the husk leaves [20]. The *Fusarium* ear rot can be differentiated into two distinct diseases that differ in their epidemiological characters: red ear rot (*Gibberella* ear red, GER) and pink ear rot (*Fusarium* ear rot, FER) [21,22]. GER is caused by species of the *Discolor* section, mainly *F. graminearum* and *F. culmorum*, however, pink ear rot is caused by representatives of the *Liseola* section, mainly *F. verticillioides*, *F. proliferatum* and *F. subglutinans* sp. In addition to the species already mentioned, there is a large number of *Fusarium* species, which are associated with both types of infestation, but isolated less frequently like *F. equiseti*, *F. poae*, *F. sporotrichioides*, *F. avenaceum* and *F. cerealis* [22]. Pathogens of the pink fusariosis colonize the ear from the tip by external infections, which appear either as randomly scattered or as groups of infected kernels. In contrast, red ear rot covers a large proportion of the entire ear. The distribution and prevalence of the different *Fusarium* species within the two kinds if ear rot disease is primarily affected by environmental conditions like temperature and precipitation as well as other factors including agrotechnical practices [23]. Infection with *F. graminearum* is favored by frequent rainfall and low temperatures during summer typically found more common in Central to Northern European areas [24,25]. FER is commonly observed in Southern to Central European areas, associated with drier and warmer climates during the grain filling period [26,27]. Root and stem infections are associated with light brown to black discolorations and the death of the heart blade with rotted stem marrow with white-pink mycelium [28,29]. Root and stalk rot are favored by dry summer, followed by a rainy autumn [30,31].

Fusarium spp. produce a large number of chemically diverse mycotoxins, which can cause considerable reduction of germination capacity of the seeds, loss of yield and loss of product quality. Species associated with FER produce various toxins including fumonisin

(FB1/ FB2/ FB3), and fusaric acid, however, trichothecenes like deoxynivalenol (DON) and nivalenol (NIV) as well as zearalenone (ZEN) are mostly detected in samples from ears infected with red ear rot [22,32–35]. Among all *Fusarium* toxins discovered, trichothecenes are the most toxigenic substances, strongly associated with acute and chronic symptoms of intoxication for humans and animals [32,34]. Trichothecenes are divided into three groups: type A trichothecenes, which include T-2 toxin and its derivatives (HT-2 toxin, T-2 triol, T-2 tetraol), and type B trichothecenes, which include DON and NIV and type C. Type B trichothecenes are less toxic compared to type A trichothecenes but occur more frequently [7,36,37]. If contaminated grain is fed to livestock, especially swine, DON contamination results in vomiting, feed refusal, decreased weight gain and fertility problems [15]. Zearalenone is produced by various *Fusarium* species, as shown in Table 1. This toxin is the most widespread *Fusarium* toxin in agriculture and is predominantly found in maize in high concentrations [10]. Zearalenone has an estrogen-like effect, which causes reproductive disorders including reduced litter size, estrogenic symptoms and male formation in particular in pigs as well as immunosuppression in humans [22,34,38]. Fumonisins are divided into 28 different types within four groups, A, B, C and P series [39]. Fumonisin B1 and B2 are the most important fumonisins in maize cultivation causing equine leukoencephalomalacia, porcine pulmonary oedemas, liver cancer in rats and are also associated with human esophageal cancer [19,40–42]. Due to these risks, the European Commission has set up a maximum tolerable daily intake for the most important *Fusarium* toxins, which are deoxynivalenol, zearalenone, fumonisin, as well as T-2 and HT-2 toxins. In addition, the legal limit in maize food and maize based products for human consumption and guidelines for the maximum levels in feed for deoxynivalenol, zearalenone and fumonisin B1 + B2 were established (Table 1).

The type of usage can therefore play an important role for feed contaminated with *Fusarium* mycotoxins. To produce silage maize, the whole maize plant is harvested, i.e. the total plant biomass contributes to the contamination with mycotoxins [43]. Maize plants used for grain maize production stay longer in the field, which subsequently leads to higher accumulation of mycotoxins in the ear. In addition, contamination is more critical when grains are used for human consumption, to feed swines and poultry due to their higher sensitivity to toxins.

Table 1: *Fusarium* species associated with ear rot in maize and their legal mycotoxin thresholds according to tolerable daily intake and limits for human food and feed contamination

***Fusarium* species**		**TDI[1] [µg/kg BW[2]]**	**Maize products for direct human consumption[3] [µg/kg]**	**Maize feed for pigs[4] [µg/kg]**
T-2 Toxin				
F. tricinctum, *F. solani*, *F. poae*, *F. sporotrichioides*, *F. acuminatum*, *F. sambucinum*	haemorrhages and necrosis of the epithelium of stomach and intestine, bone marrow, pulmonary adenocarcinomas, suppression and stimulation of immunoglobulin production	0.06	-	-
HT-2 Toxin				
F. culmorum, *F. poae*, *F. sporotrichioides*, *F. acuminatum*	Humans: vomitting, abdominal pain and diarrhea, leukopenia, bleeding from the nose and throat, depletion of the bone marrow and fever	0.06	-	-
Nivalenol (NIV)				
F. nivale, *F. graminearum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. poae*	haemolytic disorder, impairment of both humoral and cellular immune responses, haemorrhagic and emetic syndromes, human toxicosis, depletion of the bone marrow	0.7	-	-
Deoxynivalenol (DON)				
F. graminearum, *F. culmorum*	Vomiting (swine), feed refusal, weight loss and diarrhea, necrosis in various tissues	1	500	900
Zearalenone (ZEN)				
F. graminearum, *F. culmorum*, *F. cerealis*, *F. equiseti*,	hyper-estrogenism (swine), infertility and poor performance (cattle and poultry), reproductive disorder, male formation (swine), immunosuppression (human)	0.25	100	100
Fumonisin (FUM)				
F. verticillioides, *F. proliferatum*, *F. nygamai*,	Leukoencephalomalacia (horse), pulmonary edema and hepatic syndrome (swine), alteration in hepatic and immune function (cattle), esophageal cancer (human)	2	800	5000

[1] TDI = tolerable daily intake; European Commission, Option of the scientific committee on food on *Fusarium* toxins,
[2] BW = body weight,
[3] European Union, EG 1126/2007; [4] European Union, 2006/576/EG

There are three main modes of fungal entry by which fungal pathogens may enter the ear; (i) by silk channel, (ii) through wounding by insects and birds or (iii) through systemic growth from the stalk [44–47]. Which infection pathway is more important depends

on the predominant *Fusarium* spp. and the insect population in the geographical location [16].

Fusarium species survive well on maize crop residues as mycelium or through formation of survival structures like chlamydospores or thickened hyphae [48–51]. In addition, species can colonize senescent maize tissue and other crops or weed species [49] that are not considered as hosts for these pathogens [52]. From there, *Fusarium* spp. can infect the plant through the formation of ascospores or conidiospores which are dispersed by wind, insect vectors or through rain splashes [53]. The primary infection pathway by *F. graminearum* is via the silk during the first six days after silk emergency [54,55]. Fungal spores reach the silks by rain splashes or wind dispersal, germinate and grow down the silk to infect the kernels through the stylar canal [56]. Insects play a key role in the dispersal and infection of *F. verticillioides*. Severity of FER and symptomless kernel infection are closely correlated with insect injuries, primarily due to infestation with *O. nubilalis*, offering a point of entry for the pathogen [57–59]. Systemic transmission from infected stalks and seeds seems to be of lower importance and has only been reported for *F. verticillioides* [60–63] and *F. subglutinans* in maize.

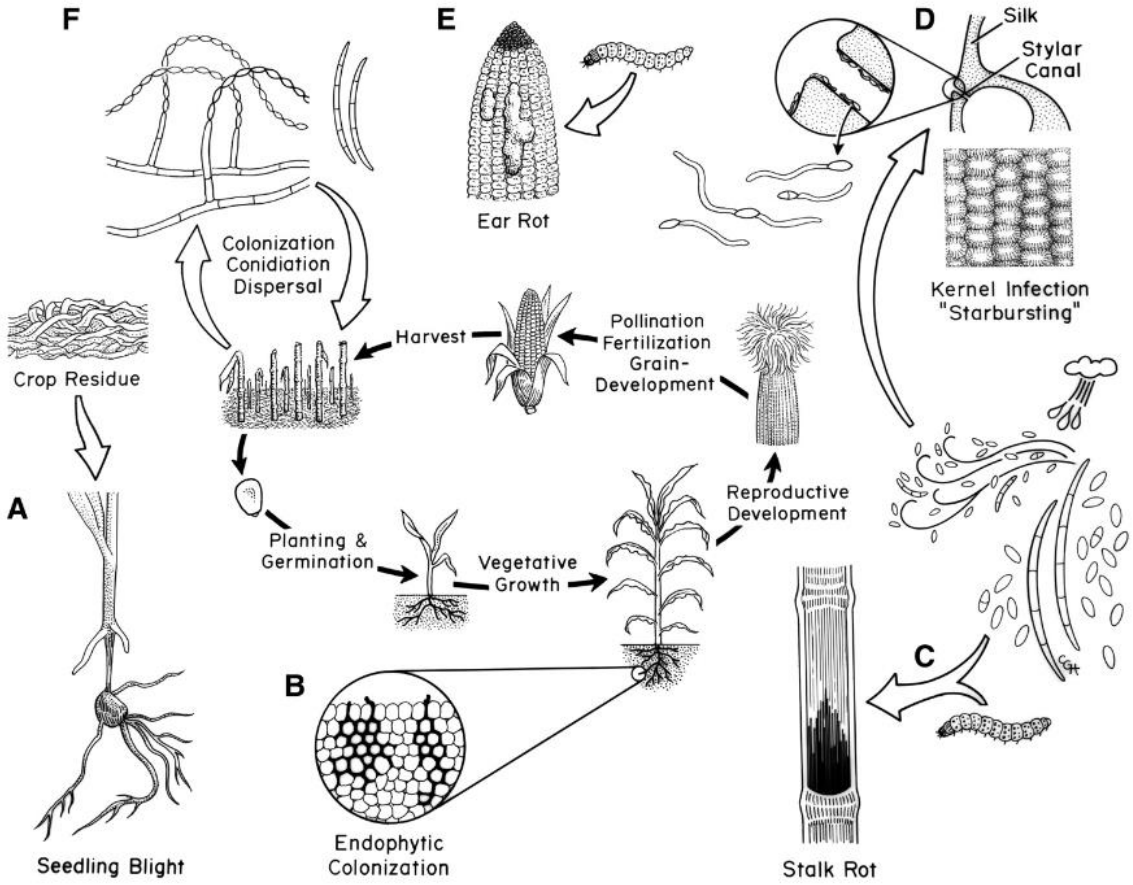

Figure 1: The *Fusarium verticillioides* disease cycle, centered on the life cycle of maize. **A** Seed germination in *Fusarium*-infested soils may develop an aggressive rot and seedling blight. **B** Endophytic colonization occurs under non disease-promoting conditions. **C** Stalk wounds from mechanical damage or insect feeding become infection courts for *F. verticillioides* and may result in stalk rot. **D** At silk stage, the fungus can colonize maize kernels via the stylar canal, giving rise to the "starbust" pattern on kernels. **E** Larvae of the European corn borer moth feed on leaves, stalks, ears, and collar tissue, providing courts for *F. verticillioides* stalk and ear rot., **F** After harvest, the fungus is capable of surviving and sporulating on crop residues, providing inoculum for subsequent infections [64].

Disease development and mycotoxin production is affected by several factors like genetic resistance of maize hybrids, weather conditions and agronomic factors. Since the European Union released legal limits for mycotoxin content in maize grains for foodstuff (EC No. 1126/2007) [65,66] and animal feed [67] (Table 1), breeding for resistance against ear rots has become an important trait for breeders, i.e. to select less susceptible inbred lines [16]. Two types of resistance have so far been identified in maize, silk channel resistance prevents the fungus from invading through the silk channel down to the kernel and kernel resistance blocks the spread of the fungus from kernel to kernel [68–70]. Different morphological factors such as pericarp thickness [71,72], surface wax layer [73] and husk covering as well as chemical factors, such as phenolic compounds, especially ferulic acid [74], have been identified to be resistance factors. Thus control of the European corn borer (*O. nubilalis*) by genetically engineered maize hybrids, containing one of the Bt-genes are known to significantly reduce *Fusarium* infection and mycotoxin concentration in maize ears and stalks [6,20,75,76].

Cultural practices such as crop rotation and tillage have been reported to influence the disease incidence and severity of *Fusarium* infection in wheat and maize [77]. Residues of previous crops serve as source of inoculum for subsequent infection [45,49] and also promote the survival of *O. nubilalis*, which may further enhance the risk of infection with *Fusarium* spp. [46,78]. Controversial effects of tillage and crop residues have been reported in previous studies. Some reports indicated no effect of alternating corn tillage practices on the incidence of ear rot pathogens [79–82], whereas others found a significant decrease in the diversity of *Fusarium* spp. in soil after conventional ploughing as compared to reduced tillage [83–85]. Other factors like the harvest date, potassium deficiency, excessive N fertilization [81], as well as the type and amount of crop residues [77,86] can affect ear rot infection. The

success of these strategies has, however, been limited owing to the broad range of *Fusarium* species and large variation in host species and their genotypes.

1.2.2 Trichoderma ear rot

Members of the genus *Trichoderma* are classified as imperfect fungi in the division Ascomycota and are ubiquitous in various types of soil. Some species of *Trichoderma* have biocontrol potential and can suppress pathogen growth by direct and indirect mechanisms [87–90]. The success of *Trichoderma* in the rhizosphere is due to their high reproduction and ability to survive under unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere and strong aggressiveness against plant pathogenic fungi [91,92]. Five modes of action are associated with biocontrol mechanism of Trichoderma; (i) competition and rhizosphere competence, (ii) antibiotic production, (iii) mycoparasitism (iv) induced defense responses in plants and (v) enzyme production [91].

They can thereby control and antagonize a broad range of economically important plant parasitic pathogens [92–94]. Several *Trichoderma* species are known with mycoparasitic potential against *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Pythium* spp. and *Fusarium* spp. [95,96]. Mycoparasitism by *Trichoderma* results in penetration of the cell wall of the host fungus and utilization of its cellular contents. Apart from the control of root and foliar pathogens, *Trichoderma* spp. enhance nutrient solubilization and uptake as well as enhanced root and root hair development [97]. This implies the increase in plant resilience against dry conditions and promotes shoot and root growth [98]. Harman et al. (2004) [95] reported a significant yield increase in maize due to *Trichoderma* treatments.

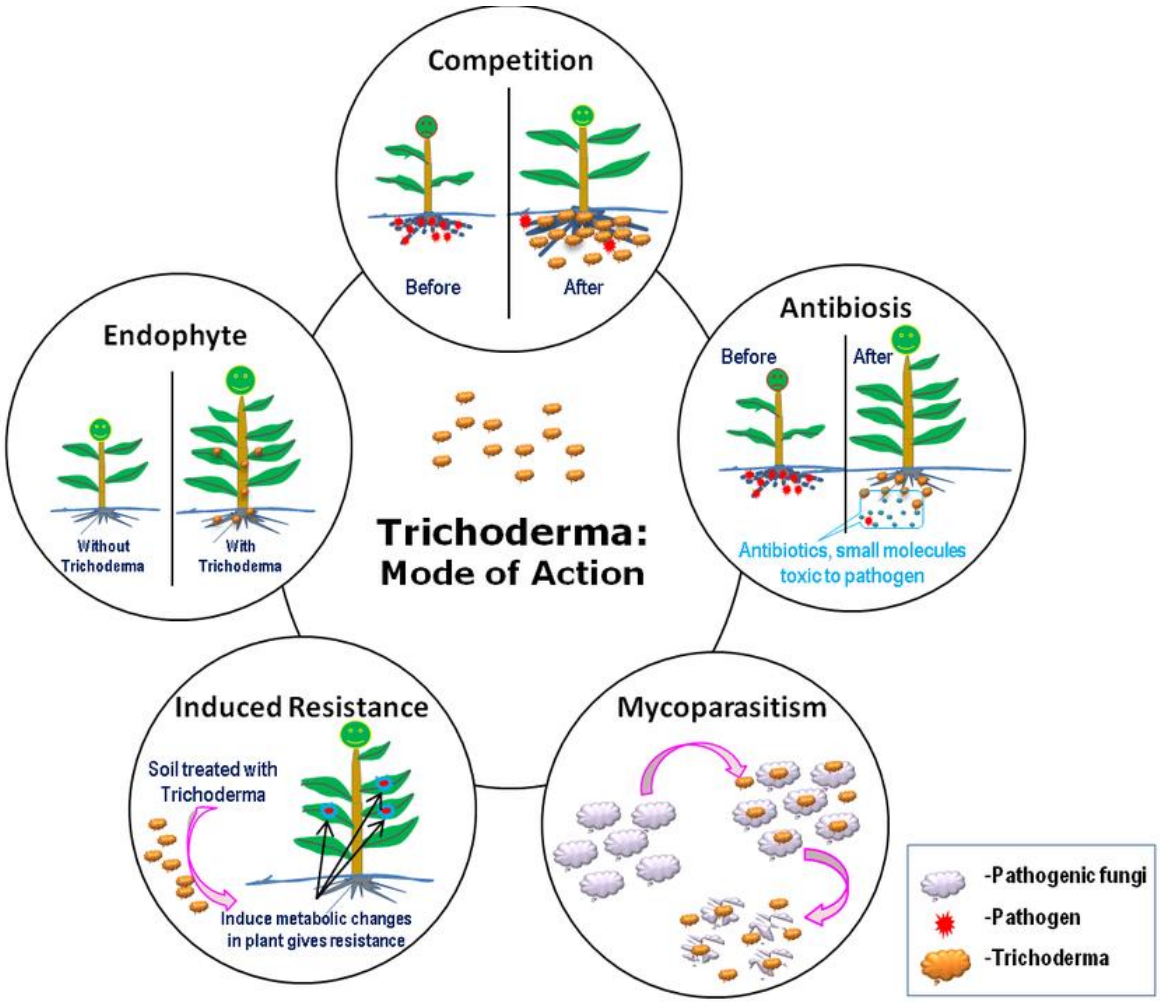

Figure 2 Overview on different modes of action of *Trichoderma* spp. antagonisms against pathogens and for plant growth improvement [99].

Trichoderma spp. produce several secondary metabolites that have different functions and potential applications in agriculture, biotechnology and medicine. Some are known to have antifungal activity like ergokonin A, trichodermin, alkylated pyranones, harzianopyridone, viridin and β-1,6-glucanases against well known pathogens like *Botrytis*, *Rhizoctonia*, *Sclerotinia*, *Penicillium* and *Aspergillus* and *Fusarium* [91]. Although *Trichoderma* species have been described as opportunistic, basically avirulent plant symbionts in soil [100], however, a few reports have mentioned *Trichoderma* as an ear rot pathogen on maize in the US [8,101–103]. *Trichoderma* ear rot infection is characterized by the occurrence of dark, blue-green layers of conidia on and between the kernels of infected ears causing premature germination of the kernels [102]. In addition, the dry matter content of ears infected with *Trichoderma* was strongly reduced compared to uninfected ears.

1.3 Aims of the study

The present study has four research objectives:

I. To determine the impact of environmental conditions and agronomic practices on the prevalence of *Fusarium* species associated with ear and stalk rot in maize. For this purpose, we evaluated the effect of weather conditions (air temperature and precipitation) and cultural practices (tillage and previous crop) on the frequency of local *Fusarium* species from naturally infected maize cobs and stalks in Germany from 2016 to 2018.

II. To determine the occurrence, the mycotoxin production and the pathogenicity of *Fusarium temperatum* from maize in Germany. For this purpose, we focused on (i) the occurrence of *F. temperatum* and *F. subglutinans* in Germany in 2017 and 2018, (ii) compared the aggressiveness to other common *Fusarium* species on maize ears and stalks, (iii) investigated the potential pathogenicity on winter wheat, (iv) determined the effect of temperature on disease severity induced by *F. temperatum* in comparison to *F. subglutinans* and (v) assessed the mycotoxin production.

III. To investigate (i) the aggressiveness of *F. graminearum*, *F. verticillioides* and *F. temperatum* on twenty maize hybrids in four locations in Germany, (ii) to correlate genotype resistance towards the respective *Fusarium* species and (ii) to determine genotype resistance to different inoculation methods (silk channel vs. kernel stab inoculation).

IV. To identify and verify *Trichoderma* as a new pathogen causing ear rot disease on maize in Europe. For this purpose, cobs infected with *Trichoderma* were sampled from ten locations in Germany and France. Isolates were cultured, microscopically examined and analyzed by sequencing the gene for translation elongation factor-*1α*. Furthermore, pathogenicity of *Trichoderma* isolates and the impact of infection on dry matter content of maize cobs was tested after artificial inoculation in the greenhouse at flowering.

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Chapter II: Impact of environmental conditions and agronomic practices on the prevalence of *Fusarium* species associated with ear- and stalk rot in maize

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Article

Impact of environmental conditions and agronomic practices on the prevalence of *Fusarium* species associated with ear- and stalk rot in maize

Annette Pfordt *[1], Lucia Ramos Romero[1], Simon Schiwek[2], Petr Karlovsky[2] and Andreas von Tiedemann[1]

[1] Plant Pathology and Crop Protection, University of Goettingen, Goettingen, Germany

[2] Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Goettingen, Germany

* Correspondence: annette.pfordt@uni-goettingen.de

Abstract:

Fusarium species are common pathogens on maize and reduce the product quality through contamination with mycotoxins thus jeopardizing safety of both animal feed and human food products. Monitoring of *Fusarium* infected maize ears and stalks was conducted in Germany to determine the range of *Fusarium* species present in the field and to assess the impact of tillage, crop rotation and weather conditions on the frequency of *Fusarium* species. From 2016 till 2018, a total of 387 infected ears and 190 stalk segments from 58 locations in Germany were collected. For each sample location, site-specific agronomic data on tillage and previous crops as well as meteorological data on precipitation, air temperature and relative humidity during the vegetation period were recorded. The most frequent *Fusarium* species detected in maize ears were *Fusarium graminearum*, *F. verticillioides* and *F. temperatum*, whereas, *F. graminearum*, *F. equiseti*, *F. culmorum* and *F. temperatum* were the species prevailing on maize stalks. Differences in the local species composition were found to be primarily associated with weather variations between the years and the microclimate at the different locations. The results indicate that mean temperature and precipitation in July, during flowering, has the strongest impact on the local range of *Fusarium* spp. on ears, whereas the incidence of *Fusarium* species on stalks is mostly affected by weather conditions during September. Ploughing significantly reduced the infection with *F. graminearum* and *F. temperatum*, while crop rotation exerted only minor effects.

Keywords: *Fusarium* spp., ear rot, stalk rot, maize, monitoring, weather conditions, agronomic practice

1. Introduction

Fusarium spp. in maize occur worldwide and can cause various diseases in different growth stages of maize, such as root and seedling rot as well as stalk and ear rot [1]. *Fusarium* ear rot (FER) and *Fusarium* stalk rot (FSR) are characterized by a white or reddish discoloration with rotting symptoms on the ears and inside the stalk [2,3]. Several toxigenic *Fusarium* species are known to cause yield losses, reduction of grain quality, thus endangering the safety of both animal feed and human food products [4,5]. The dominant *Fusarium* species causing ear and stalk rot in temperate climate zones are *Fusarium graminearum*, *F. verticillioides* and *F. subglutinans,* producing numerous, chemically diverse mycotoxins, among which the most important are deoxynivalenol, zearalenon and fumonisin [6,7].

Previous studies demonstrated, that the local *Fusarium* species composition is influenced by weather conditions as well as cultural practices [8–10, 11-13]. Several routes are known by which the fungus may enter the kernels and the stalk including wounds by insects [14,15], silk infection and systemic spread after root penetration [1,16]. The major infection pathway for the infection of maize ears by most *Fusarium* species is via the silk channel with highest severity occurring at early stages of silk development [17,18]. In contrast, infection with *F. verticillioides* is often associated with injury by insects, primarily due to the feeding of the European corn borer (*Ostrinia nubilalis*), at 10-15 days after silk emergence. Stalk colonization has been reported to increase late in the season [19,20] due to an increase in tissue susceptibility when carbohydrates and other nutrients are redirected towards developing kernels [21]. The importance of infection pathways and timepoints of infection may vary among geographical regions due to differences in weather conditions and the occurrence of insects. Temperature and moisture appear to be the most important factors affecting the range of *Fusarium* species of ear and stalk rot infection. Favorable weather conditions for an infection of *Gibberella* ear rot, mainly caused by *F. graminearum* and *F. culmorum* are low temperatures and high precipitation, whereas infection with *F. verticillioides*, *F. subglutinans* and *F. proliferatum* (*Fusarium* ear rot) is promoted at high temperatures and dry conditions [22,23].

Likewise, cultural practices such as crop rotation and tillage have been reported to influence the disease incidence and severity of *Fusarium* infection in wheat and maize [24]. Residues of previous crops serve as source of inoculum for subsequent infection [25,26] and also promote the survival of *Ostrinia nubilalis*, which may further enhance the risk of infection with *Fusarium* spp. [1,16]. Controversial effects of tillage and crop residues have been reported in previous studies. Some reports indicated no effect of alternating corn tillage practices on the incidence of ear rot pathogens [10,27–29], whereas others found a significant decrease in the diversity of *Fusarium* spp. in soil after conventional ploughing as compared to reduced tillage [9,30,31].

Prevention of *Fusarium* infection focuses on cultural practices such as crop rotation and ploughing as well as improving host resistance. The success of these strategies has, however, been limited owing to the broad range of *Fusarium* species and large variation in host species and their genotypes. In addition, maize growing areas with short rotations of wheat and maize increased in recent years resulting in a higher risk of *Fusarium* ear and stalk infection and mycotoxin contamination [23,32]. The complex of *Fusarium* species may also have extended and shifted due to climate variations and more intense maize cultivation [33].

Therefore, the objective of this study was to determine the actual *Fusarium* species composition of maize fields in Germany and to estimate how the frequency of local *Fusarium* species is affected by cultural practices (tillage and previous crop) and weather conditions (air temperature and precipitation) under natural infection from 2016 to 2018.

2. Results

2.1 Fusarium species involved in ear and stalk infections.

In the three years of field investigations, a total number of 11,610 kernels and 3,483 rachis and stalk samples were analyzed to determine the local *Fusarium* spp. composition. In the years 2017 and 2018, twelve *Fusarium* species were identified. In 2016, *F. verticillioides* and *F. proliferatum* as well as *F. temperatum* and *F. subglutinans* were treated as a species complex of *F. proliferatum sp.* and *F. subglutinans sp.*, respectively (Tab. 1).

In 2016 and 2017, *F. graminearum* was the predominant species in maize ears and detected in over 60% of all tested samples. The detection frequency of *F. graminearum* differed from year to year, with 79% in 2016, 71% in 2017 and 30% in 2018. *F. verticillioides* was the prevailing species in 2018 and detected in 39% of all ears. In total, *F. verticillioides* colonized 24% of all tested ears from 38 locations. Detection frequency of *F. temperatum* ranged from 15% in 2017 up to 33% in 2016. In total, 23% of all ears analyzed were infected with *F. temperatum*. *F. poae* colonized 14% of all tested ears followed by minor species such as *F. cerealis* (9%), *F. proliferatum* (6%), *F. tricinctum* (5%), *F. avenaceum* (5%), *F. culmorum* (4%), *F. subglutinans* (2%), *F. equiseti* (2%) and *F. sporotrichioides* (2%). Similar to ears, *F. graminearum* was also prevailing on maize stalks where; it was detected in 62% of all tested samples. In 2017, *F. graminearum* was present in more than 80% of the stalks and occurred at almost each sampling location. *F. equiseti* colonized a total of 22% of the samples within two years of investigation, however, the percentage of infected stalks containing *F. equiseti* was much higher in 2018 (34%) compared to 2017 (11%). *F. culmorum* was the third most frequent species isolated from 22% of the stalks in 2017 and 16% in 2018. Infection with *F. temperatum* and *F. cerealis* was found in 17% of the stalk samples, however, *F. cerealis* was more frequent in 2017 (19%) and *F. temperatum* in 2018 (20%). *F. verticillioides*, *F. avenaceum*, *F. tricinctum*, *F. poae F. subglutinans* and *F. sporotrichioides* were detected less frequently.

Tab. 1 Percentage of ears and stalks infected with *Fusarium* species

Ears infection								Stalk infection						
	Frequency [%]				Sample sites				Frequency [%]			Sample sites		
Fusarium species	2016 n= 94	2017 n= 180	2018 n=113	**Total**	2016 n=18	2017 n=42	2018 n=18	*Fusarium* species	2017 n=110	2018 n=80	**Total**	2017 n=21	2018 n=14	
F. gramineaum	79	71	30	**60**	17	41	15	*F. graminearum*	81	43	**62**	20	11	
F. verticillioides	19[2]	13	39	**24**	11	11	16	*F. equiseti*	11	34	**22**	10	9	
F. temperatum	33[1]	15	21	**23**	11	21	15	*F. culmorum*	22	16	**19**	14	11	
F. poae	11	15	12	**14**	6	11	12	*F. temperatum*	15	20	**17**	7	13	
F. cerealis	11	12	3	**9**	6	13	2	*F. cerealis*	19	15	**17**	9	10	
F. proliferatum	**	4	13	**6**	**	3	12	*F. verticillioides*	7	9	**8**	6	3	
F. tricinctum	4	7	2	**5**	3	8	3	*F. avenaceum*	6	5	**5**	5	3	
F. avenaceum	10	5	1	**5**	4	8	1	*F. tricinctum*	5	8	**6**	4	5	
F. culmorum	1	5	4	**4**	1	9	3	*F. proliferatum*	3	11	**6**	3	5	
F. subglutinans	*	2	2	**2**	*	3	3	*F. poae*	3	5	**4**	3	3	
								F. subglutinans	1	3	**2**	1	2	
F. sporotrichioides	4	1	5	**2**	3	1	4	*F. sporotrichioides*	1	0	**1**	1	0	

[1] In 2016, there was no differentiation between *F. subglutinans* and *F. temperatum*

[2] In 2016, there was no differentiation between *F. verticillioides* and *F. proliferatum*

2.2 Effect of previous crop

The abundance of the three most frequent species on ears (*F. graminearum*, *F. verticillioides*, *F. temperatum*) and the four species prevailing on stalks (*F. graminearum*, *F. equiseti*, *F. culmorum* and *F. temperatum*) after different pre-crops is shown in Figure 1. Crop rotation had no significant effect on ear and stalk infection with *F. graminearum*, *F. temperatum*, *F. equiseti* and *F. culmorum*. The frequency of *F. graminearum* on ears was slightly reduced in maize after maize as compared to wheat, sugar beet and non-host crops like potato, strawberries or cabbage. The highest frequency of stalk infection with *F. graminearum* was observed on maize after other crops, followed by wheat, maize and sugar beet. No effects of pre-crops were found for ears infected with *F. temperatum*. Maize as previous crop slightly favored stalk infection with *F. equiseti* (30%) while colonization with *F. culmorum* was slightly increased after wheat (27%). Only *F. verticillioides* indicated strong differences in frequency of ear infection. Colonization of *F. verticillioides* was significantly favored by maize after maize in comparison to maize after sugar beet.

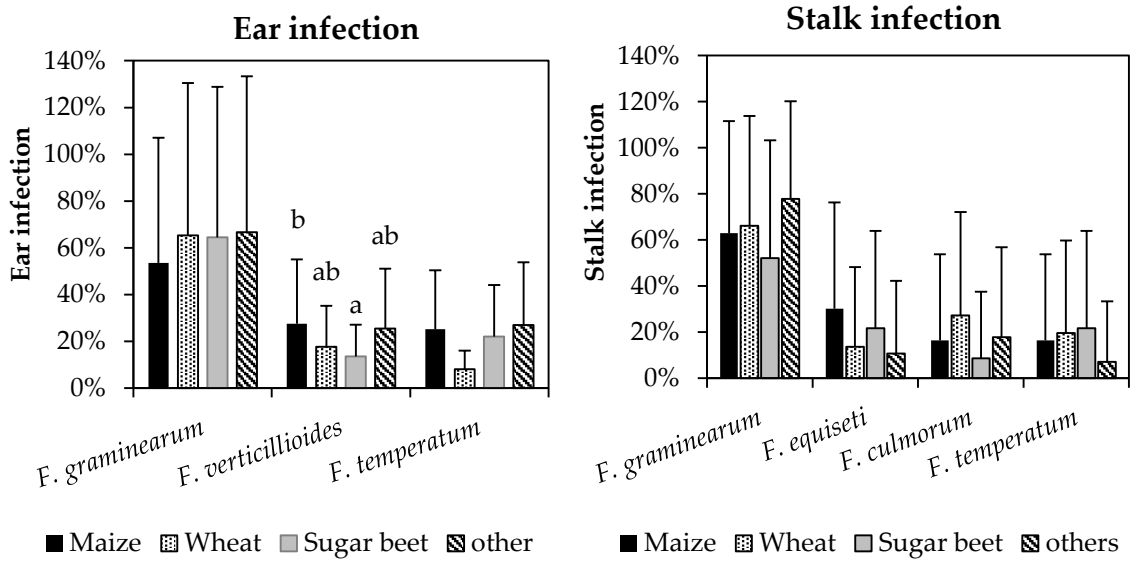


Fig. 1. Percentage of ears (left) infected with *F. graminearum, F. verticillioides* and *F. temperatum* and stalks (right) infected with *F. graminearum, F. equiseti, F. culmorum* and *F. temperatum* depending on the previous crop (maize, wheat, sugar beet, others). Vertical bars represent standard deviations. Different letters indicate significant differences ($p \leq 0.05$) within species.

2.3 Effect of tillage.

Ear and stalk infection with *F. graminearum, F. verticillioides* and *F. equiseti* were significantly affected by the type of soil tillage as shown in Figure 2. Apart from *F. verticillioides* and *F. equiseti*, reduced tillage (chisel ploughing and rotary harrow) favored infection with most *Fusarium* species compared to moldboard ploughing. Hence, colonization with *F. graminearum* in ear and stalk samples was significantly higher at reduced tillage compared to moldboard ploughing. Similarly, ear infection with *F. temperatum* was reduced after ploughing (30%) compared to reduced tillage (17%). Ploughing also reduced the frequency of *Fusarium* species in maize stalks, however, it led to higher frequencies in observations with *F. equiseti*. The percentage of ears colonized with *F. verticillioides* was significantly

higher after ploughing (24%) than after reduced tillage (12%). *F. equiseti* was equally favored by ploughing (28%) as compared to reduced tillage (7%). The type of tillage had no significant effect on stalk infection with *F. culmorum* and *F. temperatum*.

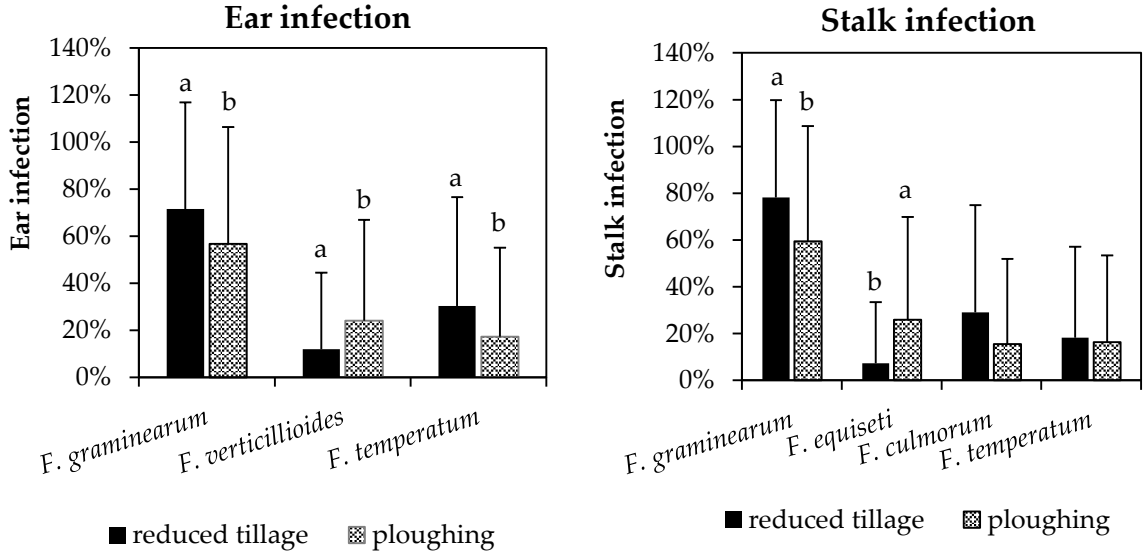


Fig. 2. Percentage of ears (left) infected with *F. graminearum, F. verticillioides* and *F. temperatum* and stalk (right) infected with *F. graminearum, F. equiseti, F. culmorum* and *F. temperatum* depending on tillage (reduced tillage vs. ploughing). Vertical bars represent standard deviation. Different letters indicate significant differences ($p \leq 0.05$) within species.

2.4 Effect of environmental conditions.

The relationship between the frequency of *Fusarium* species on ears (*F. graminearum*, *F. verticillioides*, *F. temperatum*) and stalks (*F. graminearum*, *F. equiseti*, *F. culmorum* and *F. temperatum*) and weather conditions after flowering was analyzed using Pearson correlation. While temperature and precipitation in June had no significant effect on the occurrence of the most frequent *Fusarium* species (Fig .3), temperature and precipitation during flowering in July had a medium to strong effect on the frequency of the prevailing species. Colonization of *F. graminearum* negatively correlated (r= -0.42) with temperature in July and positively correlated (r= 0.70) with precipitation in July. *F. temperatum* was favored by low precipitation (r= -0.71) and *F. verticillioides* was found to be more frequent at high temperatures (r= 0.69) and low precipitation (r= -0.71). Temperature and precipitation during August and September had minor effects on frequencies of *Fusarium* species. The correlations described above demonstrate the critical impact of temperature and precipitation in July on ear infection with the most frequent *Fusarium* species (Fig. 4). Frequency of *F. graminearum* was inversely related to temperature (r= -0.42) and positively correlated with precipitation in July (r= - 0.71). Ear infection with *F. verticillioides* significantly increased with temperature (r= 0.67) and low precipitation (r= 0.72). The temperature in July had no effect on colonization with *F. temperatum*, however, dry conditions promoted (r^2= -0.57) infections of the ear.

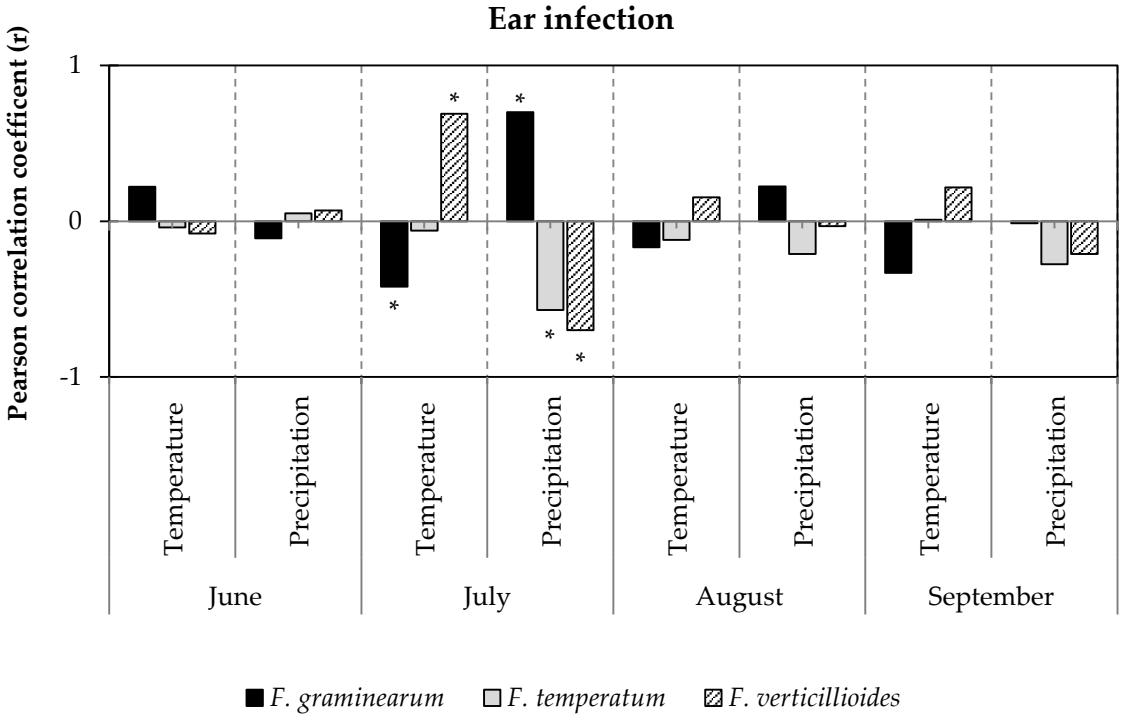
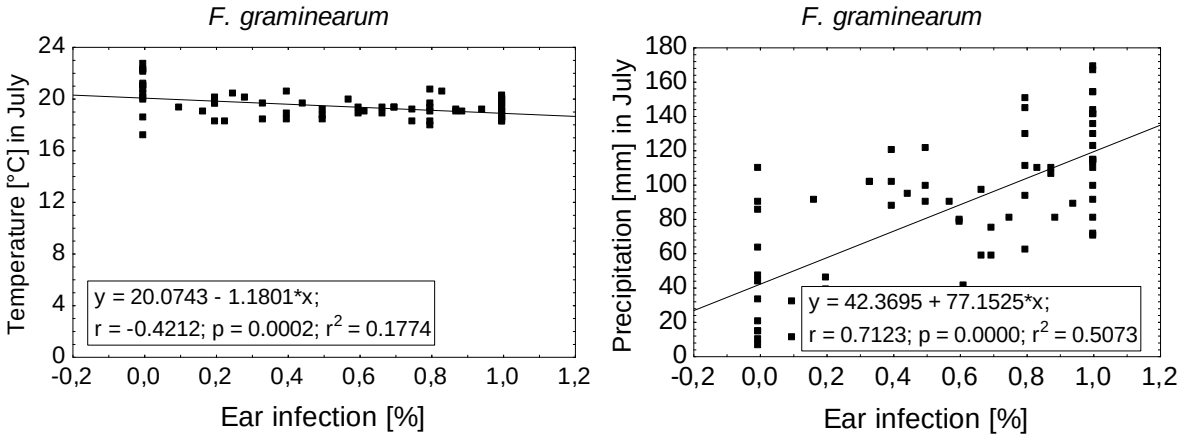


Fig. 3. Coefficients of correlation of temperature and precipitation during June, July, August and September with ear infection by *F. graminearum, F. temperatum and F. verticillioides*. Bars represent coefficients of correlation between percentage of sampled ears per location infected with *F. graminearum, F. temperatum* and *F. verticillioides* and weather data at the sampling sites recorded in 2016, 2017 and 2018 (n=387). Asterisk (*) indicates statistically significant correlation (p≤0.05).



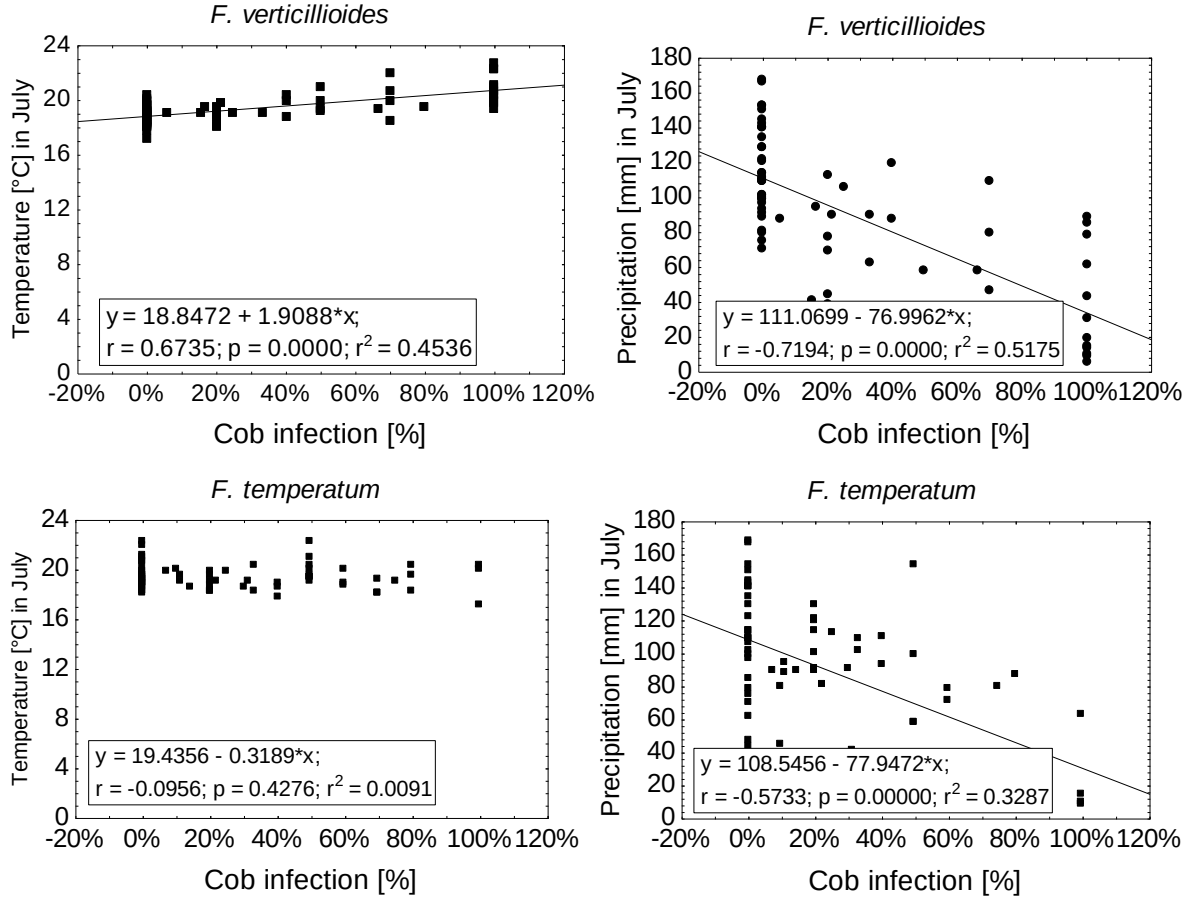


Fig.4. Relationship between ear infection [%] and temperature (left) or precipitation (right) in July of *F. graminearum, F. equiseti, F. culmorum* and *F. temperatum.* Solid line indicates a statistically significant ($p \leq 0.05$) least squares linear relationship.

In contrast to ear infection, the abundance of the most frequent *Fusarium* species on stalks (*F. graminearum*, *F. equiseti*, *F. culmorum* and *F. temperatum*) displayed significant correlation with temperature and precipitation during the month of September (Fig. 5). *F. graminearum* was significantly enhanced at low temperature (r= 0.38) and low precipitation (r= -0.54). However, *F. temperatum* (r= 0.63) and *F. culmorum* (r= 0.46) were favored by high temperature. Temperature and precipitation had no effect on the frequency of stalk infection with *F. equiseti*. The specific relationship between temperature and precipitation in September on one hand and stalk infection with the most frequent *Fusarium* species on the other hand revealed increased frequency of *F. graminearum* at low temperatures (r= -0.38) and dry conditions (r= -0.54). In turn, the percentage of ears infected with *F. temperatum* (r= 0.70) and *F. culmorum* (r= 0.46) increased at higher temperatures. Precipitation in September had no effect on stalk infection neither with *F. temperatum* nor *F. culmorum*. Stalk infection with *F. equiseti* was not influenced by temperature or precipitation during ripening.

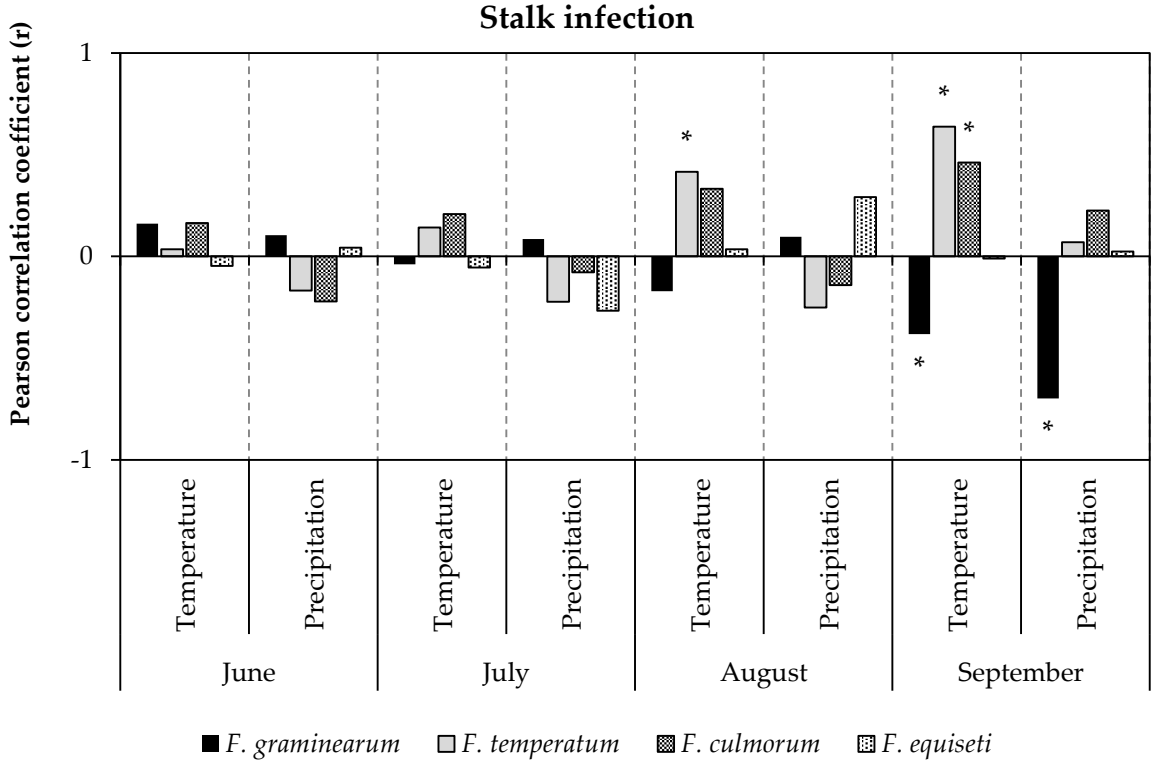
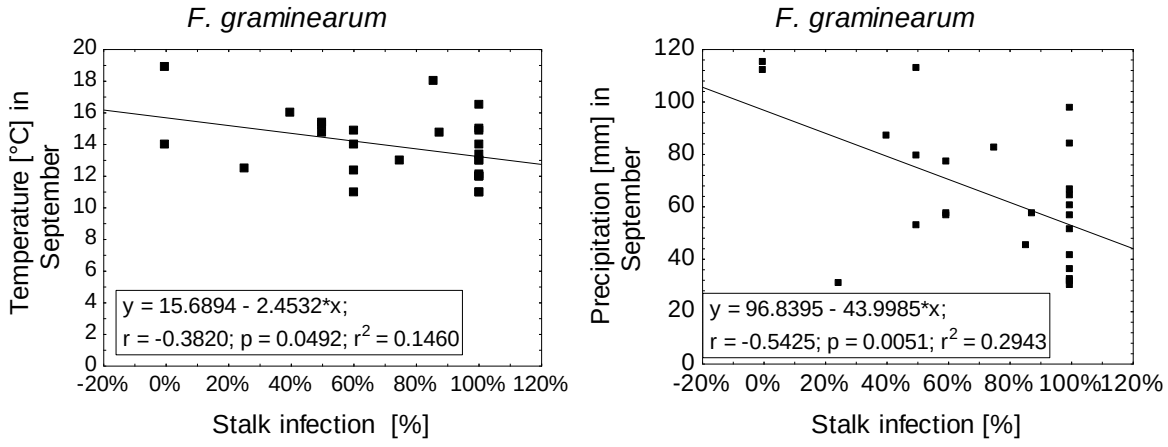


Fig. 5. Coefficients of correlation of temperature and precipitation during June, July, August and September with stalk infection by *F. graminearum, F. temperatum, F. culmorum* and *F. equiseti*. Bars represent coefficients of correlation between percentage of stalks sampled per location infected with *F. graminearum, F. temperatum, F. culmorum* and *F. equiseti* and weather data recorded at the sampling sites in 2017 and 2018 (n=190). Asterisk (*) indicates statistically significant (p≤0.05) correlation.



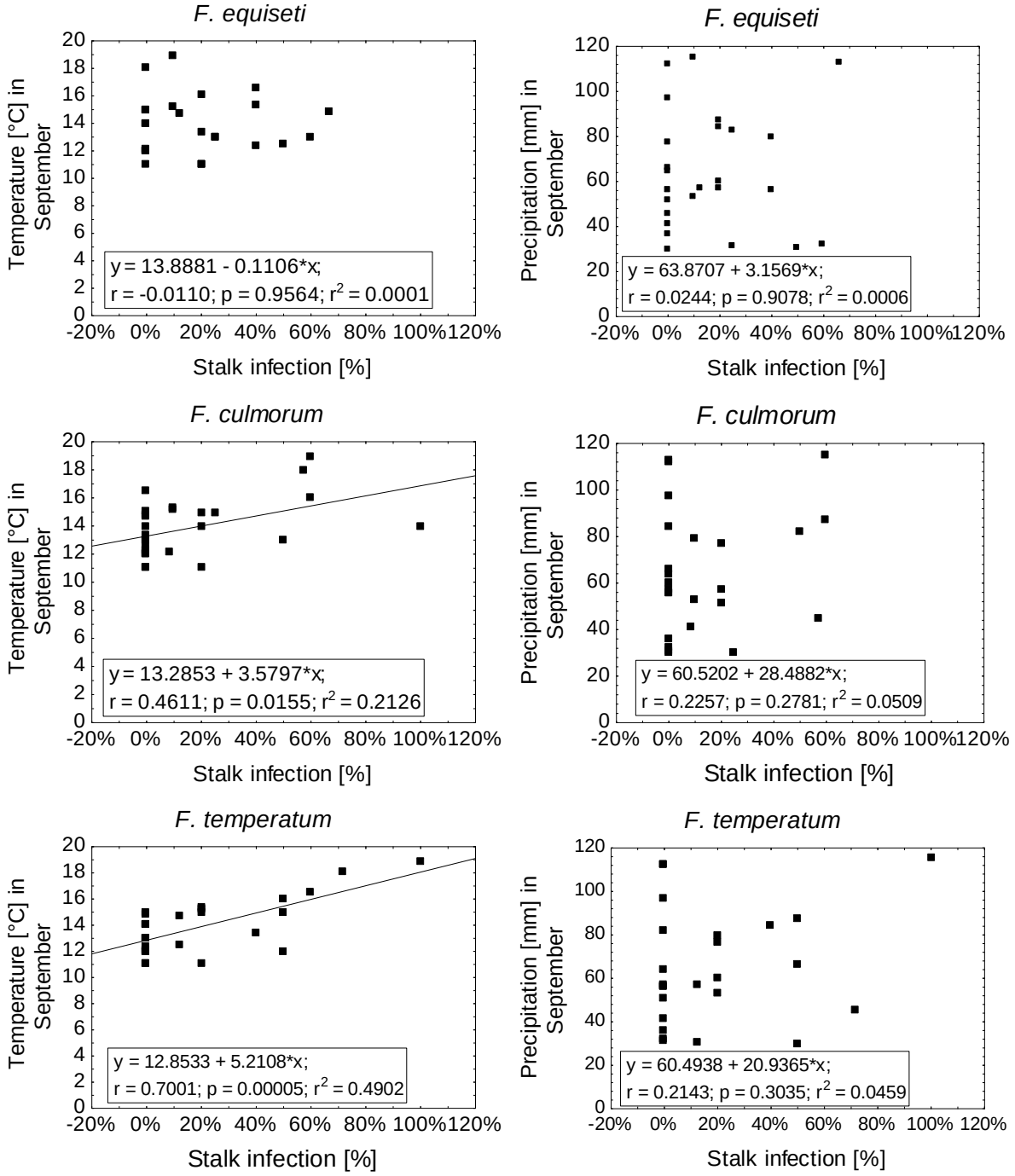


Fig. 6. Relationship between stalk infection [%] with *F. graminearum, F. equiseti, F. culmorum* and *F. temperatum* and temperature (left) or precipitation (right) in September. Solid lines indicate a statistically significant ($p \leq 0.05$) least squares linear relationship.

2.5 Relative impact of main effects.

The effects of tillage, previous crop, year and location on the percentage of ears and stalks infected with the most frequent *Fusarium* species were compared using the variance components of each factor (Fig. 7). The strongest effect was found for the sampling location, which affected the infection of maize ears with *F. graminearum* (34.9% of variance), *F. verticillioides* (26.3%), and *F. temperatum* (28.5%).

Furthermore, the frequency of *F. graminearum* was influenced by the year of sampling (24%), less by the type of tillage (3.9%) and the previous crop (1%). Similarly, the occurrence of *F. verticillioides* and *F. temperatum* were most strongly determined by the location, followed by year, previous crop and tillage. Tillage and previous crop had minor effects on ear colonization with any of the tested *Fusarium* species.

Stalk infection with *F. graminearum, F. equiseti, F. culmorum* and *F. temperatum* was mainly affected by the year and location. Stalk infection with *F. graminearum* (26.3%) strongly differed between the years, while stalk infection with *F. equiseti* (27.1%), *F. culmorum* (26.4%) and *F. temperatum* (11.8%) was mainly affected by the location. The previous crop had almost no effect on *Fusarium* species composition and tillage only slightly influenced the colonization with *F. graminearum* (6.2%), *F. equiseti* (8.9%) and *F. culmorum* (4.4%).

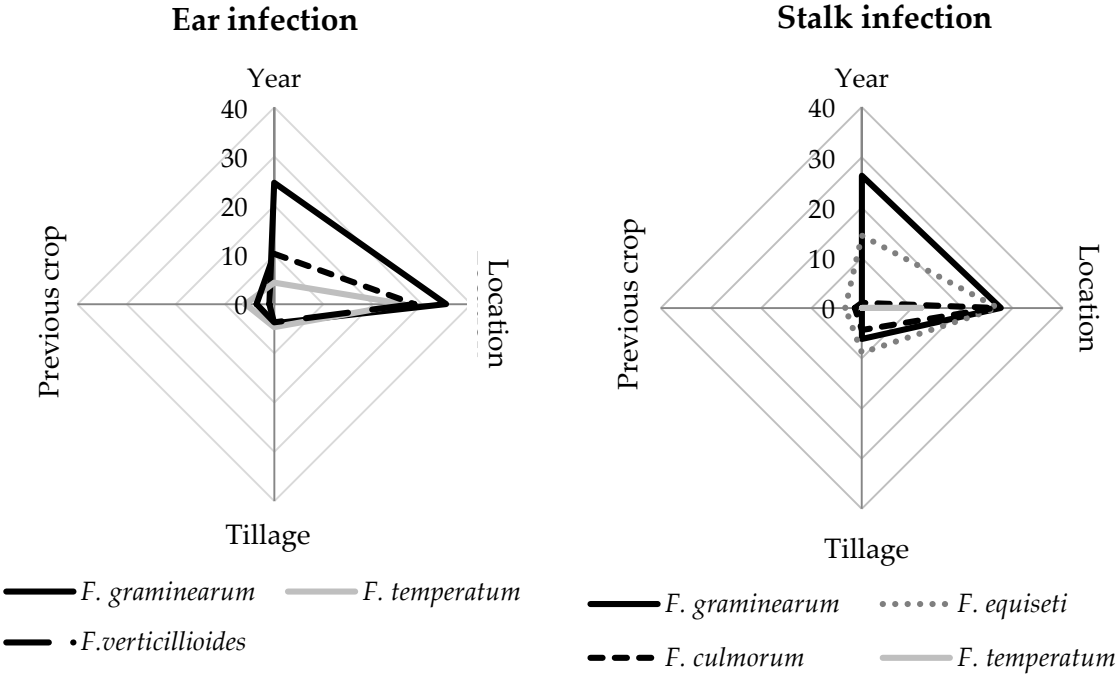


Fig.7. Relative impact of main effects (year, location, tillage, previous crop) expressed as percentage of variance of the total variance estimated with the restricted maximum likelihood model. Ear infection (left) calculated for *F. graminearum*, *F. temperatum* and *F. verticillioides*. Stalk infection (right) calculated for *F. graminearum*, *F. equiseti*, *F. culmorum* and *F. temperatum.*

3. **Discussion**

Within the three years of investigations of maize ears and stalks, twelve *Fusarium* species were isolated and identified. All the species detected are known to frequently occur on maize ears and stalks in Central Europe [34]. High year to year variability was observed for the frequency of *Fusarium* spp., which indicated a major impact of temperature and precipitation during the vegetation period. The growing seasons in 2016 and 2017 were characterized by moderate temperatures (18.8°C) and high precipitation in July (110 mm), while in 2018 high mean temperatures (20.6°C) and dry conditions (40 mm in July) prevailed. This might explain the high frequency of *F. graminearum* and *F. culmorum* in 2016 and 2017 when more than 70% of all tested ears and 80% of all tested stalks were colonized with these

two species. In contrast, *F. verticillioides* was the prevailing species in 2018, colonizing almost 40% of all ears analyzed.

Numerous studies confirm that moderate temperature and high level of moisture increases infection rates of *F. graminearum*, *F. culmorum* and *F. avenaceum* (*Gibberella* ear rot) [22,25,35], while Pink ear rot pathogens such as *F. verticillioides*, *F. proliferatum* and *F. temperatum* have often been reported from southern European regions where dry and warm conditions prevail [36]. These patterns correspond to different temperature optima of the species. The optimal growth rate for *F. graminearum* was reported 24-26°C [37] and higher moisture level, whereas optimal conditions of *F. verticillioides* are 30°C and 0.97 water activity [2,38]. The primary infection pathway for ear infection is via the silk channel (during the first 6 to 10 days after silk emergence) or insect injury of kernels (during grain filling). Under mid European conditions, silk emergence takes place between beginning of July and mid-July. At this time, weather conditions as well as insect populations strongly affect *Fusarium* spp. infection [1,16]. *Gibberella* ear rot pathogens are favored by high levels of moisture during silking, followed by moderate temperatures and high precipitation during cob maturation [25]. Shelby et al. [39] demonstrated that fumonisin levels and the occurrence of *Fusarium* ear rot pathogens were inversely correlated with rainfall in June and July. In particular, drought stress is associated with an elevated infection with *F. verticillioides* [40]. The present study confirmed that a dry period with high temperatures before and during grain filling favors ear infection with *F. verticillioides* and *F. temperatum,* while the frequency of *F. graminearum* was higher at lower temperatures and high precipitation. While temperature, precipitation and relative humidity during flowering were incorporated into forecasting models for *Fusarium* head blight on wheat [41], available risk assessment models for *Fusarium* ear rot disease were not sufficiently detailed to maize and cannot be extrapolated from the existing risk assessment models for *Fusarium* head blight [42]. Only Stewart et al. [74] were able to develop a mechanistic model relating the growth rates of *F. graminearum* and *F. verticillioides* to temperature, relative humidity and precipitation which effectively predicted ear rot severity after artificial inoculation.

In contrast to ear infection, stalk infection was mainly influenced by temperature during ripening in August and September. *Fusarium* species can enter the stalk during the whole vegetation period by systemic spread after colonization of the root [1,43], through young leaf sheaths, by seed transmission [44] and via wounds caused by hail or feeding of insects [3]. Consequently, stalk rot infection is not restricted to a specific time point and fungal infection does not correlate with seasonal weather conditions. However, temperature substantially affected the extent of invasion of *Fusarium* pathogens during ripening [30,45]. Murillo-Williams and Munkvold [45] suggested that higher temperatures in particular lead to faster maturity of the plants promoting systemic infections of species which are adapted to warmer temperature such as *F. verticillioides*. Stalk rot usually occurs at physiological maturity, in August and September, when storage products in stalks are depleted and most carbohydrates are translocated to the cob [46]. Accordingly, Dodd [21] reported that at maturity stages the root and lower stalk tissues lose their metabolic activity and thus their defense potential against stalk infection. In addition, further stresses such as drought, high plant density, leaf diseases and corn borer attacks may also favor stalk rot due to decreasing photosynthesis rate [21]. *F. temperatum*, a species recently separated from *F. subglutinans* based on its phylogeny and mycotoxin production, colonized up to 20% of all analyzed ear and stalk samples. The frequency of *F. subglutinans* within the three years of investigation was low (2%); *F. subglutinans* therefore played only a minor role in ear and stalk infection. A higher incidence of *F. temperatum* in comparison to *F. subglutinans* was also reported from maize in Belgium [47], Poland [48], France [49] and Italy [50] as well as North America [51], Korea, [52], China [53], Mexico [54] and Argentina [55]. The data of the present study demonstrate that kernel colonization with *F. temperatum* was significantly favored by low precipitation during

flowering in 2018. Moretti et al. (2018) [56] suggested that isolates belonging to group 1 (*F. temperatum*) are more frequent in cooler regions like Germany, Poland and Austria while group 2 (*F. subglutinans*) prevails in warmer and dryer regions such as Slovakia, Italy and Serbia. Czembor et al. [48] reported a similar trend of *F. temperatum* occurring more often in environments with mean temperatures of 18°C or lower in June, like in Germany.

In the present study, only low or no impact of crop rotation on ear and stalk infection with *Fusarium* spp. was observed. These findings correspond with the results from investigations by Dill-Macky and Jones [24] and Schaafsma et al. [57] indicating that similarly high disease levels caused by *F. graminearum* are found in maize grown after maize and wheat, compared to sugar beet and other pre-crops like rape seed, potato or strawberries. A similar tendency was observed by Schlüter and Kropf [29] and Gödecke [58], who reported a high disease incidence by *F. culmorum* and *F. graminearum* on wheat after non-host crops like oilseed rape and sugar beet. Mansfield et al. [9] also reported no effect of crop rotation with broadleaf crops on DON contamination of maize stalks. The most important source of inoculum for *Fusarium* spp. are plant debris, especially maize stalks. However, these fungi are also pathogenic in cereals such as wheat, barley, oats and rye as well as sugar beet. *Fusarium* spp. can survive as mycelium and other structures on residues of these crops as well as on senescent tissue of other crops or weed species, which may later serve as primary inoculum for infection [59]. Resting structures such as chlamydospores and thick-walled hyphae can survive up to ten years breaks between host crops on plant residues buried at 30 cm depth or left on the soil surface [25,60]. Long-term survival studies by Cotton and Munkvold [26] indicate an equal survival of *Fusarium* species in buried residues and surface residues after 343 days and suggest that surface residues may act as a reservoir of recolonization and spore production for airborne inoculum and spread into the next vegetation period.

Therefore, management of surface residues by tillage and deep burial are suggested as an important strategy to control ear and stalk rot diseases [61]. The results of this study indicate that, the incidence of local *Fusarium* species on ears and stalks is highly affected by conventional ploughing compared to chisel ploughing or no tillage. In particular, the frequency of *F. graminearum*, *F. temperatum* and *F. culmorum* was reduced after conventional ploughing, however, *F. verticillioides* and *F. equiseti* were enhanced by ploughing. Our study confirms the results reported by Dill-Macky and Jones [24] and Steinkellner and Langer [62], which demonstrated that most *Fusarium* species were reduced after moldboard ploughing as compared to reduced tillage. Covering crop residues with soil accelerates their decomposition by enhancing microbial activity and this reduces inoculum density [63,64]. However, investigations by Byrnes and Carroll [73] confirmed higher severity of *F. equiseti* infection after conventional tillage, whereas the concentration of DON produced by *F. graminearum* and the population density of *F. subglutinans* were reduced by conventional tillage [30,11,13]. Steinkellner and Langer [62] emphasized that ploughing compared to chisel plough and rotary tiller treatments reduced the number of colony forming units (CFU) per g of soil and the frequency of *Fusarium* species in upper soil layers. However, *Fusarium* species composition differed between different soil layers due to different survival structures of the species [31]. Especially *F. verticillioides* survived best in maize stalks at 30 cm depth due to higher moisture content and poor decomposition of plant tissue [60]. According to this study and previous research, weather conditions had the largest influence on the local *Fusarium* species composition and disease incidence in maize, however, prevention and management practices including crop rotation and tillage types may also affect ear and stalk rot diseases and mycotoxin accumulation [2,9,12,65,75].

In the three years of investigation (2016-2018), *F. graminearum*, *F. verticillioides* and *F. temperatum* were the most frequent *Fusarium* species on maize in Germany. A high year-to-year variability was observed in the shift of species composition towards a high occurrence of

F. verticillioides. Increasing temperatures and dry periods in summer can affect the *Fusarium* species complex and increase the risk of contamination with fumonisin-producing species such as *F. verticillioides* and *F. temperatum* [48]. In addition, feeding of the European corn borer (*Ostrinia nubilalis*) and the Western corn rootworm (*Diabrotica virgifera*) in Germany will likely further enhance disease incidence and mycotoxin contamination of ears and stalks as well as root rots in maize [11,16,66]. The current expansion of maize acreage and shorter crop rotations with other small grain cereals due to renewable energy policy will further increase the risk of *Fusarium* infection and mycotoxin contamination.

The current results emphasize the importance of further studies of the impact of changing climatic conditions and its interplay with cultural practices on the development of *Fusarium* population and the mycotoxin contamination of maize crops.

4. Materials and Methods

4.1 Sampling and isolation.

Naturally *Fusarium*-infected maize ear and stalk samples were collected from silage and grain maize fields in Germany in 2016 (94 ears from 18 locations), 2017 (180 ears from 42 locations and 110 stalks from 21 locations) and 2018 (113 ears from 18 locations and 80 stalks from 14 locations). For each sample site agronomic data like soil tillage and previous crop as well as meteorological data such as precipitation, air temperature and humidity during the vegetation period were recorded. Five to nine *Fusarium*-infected ears or stalks per location were placed in paper bags and sent to the Plant Pathology and Plant Protection Division in Göttingen, Germany for further analysis. Disease severity on kernels and rachis was scored according to EPPO Guidelines [67].

Thirty randomly chosen kernels of each ears were surface sterilized for 10 min with 0.1% silver nitrate and incubated on moist sterile filter paper for two days at room temperature. Afterwards, kernels with outgrowing *Fusarium* mycelium were placed on potato dextrose agar (PDA). The rachis was cut in nine slices, three from the base, three from the middle part and three from the tip of the ears. The slices were surface sterilized as described above and placed directly on PDA plates. The stalk samples were cut in nine slices, three from the lower nodium, three from the internodium and three from the upper nodium. The samples were surface sterilized and placed on PDA plates as the rachis slices. After two days, presumed *Fusarium* mycelium outgrown from the samples was transferred to synthetic low nutrition agar (SNA) to produce single spore cultures. The isolates were stored as single spore cultures on synthetic SNA plates at 4°C.

The ear and stalk infection were calculated by the following equation:

$$\text{Ear infection [\%]} = \frac{\text{Number of } \textit{Fusarium} \text{ infected kernels per cob}}{\text{Number of kernels (n=30)}} \div \text{Cobs per location}$$

$$\text{Stalk infection [\%]} = \frac{\text{Number of } \textit{Fusarium} \text{ infected slices per stalk}}{\text{Number of slices (n=9)}} \div \text{Stalks per location}$$

4.2 Species identification.

In-vitro cultures of *Fusarium* were identified macroscopically by colony characters on PDA and microscopically on SNA [68]. Total DNA was extracted from lyophilized mycelium from single spore cultures by using a CTAB-based protocol as described previously [69]. Standards of genomic DNA were obtained from *F. temperatum* MUCL52463 and *F. subglutinans* CBS215.76 [44]. The quality and quantity of extracted DNA were assessed after electrophoretic separation in agarose gels (0.8% (w/v) stained with ethidium bromide. The electrophoresis was carried out for 60 min at 4.6V/cm.

Partial translation elongation factor 1-alfa (*tef1α*) nucleotide sequence was used to differentiate between *F. temperatum* and *F. subglutinans*. Amplification was performed in a peqSTAR96 thermocycler (PEQLAB, Erlangen, Germany) using 1:100 dilutions of DNA extracts in a total reaction volume of 25µl.

The *tef1α* gene was amplified using primers EF1 (ATGGGTAAGGARGACAAGAC) and EF2 (GGARGTACCAGTSATCATGTT) [70] in a PCR reaction consisting of *Taq* reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 at 25°C), 100 µM of each deoxyribonucleoside triphosphate, 0.3 µM of each primer, 0.62 U HotStart-polymerase (NEB) and 1 µL template DNA solution. The final $MgCl_2$ concentration was adjusted to 2 mM. The PCR cycling conditions for the amplification of *tef1α* included an initial denaturation for 30s at 95 °C; 30 cycles consisting of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 68 °C; and final extension for 5 min at 68 °C. *Fusarium* species were identified by multiple alignment of each sequence with the sequences of standard strains and other reference sequences using ClustalW [71] in MEGA version 7.0.2 [72].

4.3 Meteorological and agronomical data.

The meteorological data were obtained from the closest weather stations (<10 km) to the sample location. In Bavaria, meteorological data were received from AgrarMeterologie of the Bavarian state research center for agriculture (https://www.wetter-by.de). The air temperature and the relative air humidity were recorded as daily means and precipitation as monthly sum from May to September. The agronomical field data of tillage and previous crop were obtained from breeding companies and farmers. The previous crop was assigned to four categories, in maize (silage maize and grain maize), wheat (winter wheat), sugar beet and other crops (potato, cabbage, strawberry, rye, barley). Soil tillage was differentiated into two groups; ploughing (moldboard ploughing) and no ploughing or reduced tillage including chisel ploughing and rotary harrow.

4.4 Statistical analyses.

Statistical analyses were performed using Statistica version 13.3 (TIBCO® Data Science, California, USA). Non-parametric data of average infection, tillage treatments and previous crop were statistically analyzed using Mann-Whitney-U-Test and Kruskal-Wallis-ANOVA. Tests were performed at a probability level of 95%. Pearson´s correlation coefficients were used to examine the relationship between temperature and precipitation in June, July, August and September and infection with predominant *Fusarium* species occurring on ear and stalk samples. In addition, a multiple regression was performed to determine the relationship of ear and stalk infection with temperature and/ precipitation in July for each sample location. The impact of weather conditions, soil tillage or pre-crops on the occurrence of *Fusarium* species was analyzed by variance components derived from the overall variance estimated with the restricted maximum likelihood model.

Impact of environmental conditions and agronomic practices on the prevalence of *Fusarium* species associated with ear- and stalk rot in maize

Supplementary Materials:

Table S1: Mean monthly air temperature [°C] (AT), mean relative humidity [%] (RH) and cumulative monthly precipitation [mm] (PP) in June, July, August and September in the year 2016, 2017 and 2018 within the sampling locations.

2016	**June**			**July**			**August**			**September**		
Location	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**
Altötting	16.8	86	132	19.1	74.3	153.6	17.6	85.8	65.1	15.9	90.1	149.6
Braunau am Inn	18	77	163	19.9	75.1	140	18.9	75.3	92.5	17.5	77.3	103.6
Bad Lauchstädt				17.8	71.2	52.2						
Einbeck				18.57	75.71	65.2						
Frankendorf				18.9	77	70.6						
Fraunberg-Grucking				18.9	77	71						
Grucking	16.5	79	104	18.8	77.2	70.6	17.7	75.6	61.1	15.5	79.8	58.8
München/Bockhorn	16.8	78	98.4	19.1	74.8	88.2	17.8	76.8	101	15.7	80.9	70.8
Löningen				18.5	76	33						
Osterhofen	17.5	80	123	19.0	77.35	80.4	18.4	78.3	47	16.9	80.3	82.5
Ostbevern				18.8	77	78						
Reith				19.5	76.4	129.3						
Pocking 1	17.9	85	194	19.7	84.5	168	18.2	86.9	76	16.1	89.1	121.7
Pocking 2	17.9	85	194	17.9	76.5	129.3	18.2	86.9	76	16.1	89.1	121.7
Rustenhard				16.4	78.7	87.2						
Tönisvorst				19.5		35.9						
Thenn	16.5	79	104	19.1	74.8	88.2	17.7	75.6	61.1	15.5	79.8	58.8
Unterneukirchen				19.4	74	153						
Wadersloh	17.4	78	130	19.31	71.7	43	18.6	70.4	55	18.9	64.4	16.2
Weihmörting	17.9	85	194	19.6	76	129	18.2	86.9	76	16.1	89.1	121.7
Wesel				19.2		62.8						
MEAN	17.3	80.8	138.1	18.9	76.1	91.8	18.1	79.1	70.5	16.5	81.2	87.1

2017	**June**			**July**			**August**			**September**		
Locations	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**
Altötting	20	66	59	19.2	82.2	166.4	20	74	84	13	83	31
Bad Lauchstädt	18	68		19	73		19	72		14	80	
Bernburg	19.1	63.4	67.5	19.2	70.5	89.7	19.2	70.2	99.8	14.7	73.7	39.7
Borken	18	69.8	46.8	18	78.1	121.2	22.9	81.4	55.7	18.9	82.6	115.1
Cloppenburg	17.5	70.6	93.2	17.5	77.0	150.4	17.4	75.9	58.8	13.7	82.3	85
Dinkelsbühl	18	67.3	119.9	18	73.4	134.5	19	75.1	77.4	12	81.7	71
Einbeck				18.2	80.2	174						
Geldern	18.9	69.8	68.5	18.9	75.8	109.7	18.1	79.7	67	14.3	85.3	85.4
Giebelstadt	18.5	67.2	70.5	18.9	72.2	105.9	18.7	74.0	71.5	13.0	78.1	74.6
Gondelsheim				20.5	74.1	109.3						
Greven				18.3	79							
Grucking	19	68		18.7	74.5	140	19	76		12	81	
Haselünne	18	69		17.8	83.4	89.2	17	77		13	84	
Heilbronn	20	64		20.1	73.7	114.2	19	76		13	82	
Herzlake				17.8	83.49	89.2	17	77		13	84	
Ichenhausen				18.2	82.4	113.3						
Kleinwanzleben				19.1		98.6						
Löningen	17.7	73.1	73.1	18	78.3	100.6	17.4	78.9	65.6	13.4	88.4	83.9
Moosham	19	64.4	56.1	19	70.9	80.4	19.2	74.6	103.2	12.1	84.1	41.1

Möttingen	18.9	66.2	62.8	19.1	71.1	99.1	19.0	75.5	116	12.8	78.9	72.3
Münzesheim	23	54		20.5	74.6	109.3	22	66		16	73	
Neumarkt (St.Veit)		70.2	65.7	18.8	72.3	96.6		78.3	91.5		84.1	56.9
Neupotz				20.7	75.8	61.8						
Neuhaus am Inn		64	47.6	19	72.4	93.3	19.2	75.1	112.9	12.3	85.2	55.9
Ostbevern				18.8		119.7	18	77		14	84	
Osterhofen		67		18.9	76	93.5	19	75		12	82	
Plessa		64.9	64.7	19.5	70.9	90.7		70.9	65.9		78	31.1
Pocking	21	58		19.5	81.8	94.3	21	67	126	13	77	32
Pritzwalk		77.3	113.5	17.1	79.8	84.6		77.6	29.9		83.4	54.8
Reith				19.5	72.8	101.2			81			60
Rheine	18.3	66.3	71	18.2	74.7	142.9	17.7	75.1	71.5	13.8	81.9	93.3
Saerbeck												
Sankt Peter am Hart				19.5	82.8	121.6						
Soest-Epsingen				18.5	74.7	109						
Tönisvorst				18.1								
Ulm			75	18.3	82.4	113.4			92			56
Wadersloh-Liesborn	18.6	66.8	55.7	18.6	74.4	144.4	18.0	76.9	94.2	13.9	81.1	108
Welbhausen			69	18.9	75.4	112.2			53.1			60.4
Wesel				18	82.4	90.1						
MEAN	**19.0**	**66.8**	**71.1**	**18.8**	**76.5**	**110.4**	**19.0**	**75.1**	**80.9**	**13.6**	**81.6**	**65.4**

2018	**June**			**July**			**August**			**September**		
Location	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**	**AT [°C]**	**RH [%]**	**PP [mm]**
Altötting	17.9		118.9	19.9	71	68.5	19.2		146.7	14.9		76.7
Braunau	18.3		63.2	21.1	-	79	20.4		86.4	15.2		55.7
Bernburg												
Einbeck				22.6	63.4	56.2						
Gondelsheim	19.4	75.1	60.8	22	64.6	46.9	21.7	66.8	38.3	16.6	71.6	23.9
Grucking	17.7	78.6	123	19.3	76.65	57.9	19.8	78.3	66.7	14.7	85.2	56.9
Hohenheim				21	-	-						
Kleinwanzleben				22.2	-	29.4						
Künzing	18.2	75.2	68.3	19.3	71.0	30.9	20.4	70.2	38.7	14.8	81.0	64.2
Löningen				20.3	-	14.4						
Mariaposching	18.9	71.5	90.5	20.4	67.7	24.3	21.3	67.1	46	15.5	80.5	71.7
Mintraching	18.9	69.2	80.2	20.5	66.9	38.6	21.2	67.2	43.4	15.2	79.8	52.7
Neuhaus am Inn	18.9	77.4	85.2	20.0	77.1	35.3	21.0	77.7	87.4	15.3	85.7	79.2
Ostbevern				21.5	-	8.9						
Osterhofen	19.6	66.9	37.7	19.2	76	74.8	19.1	77.3	79.7	12.5	86.2	30.4
Pocking	18.9	77.4	85.2	20	77.1	35.3	21	77.4	87.4	15.3	85.7	79.2
Prenzlau	18.0		43.1	20.4		61.9	20.8		17.3	16		11.3
Reith	16.5	85.1	124.2	18.9	79.2	69.8	19.6	80.0	111.8	14.7	88.5	112.4
St. Andreas-berg				-	-	-						
Tönisvorst				22.2	-	5.8						
Triftern	18.3	76.2	63.2	19.5	75.8	79	20.4	77.6	86.4	15.2	86.8	55.7
Ulm	17.4	83.1	47.1	19	79.3	40.9	19.4	79.2	46	14.9	83.4	29.8
Wesel												
MEAN	**18.3**	**75.8**	**81.7**	**20.6**	**72.1**	**40.4**	**20.3**	**73.7**	**71.9**	**14.9**	**82.4**	**59.4**

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Chapter III: Occurrence, pathogenicity and mycotoxin production of *Fusarium temperatum* related to other *Fusarium* species on maize in Germany

pathogens

MDPI

Article

Occurrence, pathogenicity and mycotoxin production of *Fusarium temperatum* in relation to other *Fusarium* species on maize in Germany

Annette Pfordt [1,†], Simon Schiwek [2,†], Anna Rathgeb [2], Charlotte Rodemann [1], Nele Bollmann [1], Matthias Buchholz [1], Petr Karlovsky [2,*] and Andreas von Tiedemann [1,*]

[1] Plant Pathology and Crop Protection, University of Goettingen, Goettingen 37077, Germany; annette.pfordt@uni-goettingen.de

[2] Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Goettingen 37077, Germany; simon.schiwek@uni-goettingen.de

[†] these authors contribute equally to this paper

[*] Correspondence: annette.pfordt@uni-goettingen.de (A.P.); simon.schiwek@uni-goettingen.de (S.S.)

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Abstract: *Fusarium subglutinans* is a plant pathogenic fungus infecting cereal grain crops. In 2011, the species was divided in *Fusarium temperatum sp. nov.* and *F. subglutinans sensu stricto*. In order to determine the occurrence and significance of *F. temperatum* and *F. subglutinans* on maize, a monitoring of maize ears and stalks was carried out in Germany in 2017 and 2018. Species identification was conducted by analysis of the translation elongation factor *1α (TEF-1α)* gene. Seventy-nine isolates of *F. temperatum* and seven isolates of *F. subglutinans* were obtained during two years of monitoring from 60 sampling sites in nine federal states of Germany. Inoculation of maize ears revealed a superior aggressiveness for *F. temperatum*, followed by *F. graminearum, F. verticillioides* and *F. subglutinans*. On maize stalks, *F. graminearum* was the most aggressive species while *F. temperatum* and *F. subglutinans* caused only small lesions. The optimal temperature for infection of maize ears with *F. temperatum* was 24 °C and 21 °C for *F. subglutinans*. All strains of *F. temperatum* and *F. subglutinans* were pathogenic on wheat and capable to cause moderate to severe head blight symptoms. The assessment of mycotoxin production of 60 strains of *F. temperatum* cultivated on rice revealed that all strains produced beauvericin, moniliformin, fusaric acid, and fusaproliferin. The results demonstrate a higher prevalence and aggressiveness of *F. temperatum* compared to *F. subglutinans* in German maize cultivation areas.

Keywords: *Fusarium temperatum*; *Fusarium subglutinans*; pathogenicity; maize ear rot; *Fusarium* head blight; beauvericin; translation elongation factor *1α*

Occurrence, pathogenicity and mycotoxin production of *Fusarium temperatum* in relation to other Fusarium species on maize in Germany

1. Introduction

Fusarium ear and stalk rot are ubiquitous diseases of maize with high economic impact in agriculture [1]. Several *Fusarium* species infecting maize are known to produce toxic secondary metabolites, called mycotoxins, which impair grain quality and threaten the safety of animal feed and food products [2,3]. Among the most important *Fusarium* species inciting pre- and post-harvest ear rot of maize are *F. graminearum* and *F. verticillioides* [4–6], but also other species such as *F. poae* [7,8], *F. proliferatum* [1], *F. subglutinans* [9] and *F. temperatum* [10] are frequently reported. Ear infection is typically characterized by the growth of white or reddish mycelium with rot induced on the cob and on stored grains.

F. subglutinans, which is a member of the *Fusarium fujikuroi* species complex (FFSC), predominantly occurs in temperate climate regions. *F. subglutinans* was elevated to the species level in 1983 by Nelson et al. [11], after separation from *F. verticillioides*. Later on, in 2002 Steenkamp et al. [12] reported two cryptic species within a set of isolates of *F. subglutinans*, obtained from several locations, based on phylogenetic concordance analyses of six nuclear regions, and suggested that both subspecies justify separation into two individual taxa. Phylogenetic separation was further supported by mycotoxin analyses revealing the production of beauvericin (BEA), which was exclusively produced by European isolates belonging to the so-called group 1 of *F. subglutinans* [13]. Furthermore, separation of strains was based on differences in climatic requirements, since isolates of *F. subglutinans* group 1 were frequently collected in cooler regions like Germany, Poland and Austria while *F. subglutinans* group 2 prevailed in warmer and dryer regions such as Slovakia, Italy and Serbia [13]. Several other studies also reported the detection of mycotoxins, such as beauvericin (BEA) [14,15], fusaproliferin (FUSA) [16], moniliformin (MON) [17] and rarely fumonisin B1 [18], produced by the subgroup of *F. subglutinans*, presumably *F. subglutinans* group 1. In 2011, this cryptic subgroup was classified as a novel species, establishing *F. temperatum* as *species nova*, corresponding to the formerly known group 1 of *F. subglutinans* [10]. The mycotoxin profile of *F. temperatum* is not yet fully clarified. While the production of beauvericin was consistently found in all strains of *F. temperatum*, moniliformin and fumonisin was produced only by a single isolate of *F. temperatum*as reported by Scauflaire et al. [19]. Similarly, production of beauvericin, moniliformin, fusaproliferin and fumonisins was reported for *F. temperatum* strains from Argentina [20]. Further studies report on *F. temperatum* infection on maize causing seedling blight and root rot [21] and ear rot, as well as head blight on wheat [22].

The aim of the study was to determine the occurrence of *F. temperatum* and *F. subglutinans* on maize ears and stalks in Germany and to assess their pathogenicity relative to each other and to other common *Fusarium* ear rot and head blight pathogens. In addition, the mycotoxin profiles of *F. temperatum* and *F. subglutinans* were compared.

2. Results

2.1. Occurrence of Fusarium species on Cobs and Stalks

In 2017 and 2018, ninety isolates of *F. temperatum* and seven isolates of *F. subglutinans* (Table S1) were obtained from diseased cobs collected across eight federal states of Germany (Figure S1). *F. temperatum* was isolated from 17 % of all analyzed samples, making it the third most often occurring *Fusarium* species following *F. graminearum* (57 %) and *F. verticillioides* (22 %) in cobs (n=293) and the fourth most often isolated species on stalks (n=190) (Table 1). The frequency of *F. temperatum* isolated from ears ranged from 15 % in 2017 to 21 % in 2018. *F. subglutinans* was only detected in 2 % of all analyzed cobs, at two locations, and in 3 % of the stalk samples, at one location.

Table 1. Relative occurrence of *F. temperatum, F. subglutinans, F. graminearum* and *F. verticillioides* on naturally infected cobs and stalk samples in the field in 2017 and 2018.

Fusarium species	Cobs					Stalks				
	Infected [%]			Sample sites		Infected [%]			Sample sites	
	2017 n=180	2018 n=113	**Mean[1]**	2017 n=42	2018 n=18	2017 n=110	2018 n=80	**Mean[1]**	2017 n=21	2018 n=14
F. temperatum	15	21	**17**	21	15	15	20	**17**	7	13
F. subglutinans	2	2	**2**	3	3	1	3	**2**	1	2
F. graminearum	71	30	**57**	41	15	81	43	**65**	20	11
F. verticillioides	13	39	**22**	11	16	22	16	**19**	14	11

1 Mean of the percentage of infections in the years 2017 and 2018.

2.2. Pathogenicity on Maize cobs under Field Conditions

Weather conditions in both years of investigation were conducive for the development of *Fusarium* ear rot infection at the five field sites. Disease severity was strongly affected by pathogen species, year, inoculation method, location and maize variety as well as the interactions of all factors. According to the mean of squares, pathogen species effects had the highest impact on disease severity (DS), followed by variety and the interaction of both (Table S2). On the average of field experiments, *F. temperatum* was the most aggressive species (31 % DS), followed by *F. graminearum* (20 % DS), *F. subglutinans* (15 % DS) and *F. verticillioides* (11 % DS) (Table 2). On the average of the four *Fusarium* species tested, disease severity was favored by silk channel inoculation (26 %) compared to needle pin inoculation (13 %).

Table 2. Disease severity (%) on maize cobs after inoculation with *F. graminearum, F. temperatum,* and *F. verticillioides* in 2018 and 2019 at five locations in Germany and France as well as with *F. subglutinans* in 2019 in Goettingen using needle or silk channel inoculation.

	Disease severity [%]			
	F. graminearum	***F. temperatum***	***F. verticillioides***	***F. subglutinans***
2018	28 ±[1] 30 a[2]B[3]	40 ± 34 aC	12 ± 15 aA	-
2019	15 ± 22 bC	25 ± 26 bD	11 ± 20 bA	15 ± 19 B
Needle pin	19 ± 26 aB	19 ± 14 aB	5 ± 8 aA	10 ± 7 aA
Silk channel	22 ± 26 aA	44 ± 37 bB	18 ± 22 bA	20 ± 25 bA
Bernburg	25 ± 28 cB	30 ± 31 bC	9 ± 19 aA	-
Kuenzing	21 ± 25 bcB	37 ± 30 bC	13 ± 19 aA	-
Liesborn	18 ± 22 bB	37 ± 35 bC	11 ± 19 aA	-
Rustenhart	27 ± 29 cB	32 ± 27 bC	12 ± 14 aA	-
Goettingen	14 ± 24 aA	21 ± 27 a B	-[4]	15 ± 19 A
MEAN	20 ± 26 B	31 ± 31 C	11 ± 18 A	15 ± 19 A

[1]Plus-minus sign (±) represents variation according to standard deviation. [2]Small letters indicate significant differences between treatments ($p \leq 0.05$). [3]Capital letters indicate differences ($p \leq 0.05$) between *Fusarium* species. [4]No data has been collected.

2.3. Pathogenicity on maize stalks under greenhouse conditions

Under greenhouse conditions, *F. graminearum, F. crookwellense (syn. F. cerealis), F. culmorum, F. temperatum, and F. subglutinans* were able to induce necrotic lesions in the stem tissue and on the surface of the stalks after needle pin inoculation. *F. graminearum* was the most aggressive species on

stalks, according to the internal and external lesion length, followed by *F. crookwellense* and *F. culmorum*. Severity of infection, recorded as lesion length, was significantly higher with *F. graminearum* than with *F. temperatum* and *F. subglutinans* (Table 3).

Table 3. Lesion length inside the stalk and on the stalk surface of maize at BBCH 80, 35 days post inoculation with *F. graminearum, F. crookwellense, F. culmorum, F. temperatum, F. subglutinans* and control. Different letters indicate significant differences between treatments (p≤0.05).

Inoculated species	Lesion length inside the stalk [mm]	Lesion length on the stalk surface [mm]
F. graminearum	78.3 ±[1] 4.1 c	57.7 ± 2.5 c
F. crookwellense	75.5 ± 3.7 bc	43.6 ± 1.6 bc
F. culmorum	65.7 ± 3.6 bc	40.9 ± 1.7 b
F. temperatum	55.5 ± 1.4 b	38.0 ± 1.3 b
F. subglutinans	53.0 ± 1.9 b	32.7 ± 0.9 b
Control	17.7 ± 0.6 a	15.0 ± 1.1 a
MEAN	58 ± 3.4 B	37 ± 2.0 A

[1]Values after plus-minus sign (±) represents standard deviation.

All *F. temperatum* and *F. subglutinans* isolates were able to cause typical symptoms of stalk rot in maize after toothpick inoculation on 49-days old plants (Table 4). Necrotic lesions caused by individual isolates significantly differed from the control treatment. Lesion lengths inside the stalk ranged from 15 mm to 45 mm and outside the stalk from 10 mm to 20 mm. Lesions induced by infection with *F. temperatum* were significantly longer than after inoculation with *F. subglutinans*.

Table 4. Lesion length inside the stalk and on the surface of maize stalks 14 days post inoculation in the greenhouse with eight strains of *F. subglutinans* and *fourteen strains* of *F. temperatum* compared to a water control. Different letters indicate significant differences between treatments (p≤0.05).

Isolate	Lesion length inside the stalk [mm]	Lesion length on the stalk surface [mm]
	Control	
Water	3.4 ±[1] 0.4 aA	3.6 ± 0.4 aA
	F. subglutinans	
Fs 187.1	15.5 ± 1.1 b	10.3 ± 0.5 b
Fs 262.1	16.3 ± 0.5 abc	10.3 ± 0.5 ab
Fs 126.2	22.9 ± 1.1 bc	13.0 ± 0.8 b
Fs 215.6	24.3 ± 0.5 bc	11.2 ± 0.2 b
Fs 209.4st	25.7 ± 0.8 bc	11.5 ± 0.2 b
Fs 261.2	27.8 ± 1.3 c	11.8 ± 0.2 b
Fs 28.4sp	28.0 ± 1.2 c	20.8 ± 0.6 c
MEAN	22.7 ±1.5 B	13.1 ± 0.6 B
	F. temperatum	
Ft 18.5	19.6 ± 1.4 b	8.8 ± 0.4 ab

Ft 22.4st	25.5 ± 1.7 bc	8.8 ± 0.3 ab
Ft 184.2	28.2 ± 1.5 bc	20.8 ± 1.3 c
Ft 106.4st	30.2 ± 1.5 bcd	14.2 ± 0.9 bc
Ft 98.4st	30.4 ± 1.4 bcd	16.7 ± 0.8 bc
Ft 65.2	30.5 ± 1.3 bcd	15.8 ± 0.5 bc
Ft 188.2	30.8 ± 1.5 bcd	17.7 ± 1.2 bc
Ft 91.1st	32.3 ± 1.3 bcd	16.2 ± 0.7 bc
Ft 81.4st	33.3 ± 1.7 bcd	16.1 ± 0.3 bc
Ft 127.2sp	33.2 ± 1.2 bcd	18.8 ± 0.9 c
Ft 99.3st	39.0 ± 1.9 cd	19.9 ± 0.9 c
Ft 100.3st	41.1 ± 1.3 cd	12.9 ± 0.7 bc
Ft 50.2	45.4 ± 1.4 d	20.8 ± 1.0 c
MEAN	32.3 ± 1.6 C	15.9 ± 0.9 C

[1]Plus-minus sign (±) represents variation according to standard deviation.

2.4. Effect of Temperature on Ear Infection

The temperature had a significant ($p \leq 0.05$) effect on disease severity of both, *F. temperatum* and *F. subglutinans*. The highest contribution to the variance of disease severity originated from the varieties, followed by the interaction of temperature and fungal isolate (Table S3). Inoculation with *F. subglutinans* caused the highest disease severity on kernels (23 %) and rachis (46 %) at 21 °C. However, the highest disease severity caused by *F. temperatum* on kernels (24 %) and rachis (20 %) occurred at 24 °C (Figure 1).

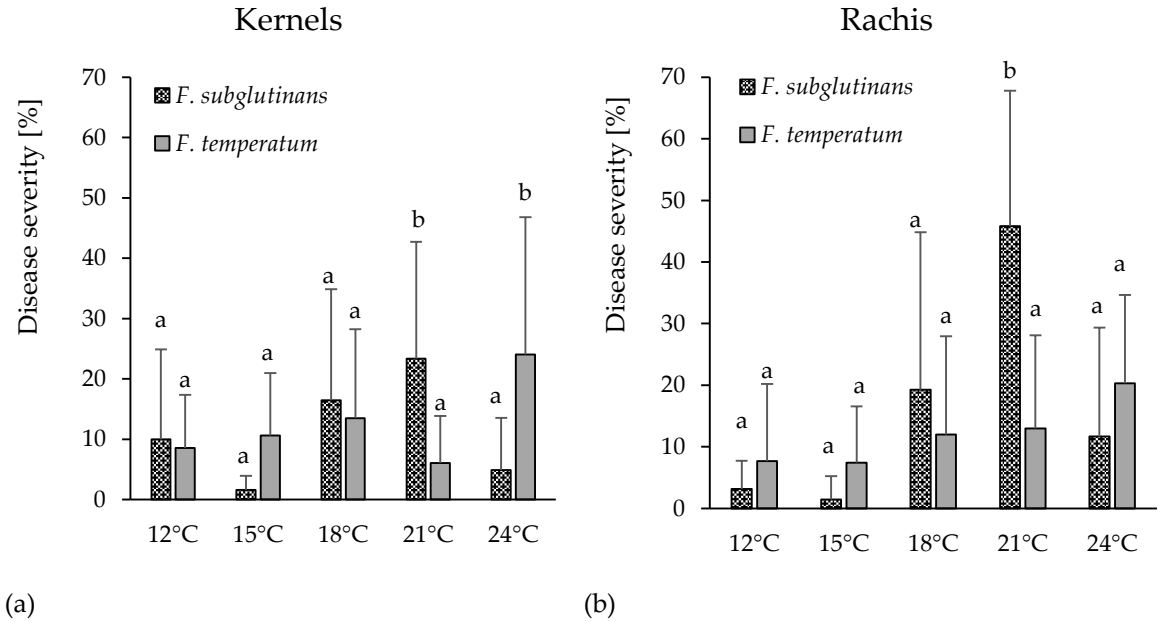

Figure 1. Disease severity induced by *F. subglutinans* and *F. temperatum* at 12 °C, 15 °C, 18 °C, 21 °C and 24 °C on kernels (a) and rachis (b) of maize cobs. Vertical bars represent standard deviations. Different letters indicate significant differences ($p \leq 0.05$).

2.5. Pathogenicity on wheat under greenhouse conditions

All *F. subglutinans* and *F. temperatum* isolates were able to infect winter wheat and to cause symptoms of *Fusarium* head blight (Table 5). The highest disease severity was caused 21 days after

infection by *F. graminearum* (52 %), followed by *F. temperatum* (44 %) and *F. subglutinans* (40 %). However, no significant differences were observed between *F. graminearum* and *F. temperatum* regarding disease severity and kernel weight.

Table 5. Disease severity and thousand-kernel weight (TKW) of winter wheat inoculated in the greenhouse at flowering stage with three different *Fusarium* species. Different letters indicate significant differences within the columns (p≤0.05).

Species	Disease severity [%]			TKW [g]
	7 dpi[1]	14 dpi	21 dpi	
Control	3 ±[2] 6 a	10 ± 11 a	17 ± 15 a	33 ± 10 a
F. subglutinans	7 ± 8 ab	19 ± 15 b	40 ± 23 b	34 ± 7 a
F. temperatum	7 ± 6 b	18 ± 12 bc	44 ± 24 bc	33 ± 7 ab
F. graminearum	12 ± 11 b	28 ± 18 c	52 ± 29 c	27 ± 5 b

[1] Days post inoculation (dpi). [2]Plus-minus sign (±) represents variation according to standard deviation.

2.6. Differential Identification of F.temperatum and F. subglutinans

Differential identification of species in the *Fusarium fujikuroi* species complex (FFSC) (*F. temperatum*, *F. subglutinans*, *F. verticillioides* and *F. proliferatum*,) was performed by species-specific PCR. A total number of 161 single-spore cultures isolated from naturally infected maize cobs and stalk samples in 2018 and grown in PDB were used to extract genomic DNA. The specificity of primers was validated in the studies in which they were designed (references in Table S6). Primers specific for *F. temperatum* allowed amplification of the expected DNA fragments in 44 samples of *F. temperatum*, while samples obtained from pure cultures of *F. subglutinans*, *F. verticillioides,* and *F. proliferatum* did not show amplification. Primers SUB1/SUB2 (Table S7), designed for the amplification of DNA from *F. subglutinans* [23], also generated amplicons in all samples of *F. temperatum* but did not amplify *F. verticillioides* or *F. proliferatum.* Three samples were identified as *F. subglutinans*, as they showed no amplification with primers specific for *F. temperatum* but were positive in the species-specific assay for *F. subglutinans*. Primers for *F. verticillioides* and *F. proliferatum*, the specificity of which has been extensively validated [24], enabled the differential identification of these species in 66 and 48 isolates, respectively.

Following amplification and sequencing of *TEF-1α*, partial nucleotide sequences (601 bp) from pre-identified isolates of *F. temperatum* and *F. subglutinans* were aligned with additional references of *F. verticillioides*, *F. proliferatum* and *F. pseudograminearum* using ClustalW [25] in MEGA7 [26] (Figure 2). All sequences of *F. temperatum* (n=72) and *F. subglutinans* (n=7) clustered into two groups. The separation between *F. temperatum* and *F. subglutinans* was based on 20 single nucleotide polymorphisms (SNP) within *TEF-1α*. We found 30 isolates of *F. temperatum* with 100 % sequence identity with the reference strains MUCL52436, 24 isolates with the same *TEF-1α* sequences as MUCL52450, 5 isolates with *TEF-1α* identical with MUCL52445 and 4 isolates with *TEF-1α* sequence identical with MUCL52454. These sequences formed a separate clade in the phylogenetic tree (Figure 2). Only 6 samples, identical in their sequence to MUCL52462, were grouped in a further separate clade. The assignments of isolates to individual reference strains are presented in more detail in Table S4. The differentiation among the isolates of *F. temperatum* was mostly based on single SNPs. Thus, despite resampling a relatively uniform population, the phylogenetic analysis showed that isolates of *F. temperatum* can be further divided into two subgroups, which was strongly supported by bootstrap values. Group 1 included the majority of 68 sequences, sharing a high sequence similarity with the reference strains MUCL52436 and MUCL52450. Group 2 is represented by only 7 sequences, including MUCL52462. Sequence variation was observed at five positions within the segment of *TEF-1α*, further dividing the species in 6 genotypes. The grouping within the *F. subglutinans* clade was not supported by bootstrapping.

Analysis of partial *RPB2* was performed for a subset of six isolates, representing the two clades that were observed during investigation of *TEF-1α*. All tested isolates were assigned to a single phylogenetic group, together with reference strain MUCL52463 (Figure S2). Newly obtained sequences were submitted to Genbank; the accession numbers are provided in Tables S1 and S5.

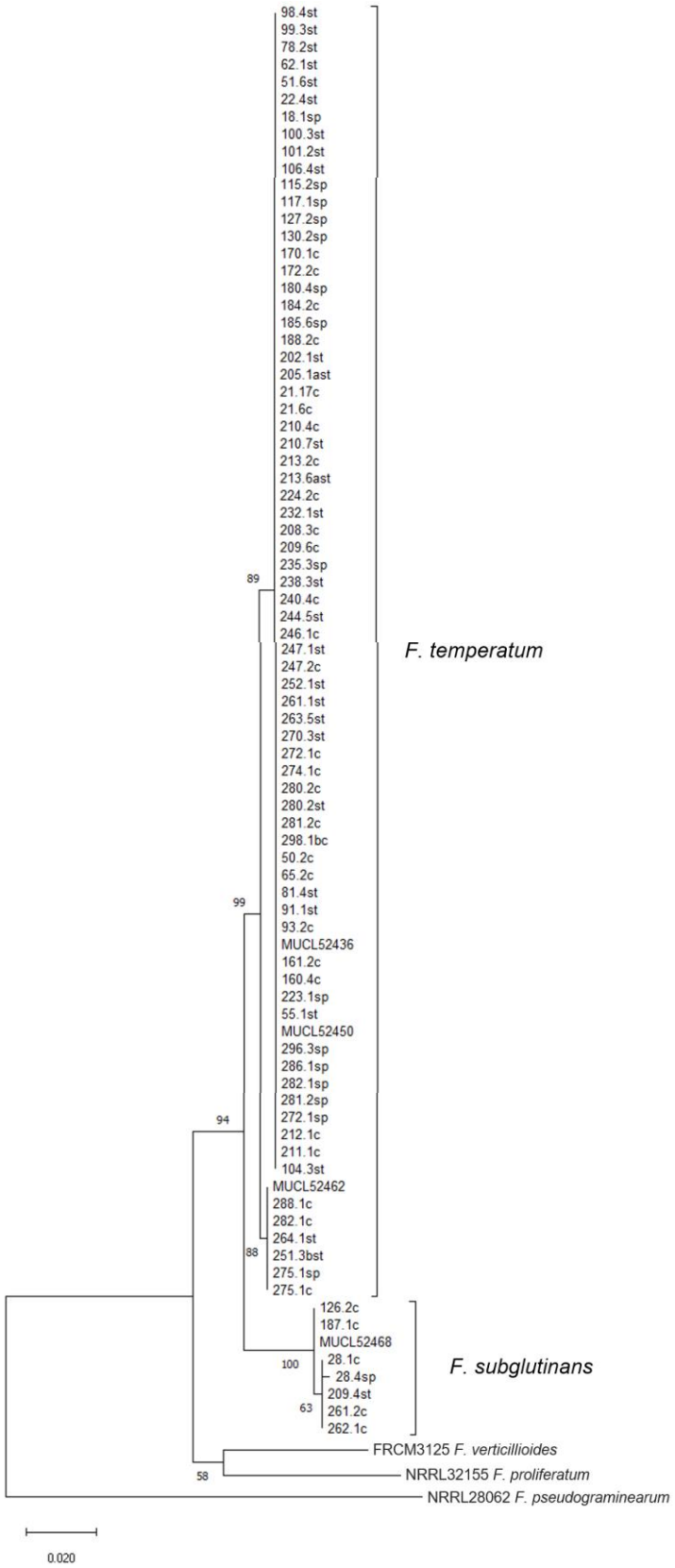

Figure 2. Molecular phylogenetic analysis of translation elongation factor 1 alpha (*TEF-1α*) by the maximum likelihood method (1000 bootstrap replicates) [27]. Analysis was performed with ClustalW [25] in MEGA version 7.0.26 [26] with partial *TEF-1α* sequences of 72 isolates of *F. temperatum* and 7 isolates of *F. subglutinans* (Table S3), and references for *F. temperatum* MUCL52436, MUCL52450, MUCL52462 and *F. subglutinans* MUCL52468

[10]. We added additional references for *F. proliferatum* NRRL32155, *F. verticillioides* FRCM3125 and *F. pseudograminearum* NRRL26062 to scale phylogenetic separation. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are presented next to the nodes. Nucleotide sequences have been subjected to Genbank, the accession numbers are presented in Table S1 and S5.

2.7. Mycotoxin analysis

We tested 61 isolates of *F. temperatum* and six isolates of *F. subglutinans*, obtained from 67 naturally infected maize cobs or stalk samples (limited to a single isolate per sample to prevent repeated isolation of the same strains), in cultures of polished rice for the production of the following mycotoxins: fumonisin B1 (FB1), fumonisin B2 (FB2), beauvericin (BEA) fusaric acid (FA), moniliformin (MON), enniatin B (ENNB), enniatin A1 (ENNA1), and fusaproliferin (FUSA). Furthermore, the reference strain MUCL52463 (*F. temperatum*) and isolates of *F. verticillioides* and *F. proliferatum*, identified by sequencing of partial nucleotide sequence of *TEF-1α* (section *3.6*), were included because they have distinct mycotoxin profiles. The results are summarized in Table 6. *F. temperatum* isolates only produced BEA, FA, MON and FUSA. In *F. subglutinans*, FA, MON and FUSA but no BEA were detected in all cultures. One isolate of *F. temperatum* and one isolate of *F. subglutinans* produced FB1 and FB2, similarly to *F. verticillioides* and *F. proliferatum*, but the results likely resulted from a contamination because the strains did not possess the fumonisin biosynthetic gene *FUM1* [28] (see below). Rice cultures of *F. verticillioides* and *F. proliferatum* accumulated FB1, FB2 and FUSA, while BEA, FA and MON were only produced by *F. proliferatum*. No enniatins (ENNB, ENNA1) were detected in any of the analyzed cultures.

Table 6. *M*ycotoxin production in rice cultures of *F. temperatum, F. subglutinans, F. verticillioides,* and *F. proliferatum*, isolated from naturally infected maize cobs and stalk samples.

Species	No. of isolates	Toxins						
		FB1	FB2	BEA	FA[3]	MON	FUSA[4]	ENNs[5]
F. temperatum[6]	60	-[1]	-	++[2]	++	++	yes	-
F. subglutinans[6]	5	-	-	-	+[2]	+	yes	-
F. verticillioides	4	+++	+++	-	-	-	yes	-
F. proliferatum	4	+++	+++	+	+	++	yes	-

[1]Metabolite could not be detected (values were below LOD, Table S6). [2]Average concentration of mycotoxins (FB1, FB2, BEA, MON, ENNB, ENNA1): +++ more than 1 g/kg, ++ 0.1 to 1.0 g/kg, + less than 100 mg/kg. [3]Average for FA concentration: +++ more than 10 mg/kg, ++ 1 to 10 mg/kg, + less than 1 mg/kg. [4]FUSA was qualitatively evaluated; yes, indicates the presence of FUSA in the tested sample. [5]ENNB and ENNA1. [6]Fumonisins were detected in a single culture of each *F. temperatum* and *F. subglutinans* (see main text).

2.8. Search for FUM1*-analogues in F. temperatum and F. subglutinans*

The capacity to produce fumonisins was investigated by the amplification and sequencing of a segment of the *FUM1* gene. In a set of isolates, including one isolate of *F. temperatum* and one isolate of *F. subglutinans* that appeared to produce fumonisins*in-vitro*, we sequenced the DNA fragment amplified using primers FUM1F1 and FUM1R2, designed by Stepien et al. for the *FUM1* gene [28]. The amplicons were approximately 860 bp long. Amplification of DNA from two randomly chosen isolates of *F. verticillioides* and *F. proliferatum* yielded a single fragment of about 1.1 kb from each isolate. Sequencing and BLAST-analysis revealed the identity of the latter with the gene *FUM1* in *F. verticillioides* (KC188787.1) and *F. proliferatum* (KU180047.1), encoding a polyketide synthase. No similarity at nucleotide or amino acid sequences level could be found between the amplicons generated from *F. temperatum F. subglutinans* DNA with the primers FUM1F1/FUM1R2 [28] and the

genomes of *F. verticillioides* or *F. proliferatum*. We also tried out primers Rp32: 5′-ACAAGTGTCCTTGGGGTCCAGG-3′ and Rp33:5′-GATGCTCTTGG AAGTGGCCTACG-3′, specific for the amplification of the *FUM1* gene from *F. verticillioides* [29], but we could not amplify the gene from *F. temperatum* or *F. subglutinans*.

Multiple alignment of the sequences revealed high similarity between the genes from *F. subglutinans* and *F. temperatum,* yet no obvious similarity with the *FUM1* gene (Fig. 3). Search of NCBI database (BLASTX [74]) with the combined nucleotide data set for four isolates of *F. temperatum* (50.2c, 93.2c, 202.1st and 264.1st) and one isolate of *F. subglutinans* (262.1c) as a query yielded a single hit in the recently published whole genome shotgun of *F. anthophilum* (strain NRRL 25214). The respective gene of *F. anthophilum* was assigned to a hypothetical protein FANTH_8583. The newly sequenced gene loci in *F. temperatum* and *F. subglutinans*, amplified with primers FUM1F1 and FUM1R2, designed by Stepien et al. [28], were tentatively named FTEMP8583 and FSUBG8583, respectively. Following translation, the amino acid sequences were tested for similarity to known protein domains using the SMART-tool [75]. No similar protein domain was found but a putative coiled coil region was identified. The region started at position 32 and ended at position 68 in the sequences of *F. temperatum,* and started at position 11 and ended at position 47 in the sequences of *F. subglutinans*. The alignment of amino acid sequences is presented in Figure 3. All nucleotide sequences have been subjected to Genbank; the accession numbers are presented in Table S1.

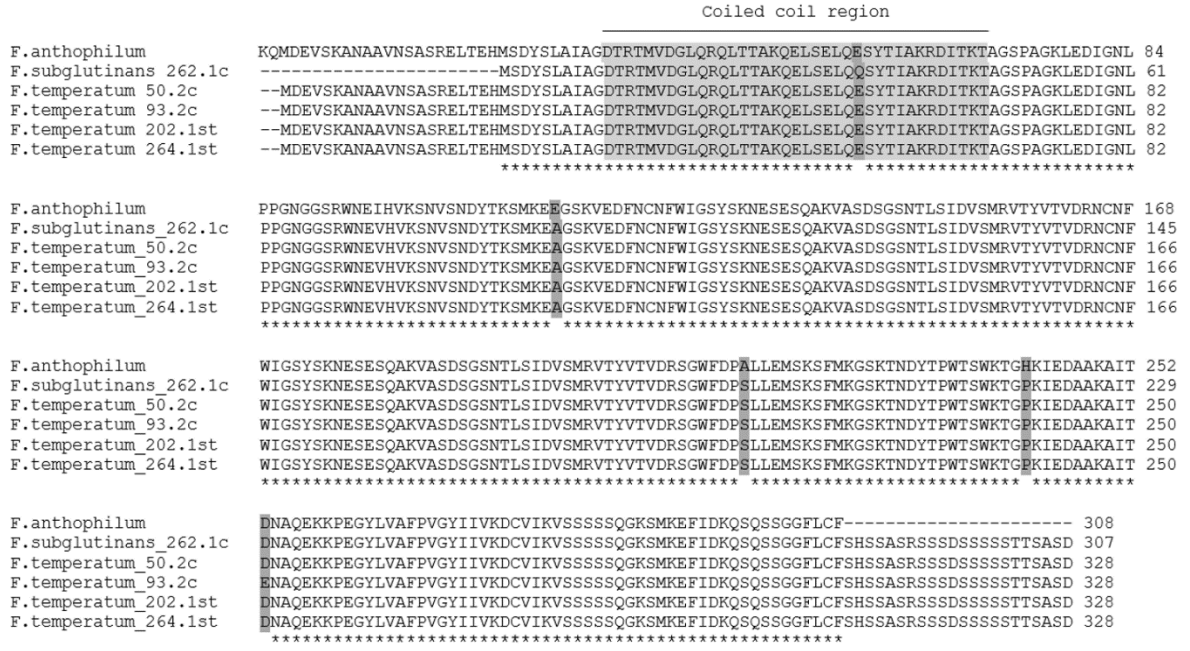

Figure 3. Comparison of the amino acid sequence of a putative *FUM1* analogous gene locus in *F. anthophilum* (FANTH8583), *F. temperatum* (FTEMP8583) and *F. subglutinans* (FSUBG8583). Alignment of amino acid sequences and reference with highest nucleotide sequence similarity after BLASTx search: *F. anthophilum* NRRL25214 (Accession: JABEVY010000206.1). Light grey was used to highlight a coiled coil region, inferred from SMART-analysis [75]. Dark grey highlights amino acid residues differing among the sequences. The alignment was made with ClustalOmega [76]. Symbol* below the alignment indicates identical amino acids residues. Nucleotide sequences were submitted to Genbank; accession numbers are provided in Tables S1 and S5.

3. Discussion.

In the monitoring of 2017 and 2018, *F. temperatum* was found to be the third most often occurring *Fusarium* species on maize cobs in Germany. Among all samples, 17 % of cob and 17 % of stalk samples were infected with *F. temperatum* while only 2 % samples were infected with *F. subglutinans*.

Similar findings have recently been reported from several European countries, including Poland [30], France [31] and Belgium [10], as well as China [22] and South Korea [32].

Environmental factors like precipitation and humidity are known to strongly affect the occurrence and disease severity of several *Fusarium* species [1,11,33,34]. During the two years of monitoring, weather conditions in July differed considerably. In 2017, high precipitation (110.4 mm) occurred and mean temperatures were around 18.8 °C, while in 2018 precipitation was low (40.4 mm) and temperatures were high (20.6 °C). Nonetheless, the frequency of *F. subglutinans* did not change between the years, while the occurrence of *F. temperatum* slightly increased in 2018, suggesting warmer temperatures and low precipitation to be favorable for the latter. Similar tendencies were observed in the inoculation experiments in climate chambers. Disease severity on ears inoculated with *F. temperatum* was highest at 24 °C, while infection with *F. subglutinans* peaked at 21 °C. Even before *F. temperatum* and *F. subglutinans* were defined as individual species, Moretti et al. [13] suggested that a separation into two subgroups may be based on different temperature and humidity requirements, which may have resulted from physiological changes in their sites of origin. Similarly, numerous studies related the occurrence of *F. temperatum* and *F. subglutinans* to temperature and humidity conditions. Moretti et al. [13] observed that *F. subglutinans* occurred more often in warmer and drier regions such as Italy, Slovakia and Serbia, while *F. temperatum* was reported more often from Germany, Poland, Austria and Switzerland. These findings correspond to several studies from Belgium [10], China [22] and Argentina [20] indicating higher frequencies of *F. temperatum* in moderate to cool and moist regions with mean temperatures of 18 °C or lower, while other studies reported *F. temperatum* more often in Poland [30] and Germany [35] following dry conditions. Marin et al. [36] demonstrated that the growth rate of most *Fusarium* ssp. increased with increasing water activity (a_w value), however the growth rate of *F. proliferatum* and *F. subglutinans* decreased at 25 °C when the a_w value increased from 0.980 to 0.995. Further studies are needed to clarify the effect of temperature and precipitation on the occurrence of and disease incidence caused by *F. temperatum* and *F. subglutinans*.

Field inoculation studies at five locations in Germany in 2017 and 2018 showed *F. temperatum* to be the most aggressive *Fusarium* species in maize, even as compared to *F. graminearum* and *F. verticillioides.* However, no significant differences in pathogenicity were observed between *F. temperatum* and *F. subglutinans* at a field site in 2018. This observation confirms the particular importance of *F. temperatum* as an ear rot pathogen in maize cultivation in Germany and other locations with similar climate. The low visual infection rate of *F. verticillioides* may be explained by symptomless infection and endophytic colonization of maize ears; therefore disease symptoms may not reflect plant colonization and mycotoxin concentration accurately [37–40].

Inoculation of maize stalks with toothpicks showed that *F. temperatum* and *F. subglutinans* are pathogenic on the stalks. However, compared to common stalk rot pathogens like *F. graminearum*, *F. culmorum*, and *F. crookwellense*, disease severity was relatively low. This corresponds to the results of Levic et al. [41] and Scauflaire et al. [19], who reported the formation of necrotic lesions and symptoms like wilting, stunting, rotting on stalks and leaf sheaths by *F. temperatum* and *F. subglutinans* yet lower disease severity as compared to *F. crookwellense*, *F. verticillioides*, *F. culmorum* and *F. graminearum* [21].

Crop residues of maize infected with *Fusarium* spp. are considered a major inoculum source for *Fusarium* diseases in small grain cereals in Europe, such as seedling and root rot at the seedling stage and *Fusarium* head blight during anthesis [42,43]. *Fusarium* head blight of wheat is mainly caused by *F. graminearum*, *F. culmorum, F. poae, F. tricinctum,* and *F. avenaceum* [45,46]. In addition, *F. subglutinans* was reported to rarely infect wheat, causing contamination with MON in small grain cereals from central to north-east European countries [45,47]. *F. proliferatum* occasionally infects wheat, causing contamination with fumonisins and BEA [72,73]. In our study, all *F. temperatum* and *F. subglutinans* isolates were able to infect winter wheat and cause *Fusarium* head blight at anthesis. The severity of disease caused by *F. graminearum* was highest and the colonization of the plant

advanced with the highest rate, while 21 days post infection no significant differences between *F. temperatum* and *F. graminearum* were found with regard to disease severity and thousand-kernel weight. In line with our results, the investigations of Wang et al. [22] demonstrated pathogenicity of *F. temperatum* and *F. subglutinans* on wheat, however, the aggressiveness of tested strains was significantly lower than the aggressiveness of a control strain of *F. asiaticum.*

Identification of *F. temperatum* and *F. subglutinans* was carried out by species-specific PCR assays [23,48] and strengthened by the analysis of the marker gene *TEF-1α*, as previously reported [10]. Phylogenetic analysis enabled the assignment of all obtained isolates to their respective species, as supported by bootstrap values. Separation of isolates and references suggest a rather uniform population of *F. temperatum*, when compared with phylogenetic investigations recently published [9,31,33,49–51]. The analysis showed that the isolates of *F. temperatum* are genetically divided into two groups, as supported by a high bootstrap values. This has also been reported by Shin et al. [50] for isolates of *F. temperatum* from Korea, even though the isolates were obtained from a single location. We were unable to link this grouping to morphological characteristics nor to mycotoxin profiles of the respective isolates. The analysis of *TEF-1α* is highly recommended for taxonomical identification of species in the genus *Fusarium* but reliable separation shall be verified by the investigation of additional informative loci [52]. Hence, we selected the gene for the DNA-directed RNA polymerase II subunit (*RPB2*) for verification of distinct groups that we observed in the analysis of *TEF-1α.* The formation of clades could not be reproduced with *RPB2* amplified according to Lofgren et al. [53], using a 763 bp portion of the gene located at the 5′ end. The associated phylogram, based on multiple sequence alignments for a small set of isolates and reference strains, is provided in Figure S6. Therefore, we assume that the grouping of nucleotide sequences of *TEF-1α* does not reflect the genetic relatedness among the strains.

In order to evaluate the risk of mycotoxin contamination upon infection with *F. temperatum* and *F. subglutinans*, *in-vitro* cultures were screened for the presence of eight mycotoxins (BEA, MON, FA, FUSA, ENNB, ENNA1, FB1, FB2), selected according to Scauflaire et al. [19] and others [14,17,18]. We detected BEA in 58 cultures exclusively of *F. temperatum*, supporting the suitability of BEA production for the separation of *F. temperatum* from *F. subglutinans,* similarly as BEA production separated *F. verticillioides* from *F. proliferatum* [24]. The production of MON, FA, and FUSA was confirmed in almost all tested cultures of *F. temperatum* and *F. subglutinans*. Previous studies suggested that MON may not be produced universally by *F. temperatum* [19]. Based on our results, both *F. temperatum* and *F. subglutinans* produced MON but the amounts varied among isolates. The production of MON and FA by some isolates was so low that it could escape detection. As we found only single isolates showing low production of MON and FA, further studies need to be conducted to clarify this finding. The amounts of FA were low, as reported from other studies [54]. Even though FA exerts low toxicity at levels normally detected in natural infections, synergistic effects have been reported between DON and FA in pigs and FB1 and FA in chicken eggs [55]. Although DON is not produced by the species of the FFSC, both DON and FB1 are common in maize grains and contamination with multiple mycotoxins may occur. FA thus potentially increases the risk of mycotoxin exposure via maize consumption.

In the present study, all strains of *F. temperatum* and *F. subglutinans* were FUSA-producers. Even though contamination with FUSA and also BEA are rarely reported in literature [56], a significant role of these toxins in the natural toxicity of the producing species, also in association with other toxins, such as MON, was suggested [16]. The biological activity of FUSA remains to be fully elucidated. We were not able to detect ENNB or ENNA1 in any analyzed rice culture; however, low amounts of ENNB were detected in three maize cobs naturally infected with *F. temperatum,* harvested in 2017 (data not shown). These maize cobs were co-infected with *F. temperatum* and *F. avenaceum*, which were likely responsible for the production of ENNB [6]. Production of any enniatins has shown to be a rare event among isolates of *F. temperatum* so it may not be considered a mycotoxin characteristic of *F. temperatum* [19]. Even though the ability to synthesize enniatins is a common

feature of some trichothecene producing species of *Fusarium*, such as *F. avenaceum*, [6,57] it has rarely been observed for any species of the GFSC [13]. Enniatins are less toxic than trichothecenes, such as deoxynivalenol. Their function in pathogenesis on maize is still unknown.

The production of fumonisins has been reported in a few cases for both *F. temperatum* and *F. subglutinans*, even though the associated *FUM*-cluster could not be detected in their genomes. The production of these toxins by both species in maize plants [32] can be explained by spontaneous infection of the plants with other species. Wang at al. [22] reported production of fumonisins by *F. temperarum* but because they analyzed grains from the field rather than axenic cultures and have not described the analytical method adequately, their results have not been considered here. We detected fumonisins in only one culture of *F. temperatum*, likely due to contamination, supporting the current view that *F. temperatum* does not produce fumonisins. FB1 and FB2 were also detected in one culture of *F. subglutinans.* Even though *F. subglutinans* was occasionally reported to produce fumonisins [58], which is a common feature among members of the FFSC, both classical [18] as well as modern studies [62-65] convincingly showed that *F. subglutinans* does not produce fumonisins. Mycotoxin production found in a small set of cultures of *F. verticillioides* and *F. proliferatum* confirmed the established mycotoxin spectra of these species [54,59–61].

Gene clusters required for synthesis of sphinganine-analog metabolites, such as fumonisins, in *Fusarium* spp. are conserved [63-65]. Sequence analysis of the gene amplified from *F. subglutinans* and *F. temperatum* with primers for the gene *FUM1* revealed that the product was unrelated to *FUM1*. Interestingly, a gene with a high sequence similarity to the product was found to the genome of a closely related species *F. anthophilum*. The amino acid sequence predicted a coiled coil region (Figure 2), possibly indicating involvement of a hypothetical protein in the regulation of gene expression. *F. anthophilum* is a member of the American clade of the FFSC, which includes fumonisin-nonproducing species *F. temperatum* and *F. subglutinans* and fumonisin-producing species *F. anthophilum* and *F. bulbicola* [63].The authors assume a combination of loss of the respective genes during species divergence and horizontal gene transfer, leading to the loss or retention of fumonisin synthesis.

The results obtained in the present study indicate a high degree of variability in BEA, MON, FA, and FUSA production among isolates of *F. temperatum*. We found isolates with a comparably low toxicity, producing low amounts of FA, BEA, MON and FUSA, and highly toxic isolates. Our results support the assumption of lower toxigenic risk due to infection of maize with *F. subglutinans* as compared to *F. temperatum*, especially regarding the production of BEA.

In conclusion, the present investigations indicate that *F. temperatum* occurs more frequently on maize cobs and is more aggressive than previously known and thus represents an elevated threat of food and feed contamination to growers, processing industries and consumers. In addition, *F. temperatum* may enhance the risk of head blight on wheat if grown in rotation with maize.

4. Materials and Methods

4.1. Fungal isolation and cultivation

Fusarium isolates were obtained from 293 naturally infected maize cobs and 190 stalk samples, which were collected from silage and grain maize at 72 field sites in Germany in 2017 and 2018. Thirty randomly chosen kernels of each cob were surface sterilized for 10 min with 0.1 % silver nitrate and incubated on moist sterile filter paper for two days at room temperature. Afterwards, kernels with outgrowing *Fusarium* mycelium were placed on potato dextrose agar (PDA) [77]. The rachis was cut in nine slices, three from the base, three from the middle part and three from the tip of the cob. The slices were surface sterilized as described above and placed directly on PDA plates. The stalk samples were cut in nine slices, three from the first nodium, three from the internodium and three from the second nodium. The samples were surface sterilized and placed on PDA plates. After two days, *Fusarium* mycelium outgrown from the sample was transferred to synthetic low nutrition agar (SNA)

[77] to produce single spore cultures. The isolates were stored as single spore cultures on synthetic SNA plates at 4 °C. Reference strains of *Fusarium* (Table S7) were grown at 25 °C in the dark.

4.2. Inoculum preparation

Spore suspension was produced according to Reid et al. [66] in liquid media containing 2 g KH_2PO_4, 2 g KNO_3, 1 g $MgSO_4$, 1 g KCL, 1 g glucose, 2 mg $FeCl_3$, 0.2 mg $MnSO_4$ and 0.2 mg $ZnSO_4$ in 1 l of water. A plug of agar medium (PDA or SNA) with a diameter of 1 cm overgrown with mycelium was added to 200 ml of the autoclaved medium in a 500 ml Erlenmeyer flask. The medium was placed on a shaker and shaken slowly for 10 days under Near-UV-light (λ = 440-400 nm). The spore suspension was filtered through gauze and spore concentration was determined with a Thoma haemocytometer. For *F. graminearum*, spore density was adjusted to $1x10^4$ spores per ml. For *F. temperatum*, *F. subglutinans*, *F. crookwellense*, *F. culmorum* and *F. verticillioides*, the inoculum was adjusted to a density of $1x10^6$ spores per ml.

4.3. Pathogenicity test on maize cobs under field conditions

The field trials in 2018 and 2019 were located in five locations in Germany and France, i.e. Liesborn (North Rhine-Westphalia, Germany), Bernburg (Saxony, Germany), Kuenzing (Bavaria, Germany) and Rustenhart (Gran Est, France). In 2019, an additional field trial was set up in Goettingen. At each location, maize plants of four susceptible varieties were inoculated by silk channel injection and needle pin stabbing with *F. graminearum*, *F. temperatum* and *F. verticillioides*. Maize plants in Goettingen were inoculated with *F. subglutinans* instead of *F. verticillioides*. Plants were grown in a randomized complete block design, with 75 cm between rows and 13.3 cm between plants (9 plants/m^2) in two repetitions. The primary ear of ten plants per row was inoculated with the pathogen, whereas another ten cobs were inoculated with water (control). The time point of inoculation was determined individually based on the time point of flowering. Silk channel inoculation was performed by a self-refilling syringe (Socorex 173, Ecublens, Swiss) seven days after 50 % silk emergence in a row. Two ml of spore suspension were injected into silk channels between the cob tip and the point where silks emerge [66]. Needle pin inoculation was conducted 15 days after silk emergence. Prior to wounding, the four stainless steel needles (18 mm long, 10 mm wide) were dipped into the spore suspension and stabbed in the center of the ear through the husk leaves. At physiological maturity, husk leaves of ten *Fusarium*-inoculated and ten control ears were removed, and disease severity was rated. Disease severity on primary ears was assessed visually as percentage (0-100 %) of surface covered with mycelium based on the EPPO Guidelines (PP 1/285) [67]. Ten *Fusarium* inoculated and five water inoculated ears per row were harvested, dried and shelled (Almaco, Iowa, USA). Temperature and rainfall data were obtained during the whole vegetation period from a weather station close to the field site (< 5 km).

4.4. Pathogenicity test on maize stalks under greenhouse conditions

Pathogenicity on maize stalk was tested at two plant growth stages in two separate experiments, after seven weeks (BBCH 13) by toothpick inoculation, and at flowering (BBCH 65) by needle pin inoculation. Toothpick inoculation was adapted from Scauflaire et al. [19]. Six wooden toothpicks per treatment were autoclaved (three times for 15 min at 121 °C) and preserved in 15 ml tubes with 5 ml of 2 % malt extract broth medium (Merck, Darmstadt, Germany). Afterwards, 1 ml of spore suspension of 13 isolates of *F. temperatum* and seven isolates of *F. subglutinans* was added to the preserved toothpicks. Following inoculation, toothpicks were incubated for two weeks at 23 °C in the dark.

Seeds of one maize hybrid were surface sterilized with 0.1 % sodium hypochlorite for 10 min and sown in 12 cm diameter pots filled with a mixture of potting soil, compost and sand (3/1/1). Pots were placed in growth chambers at 22 °C, 50 % relative humidity and a day-/night light cycle of 14/10

h. After seven weeks, stalks were inoculated by piercing a with toothpick overgrown with *Fusarium* 10 cm above the soil surface. The toothpick was cut at both sides of the stalk surface and the inoculation site sealed with Parafilm®. Six plants were inoculated per isolate. After 14 days, plants were collected and the length of necrotic lesions around the inoculation point was measured. Lesion length was measured from the stark surface, then stalk was cut in two halves and necrosis were measured inside the stalk

Pathogenicity testing on maize stalks by needle pin inoculation was conducted at the flowering stage. Maize seeds of four susceptible hybrids were seeded in a mixture of potting soil in 20 cm diameter pots. Pots were placed in the greenhouse at 23 °C at a seasonal day-/night light cycle. Stalks were inoculated with *F. graminearum*, *F. crookwellense*, *F. culmorum*, *F. subglutinans* and *F. temperatum* by dipping the needle pin into the spore suspension and stabbing in the middle of the first elongated internode of the stalk. The insertion point was sealed with Parafilm M (VWR International, Darmstadt, Germany). Ten plants per treatment were inoculated in two repetitions. Six weeks (42 dpi) after inoculation, disease severity was assessed as mentioned earlier.

4.5. Effect of temperature on ear infection

In order to assess and compare the effect of temperature on the aggressiveness of *F. subglutinans* and *F. temperatum* on maize, a climate chamber trial was performed. Plants of a susceptible maize hybrid were sown in 16 cm diameter pots filled with a mixture of potting soil, compost and sand (3/1/1) and placed in the greenhouse at seasonal temperature and a day-/night light cycle until flowering. Plants were inoculated by a syringe (Braun, Melsungen, Germany) with two isolates of *F. temperatum* (50.2c and 22.4st, Table S1 and Table 12), differing in the mycotoxin profile, and one isolate of *F. subglutinans* (28.4sp, Table S1 and Table 12), by silk channel inoculation. Inoculation was carried out ten days after silk emergence by injection of 1 ml spore suspension into the silk channel between the cob tip and the point where silks emerge from the husk. Plants were maintained in separate climate chambers at 12 °C, 15 °C, 18 °C, 21 °C and 24 °C, with a relative humidity of 70 % and day/night light cycle of 14/10 h. Experiments were carried out in duplicates; five plants and temperature were inoculated with sterile water and served as control. Plants were harvested six weeks after inoculation and disease severity was scored visually as mentioned before.

4.6. Pathogenicity test on wheat under greenhouse conditions

The pathogenicity of *F. temperatum* and *F. subglutinans* in comparison to *F. graminearum* was examined on two highly susceptible and one less susceptible winter wheat variety. Seedlings were vernalized for seven weeks at 4 °C and planted in 7 cm diameter pots filled with potting soil and compost (1/1). Pots were placed in the greenhouse at seasonal temperature and day-/night light cycle. Plants were inoculated with four isolates of *F. temperatum*, three isolates of *F. subglutinans* and one isolate of *F. graminearum* by spray and point inoculation. Ten plants in two repetitions were inoculated with the pathogen and five plants per variety were inoculated with sterile water, which served as control. Point inoculation was conducted with a syringe (Braun, Melsungen, Germany) injecting 25 µl of spore suspension into the center of two florets at anthesis. Spray inoculation was conducted at the beginning of anthesis by spraying 2 ml spore suspension (same densities as described above) from two sides on cereal heads. Ears were covered with plastic bags for 48 hours/days post inoculation. Severity of infection was scored visually as percentage (0-100 %).

4.7. DNA Extraction, PCR, sequencing and bioinformatic analysis

Mycelium was carefully scrubbed from the surface of PDA culture plates, inoculated with *Fusarium* sp. obtained from naturally infected maize cobs or reference strains (Table S4), and incubated at 25 °C in the dark for 5-7 days. DNA was extracted from lyophilized mycelium, using a CTAB-based protocol as described by Brandfass & Karlovsky [68]. Quality and quantity of the

extracted DNA were assessed on agarose gels (0.8 % (w/v) in 1 × Tris-acetate-EDTA buffer) stained with ethidium bromide. Gel electrophoresis was carried out for 60 min at 4.6 V/cm.

Species-specific PCR analysis was performed in a CFX384 Thermocycler (Biorad, Ruedigheim, Germany) in 384-well microplates (SARSTEDT AG & Co. KG, Nuembrecht, Germany) using a total reaction volume of 4 µl. Reactions were composed of 1 µl template DNA or ddH_2O for negative controls and 3 µl of reaction mixture (Table 8 and ddH_2O; 0.1X SYBR Green I solution (Invitrogen, Karlsruhe, Germany); 1 mg/mL bovine serum albumin (BSA); 0.025 u of DNA polymerase (Table 8). Individual cycler conditions are summarized in Table 9. All standards as well as the negative control were amplified in duplicates. Following amplification, melting curves were obtained. Samples were heated to 95 °C for 60 s and cooled to 55 °C for 60 s. Afterwards, the temperature was increased from 55 °C to 9 °C by 0.5 °C per cycle with continuous fluorescence measurement. Fluorescent data were obtained during the annealing phase to construct a melting curve at the end of assay. The PCR was completed by running a melting curve analysis.

Table 7. Primers used in this study.

Name	Sequence (5′-3′)	Gene	Amplicon length (bp)	Reference
SUB 1	CTGTCGCTAACCTCTTTATCCA	*cal*[1]	631	[23]
SUB 2	CAGTATGGACGTTGGTATTATATCTAA			
FtempF	AAGACCTGGCGGGC	*TEF-1α*	296	[69]
FtempR	TCAGAAGGTTGTGGCAATGG			
VER 1	CTTCCTGCGATGTTTCTCC	*cal*	578	[23]
VER 2 A	ATTGGCCATTGGTATTATATATCTA			
Fp3-F	CGGCCACCAGAGGATGTG	*igs*[2]	230	[48]
Fp4-R	CAACACGAATCGCTTCCTGAC			
EF1αF	ATGGGTAAGGARGACAAGAC	*TEF-1α*	694	[70]
EF1αR	GGARGTACCAGTRATCATGTT			
RPB2-5F2	GGGGWGAYCAG AAGAAGGC	*RPB2*	1200	[53]
RPB2-7CR	CCCATRGCTTGYTT RCCCAT			
FUM1F1	CACATCTGTGGGCGATCC	*FUM1*	1118	[28]
FUM1R2	ATATGGCCCCAGCTGCATA			

[1]calmodulin gene. [2]intergenic spacer of rDNA.

Table 8. Reaction mixtures for species-specific PCR assays.

Target species	$MgCl_2$ (mM)	Primer (µM)	dNTP[1] (µM)	DNA-Polymerase[2]	Reaction buffer[1]
F. temperatum	2	0.3	150	HotStart *Taq*	Standard *Taq* [3]
F. subglutinans	3.5	0.3	100	*Taq*	ThermoPol®[4]
F. verticillioides	2.5	0.3	100	*Taq*	ThermoPol®
F. proliferatum	2	0.3	125	*Taq*	ThermoPol®

[1]deoxyribonucleosides (Bioline, Luckenwalde, Germany). [2]purchased from New England Biolabs, Beverly, Massachusetts, USA; [3] standard *Taq* reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 at 25 °C). [4]ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton® X-100, pH 8.8 at 25 °C).

Table 9. Cycler conditions for species-specific PCR assays.

Target species	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles
F. temperatum	95 °C, 120 s	94 °C, 30 s	63 °C, 30 s	68 °C, 30 s	35
F. subglutinans	95 °C, 120 s	94 °C, 30 s	65 °C, 30 s	68 °C, 40 s	35

F. verticillioides	95 °C, 120 s	94 °C, 40 s	62,5 °C, 30 s	68 °C, 40 s	35
F. proliferatum	95 °C, 120 s	94 °C, 35 s	64 °C, 30 s	68 °C, 35 s	35

Amplification of partial genes *TEF-1α* (694 bp), *RPB2* (ca. 763 bp) and *FUM1* (1118 bp) were performed in a peqSTAR 96 universal gradient thermocycler (PEQLAB, Erlangen, Germany) using 1:100 (v/v) dilutions of the DNA extract in a total reaction volume of 25 µl. The *TEF-1α* gene was amplified using the primers EF1 and EF2 (Table 7). Partial *RPB2* region was amplified with the primers RPB2-5F2 and RPB2-7CR, according to Lofgren et al. [53]. For amplification of the *FUM1* gene, we used the primers FUM1F1 and FUM1R2 (Table 1), originally designed for amplification of *FUM1* sequences in *F. proliferatum* [28]. PCR mixtures were composed of Standard *Taq* reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3 at 25 °C; NEB), 100 µM of each deoxyribonucleoside triphosphate, 0.3 µM of each primer, 0.62 u HotStart-polymerase (NEB) and 1 µL template DNA solution. Final $MgCl_2$ concentration was adjusted to 2 mM. PCR conditions for amplification of *TEF-1α* were: initial denaturation for 30 s at 95 °C; 30 cycles consisting of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 68 °C; and final extension for 5 min at 68 °C. PCR conditions for amplification of *FUM1* were: initial denaturation for 30 s at 95 °C; 35 cycles consisting of 30 s at 94 °C, 30 s at 60 °C, and 90 s at 68 °C; and final extension for 5 min at 68 °C. The PCR cycling conditions for amplification of *RPB2* included an initial denaturation for 30 s at 95 °C; 10 cycles consisting of 30 s at 94 °C, a gradual decrease from 62 °C to 53 °C (-1 °C/cycle) for 40 s, and 1 min at 68 °C; 30 cycles of 30 s at 94 °C, 40 s at 56 °C, and 1 min at 68 °C; and final extension for 5 min at 68 °C. All PCR products were purified and sent for Sanger-sequencing to Macrogen Europe (Amsterdam, The Netherlands). Amplicons generated for the *FUM1* gene were purified from an agarose gel by using the FastgeneTM Gel/PCR Extraction kit (Nippon Genetics Europe GmbH, Düren, Germany). Results were evaluated with Chromas version 2.6.6 (South Brisbane, Australia) and used for comparative BLAST analysis. Multiple sequence alignment was then performed by using ClustalW [25] in MEGA version 7.0.26 [26].

4.8. Mycotoxin extraction and HPLC-analysis

Rice cultures [24] were inoculated with single-spore isolates (SNA, agar plugs of 0.5 cm diameter) of *F. temperatum* and *F. subglutinans*, obtained from naturally infected maize cobs, and references strain MUCL52463 (Table S4), kindly provided by Dr. Jonathan Scauflaire (Earth and Life Institute, Louvain-la-Neuve, Belgium). Controls were inoculated with blank culture medium. Tubes were incubated in the dark for 28 days, at 21 °C. Mycotoxins were extracted in 30 ml acetonitrile/water/acetic acid (84/15/1 (v/v/v)), following evaporation and sample preparation in methanol/water (20/80 (v/v)) for HPLC-MS/MS, as described elsewhere [71].

Toxin quantification was performed on an Agilent 1290 Infinity II HPLC system coupled to an Agilent 6460 QQQ (Agilent Technologies, Waldbronn, Germany). Samples were analyzed on a Phenomenex Kinetex C18 column with a particle size of 2.5 µm, 100 Å pore size and 50 x 2.1 mm (Phenomenex Ltd., Aschaffenburg, Germany). A 12-point calibration ranging from 3.9 to 2000 µg/l was used. Final analysis was performed with MassHunter B.0.8.00 (Agilent, Waldbronn, Germany). The MS/MS transitions, limits of detection (LODs) and limits of quantification (LOQs) are listed in Table S4.

4.9. Statistical analysis

Statistical analysis was conducted using STATISTICA version 13 (Statistica GmbH, Germany). Means of lesion length were estimated for inside and outside of the stalk for each *Fusarium* species and isolates using the non-parametric Kruskal-Wallis ANOVA and Mann-Whitney-U-Test by 5 % probability. Disease severity of ears and wheat heads were log (x+1) transformed to normalized data. Analysis of variance (ANOVA) for field and greenhouse experiments were carried out by Tukey-

HSD-test at 5 % probability. Thousand-kernel-weight (TKW) was analyzed by ANOVA and Tukey-Test at 5 % probability.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Figure S1: Sampling locations. Figure S2: Molecular phylogenetic analysis of DNA-directed RNA polymerase II subunit (*RPB2*). Table S1: List of isolates of *F. temperatum* and *F. subglutinans* and selected isolates of *F. proliferatum*, and *F. verticillioides* Table S2: Analysis of variance from maize cob inoculation under field conditions. Table S3. Analysis of variance from maize cob inoculation at greenhouse conditions at five different temperatures. Table S4: Sequence variations of partial *TEF-1α* gene in isolates of *F. temperatum*. Reference strains of *Fusarium* used in this study. Table S5: Accession numbers of reference sequences used in phylogenetic analysis of *TEF-1α*, *RPB2* and *FUM1*. HPLC-MS/MS analysis. Table S6: Specification of HPLC-MS/MS analysis. Table S7: Reference strains of *Fusarium*.

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Supplementary Materials:

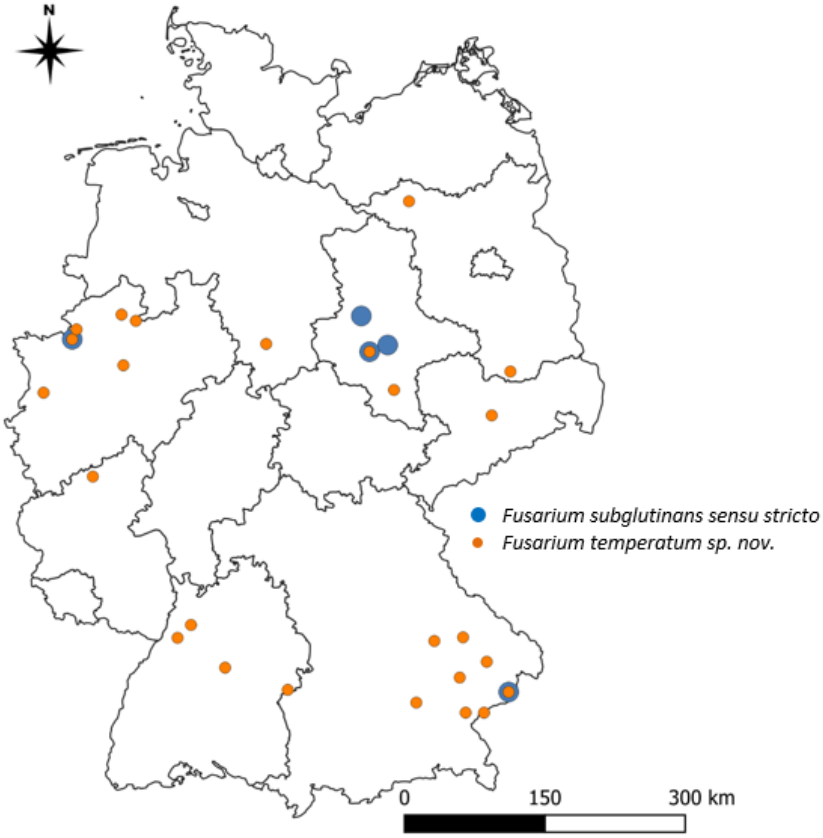

Figure S1 Sampling sites in Germany, where isolates were obtained from maize cobs with infection of *F. subglutinans* and/or *F. temperatum.*

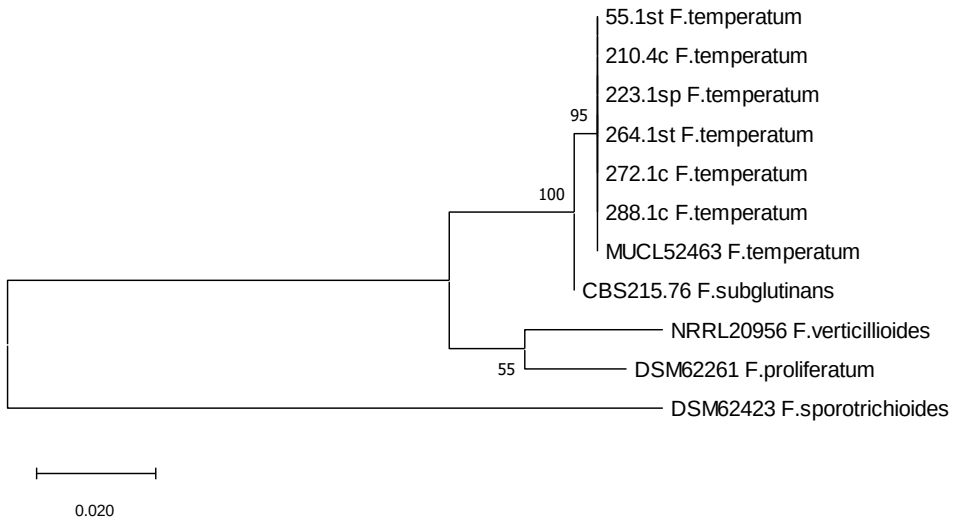

Figure S2. Molecular phylogenetic analysis of DNA-directed RNA polymerase II subunit (*rpb2*) by maximum likelihood method (1000 bootstrap replicates) [25]. Analysis was performed with ClustalW [25] in MEGA version 7.0.26 [26] with partial *rpb2* sequences of 6 isolates of *F. temperatum*, representing

phylogenetic group 1 (55.1st, 210.4c, 223.1sp, 272.1c) and group 2 (264.1st, 288.1c) (Figure 1 & Table S5) and references for *F. temperatum* MUCL52463 and *F. subglutinans* CBS215.76 (Table S4). We added additional references DSM62261 *F. proliferatum*, NRRL20956 *F. verticillioides* and DSM62423 *F. sporotrichioides* to scale phylogenetic separation. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are presented next to the nodes. Individual accession numbers are presented in Table SX. Sequence alignment and phylogenetic tree are available at TreeBASE (X).

Table S1 List of isolates of *F. temperatum* and *F. subglutinans* and selected isolates of *F. proliferatum,* and *F. verticillioides,* obtained from naturally infected maize cobs, during the present study.

Isolate[1]	Maize organ	Year	Location	Morphological characteristics	Sequencing of *tef-1α*
Ft 18.1	rachis	2017	Greven	*F. subglutinans* s. lat	*F. temperatum*
Ft 18.5	kernel	2017	Greven	*F. subglutinans* s. lat	*F. temperatum*
Ft 21.3	kernel	2017	Greven	*F. subglutinans* s. lat	*F. temperatum*
Ft 21.10	kernel	2017	Greven	*F. subglutinans* s. lat	*F. temperatum*
Ft 22.4	stalk	2017	Gondelsheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 50.2	kernel	2017	Muenzesheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 51.1	rachis	2017	Muenzesheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 51.6	stalk	2017	Nossen	*F. subglutinans* s. lat	*F. temperatum*
Ft 55.1	stalk	2017	Nossen	*F. subglutinans* s. lat	*F. temperatum*
Ft 61.2	kernel	2017	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 62.1	stalk	2017	Borken	*F. subglutinans* s. lat	*F. temperatum*
Ft 65.2	kernel	2017	Ostbeven	*F. subglutinans* s. lat	*F. temperatum*
Ft 78.2	stalk	2017	Osterhofen	*F. subglutinans* s. lat	*F. temperatum*
Ft 81.4	stalk	2017	Osterhofen	*F. subglutinans* s. lat	*F. temperatum*
Ft 91.1	stalk	2017	Reith	*F. subglutinans* s. lat	*F. temperatum*
Ft 93.2	kernel	2017	Lauchstaedt	*F. subglutinans* s. lat	*F. temperatum*
Ft 98.4	stalk	2017	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 99.3	stalk	2017	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 104.3	stalk	2017	Loenningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 100.3	stalk	2017	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 106.4	stalk	2017	Loenningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 115.2	rachis	2017	Moosham	*F. subglutinans* s. lat	*F. temperatum*
Ft 117.1	rachis	2017	Moosham	*F. subglutinans* s. lat	*F. temperatum*
Ft 127.2	rachis	2017	Borken	*F. subglutinans* s. lat	*F. temperatum*
Ft 130.2	rachis	2017	Westum	*F. subglutinans* s. lat	*F. temperatum*
Ft 160.4	kernel	2017	Osterhoven	*F. subglutinans* s. lat	*F. temperatum*
Ft 161.2	kernel	2017	Osterhoven	*F. subglutinans* s. lat	*F. temperatum*
Ft 170.1	kernel	2017	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 172.2	kernel	2017	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 175.1	kernel	2017	Loenningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 178.1	kernel	2017	Toenisvorst	*F. subglutinans* s. lat	*F. temperatum*
Ft 180.4	rachis	2017	Toenisvorst	*F. subglutinans* s. lat	*F. temperatum*
Ft 184.2	kernel	2017	Plessa	*F. subglutinans* s. lat	*F. temperatum*
Ft 185.6	rachis	2017	Plessa	*F. subglutinans* s. lat	*F. temperatum*
Ft 188.2	kernel	2017	Pritzwalk	*F. subglutinans* s. lat	*F. temperatum*

Ft 202.1	stalk	2018	Goettingen	*F. subglutinans* s. lat	*F. temperatum*
Ft 205.1	stalk	2018	Mintraching	*F. subglutinans* s. lat	*F. temperatum*
Ft 208.2	rachis	2018	Braunau	*F. subglutinans* s. lat	*F. temperatum*
Ft 208.3	kernel	2018	Braunau	*F. subglutinans* s. lat	*F. temperatum*
Ft 208.5	kernel	2018	Braunau	*F. subglutinans* s. lat	*F. temperatum*
Ft 208.6	kernel	2018	Braunau	*F. subglutinans* s. lat	*F. temperatum*
Ft 208.1	rachis	2018	Braunau	*F. subglutinans* s. lat	*F. temperatum*
Ft 209.6	kernel	2018	Hohenheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 210.4	kernel	2018	Hohenheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 210.7	stalk	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 211.1	kernel	2018	Hohenheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 212.1	kernel	2018	Hohenheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 213.2	kernel	2018	Hohenheim	*F. subglutinans* s. lat	*F. temperatum*
Ft 213.6	stalk	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 223.1	rachis	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 224.2	kernel	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 224.5	kernel	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 232.1	stalk	2018	Einbeck	*F. subglutinans* s. lat	*F. temperatum*
Ft 235.3	rachis	2018	Frauenberg	*F. subglutinans* s. lat	*F. temperatum*
Ft 238.3	stalk	2018	Frauenberg	*F. subglutinans* s. lat	*F. temperatum*
Ft 240.4	kernel	2018	Ulm Langenau	*F. subglutinans* s. lat	*F. temperatum*
Ft 244.5	stalk	2018	Ulm Langenau	*F. subglutinans* s. lat	*F. temperatum*
Ft 245.1	stalk	2018	Ulm Langenau	*F. subglutinans* s. lat	*F. temperatum*
Ft 246.1	kernel	2018	Ulm Langenau	*F. subglutinans* s. lat	*F. temperatum*
Ft 247.2	kernel	2018	Ulm Langenau	*F. subglutinans* s. lat	*F. temperatum*
Ft 247.1	rachis	2018	Ulm Langenau	*F. subglutinans* s. lat	*F. temperatum*
Ft 247.1	stalk	2018	Mintraching	*F. subglutinans* s. lat	*F. temperatum*
Ft 251.3	stalk	2018	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 252.1	stalk	2018	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 261.1	stalk	2018	Toenisvorst	*F. subglutinans* s. lat	*F. temperatum*
Ft 263.5	stalk	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 264.1	stalk	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 270.3	stalk	2018	Osterhofen	*F. subglutinans* s. lat	*F. temperatum*
Ft 272.1	kernel	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 272.1	rachis	2018	Pocking	*F. subglutinans* s. lat	*F. temperatum*
Ft 274.1	kernel	2018	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 275.1	kernel	2018	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 275.1	rachis	2018	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 275.3	kernel	2018	Wesel	*F. subglutinans* s. lat	*F. temperatum*
Ft 280.2	kernel	2018	Toenisvorst	*F. subglutinans* s. lat	*F. temperatum*
Ft 280.4	kernel	2018	Toenisvorst	*F. subglutinans* s. lat	*F. temperatum*
Ft 280.2	stalk	2018	Altoetting	*F. subglutinans* s. lat	*F. temperatum*
Ft 281.2	kernel	2018	Loeningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 281.2	rachis	2018	Loeningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 282.1	kernel	2018	Loenningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 282.1	rachis	2018	Loeningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 282.2	kernel	2018	Loenningen	*F. subglutinans* s. lat	*F. temperatum*
Ft 285.1	kernel	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 285.1	rachis	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 286.1	rachis	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 287.1	kernel	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*

Ft 287.1	rachis	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 288.1	rachis	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 288.2	kernel	2018	Ostbevern	*F. subglutinans* s. lat	*F. temperatum*
Ft 298.1	kernel	2018	Altoetting	*F. subglutinans* s. lat	*F. temperatum*
Fs 28.4	rachis	2017	Bernburg	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fs 126.2	kernel	2017	Borken	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fs 187.1	kernel	2017	Kleinwanzleben	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fs 209.4	stalk	2018	Pocking	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fs 215.6	kernel	2018	Kleinwanzleben	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fs 261.2	kernel	2018	Mintraching	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fs 262.1	kernel	2018	Pocking	*F. subglutinans* s. lat	*F. subglutinans* s.str.
Fp 201.1	kernel	2018	Mintraching	*F. proliferatum*	*F. proliferatum*
Fp 209.2	stalk	2018	Pocking	*F. proliferatum*	*F. proliferatum*
Fp 239.6	kernel	2018	Grucking	*F. proliferatum*	*F. proliferatum*
Fp 273.1	stalk	2018	Osterhofen	*F. proliferatum*	*F. proliferatum*
Fv 207.2	rachis	2018	Braunau	*F. verticillioides*	*F. verticillioides*
Fv 232.3	rachis	2018	Grucking	*F. verticillioides*	*F. verticillioides*
Fv 236.2	stalk	2018	Grucking	*F. verticillioides*	*F. verticillioides*
Fv 263.2	kernel	2018	Pocking	*F. verticillioides*	*F. verticillioides*

[1] Abbreviations of species: Ft = *F. temperatum*, Fs = *F. subglutinans*, Fp = *F. proliferatum*, and Fv = *F. verticillioides*.

Table S2. Multiple variance analyses (year, location, method, variety) and interactions on disease severity of *Fusarium* species on maize cobs under field conditions in 2018 and 2019.

Effect	Degr. of Freedom	F-value	p-value
Year	1	68,751	0,000000
Location	3	32,382	0,000000
Method	1	318,813	0,000000
Variety	19	114,409	0,000000
Year x Location	3	11,160	0,000000
Year x Method	1	15,651	0,000076
Location x Method	3	97,979	0,000000
Year x Variety	19	12,207	0,000000
Location x Variety	57	4,285	0,000000
Method x Variety	19	43,685	0,000000
Year x Location x Method	3	21,903	0,000000
Year x Location x Variety	57	3,876	0,000000
Year x Method x Variety	19	9,093	0,000000
Location x Method x Variety	57	4,942	0,000000
Year x Location x Method x Variety	57	3,048	0,000000
Error	33705		

Table S3. Multiple variance analyses (temperature, isolate, variety) and interactions on disease severity of *F. temperatum* and *F. subglutinans* on maize cobs under greenhouse conditions at five different temperatures.

	Effect on kernels infection	Effect on rachis infection

Effect	SS	FG	p-value	SS	FG	p-value
Temperature	4,3380	4	0,008151	18,4010	4	0,000000
Isolate	4,2537	2	0,000892	2,8193	2	0,010587
Variety	5,3634	1	0,000035	5,9246	1	0,000016
Temperature*Isolate	6,8303	8	0,005027	5,6802	8	0,019797
Temperature*Variety	1,4350	4	0,313567	2,7728	4	0,061330
Isolate*Variety	2,4368	2	0,018560	1,4574	2	0,093004
Temperature*Isolate*Variety	2,0514	8	0,555614	2,8282	8	0,321599

Table S4. Reference strains of *Fusarium* used in this study.

Strain ID[1]	**Formae specials**	**Isolated from**	**Country of origin**
NRRL13383	*F. graminearum* Schwabe	*Zea mays*	Iran
DSM62261	*F. proliferatum* (Matsushima) Nirenberg	*Cymbidium* hybrid	Germany
DSM62423	*F. sporotrichioides* Sherbakoff	*Pinus nigra*	Germany
CBS215.76	*F. subglutinans* (Wollenw. & Reinking)	*Zea mays*	Germany
MUCL52463	*F. temperatum* Scauflaire J. & Munaut F.	*Zea mays*	Belgium
NRRL20956	*F. verticillioides* (Saccardo) Nirenberg	*Zea mays*	USA

[1]Fungal strains were obtained from German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSM); Westerdijk Fungal Biodiversity Institute, Baarn, Holland (CBS); ARS culture collection, Peoria, IL, USA (NRRL); and Dr Jonathan Scauflaire, Earth and Life Institute, Louvain-la-Neuve, Belgium (MUCL).

Table S5. HPLC-MS/MS analysis

Toxin	Obtained from	Molecular ion	Parent ion	Product ions	LOD[A] [mg/kg]	LOQ[A] [mg/kg]
BEA	Merck (Darmstadt, Germany)	[M+H]+	784.4	244.0 262.2	2.5	20
MON	Enzo Life Sciences (Lörrach, Deutschland)	[M+H]+	97	41.1	1	2
FA	Enzo Life Sciences (Lörrach, Deutschland)	[M+H]+	180.1	162.1 134.1	0.02	0.1
FUSA	Dr. Franz Berthiller (BOKU, Vienna, Austria)	[M+H]+	445.3	367.3 385.4	-[B]	-
FB1	Merck (Darmstadt, Germany)	[M+H]+	722.4	352.2 334.2	3	6
FB2	Enzo Life Sciences (Lörrach, Deutschland)	[M+H]+	706.4	318.3 336.3	5	10
ENNA1	Merck (Darmstadt, Germany)	[M+H]+	668.4	228.2 210.2	1,5	2
ENNB	Merck (Darmstadt, Germany)	[M+H]+	640.4	527.3 196.1	1	2,5

[A]LOD and LOQ were estimated according to blank samples of polished rice, inoculated with pure culture medium.
[B]No LOD and LOQ were estimated for Fusaproliferin.

Table S6. Sequence variations of partial *tef-1α* gene in isolates of *F. temperatum*.

tef-1α		SNPs		Identical reference	Phylogenetic
Genotypes	Isolates	Position[2]	Nucleotide	and accession[3]	group[4]
1	28[1]	136	G	MUCL52436	1
		142	A	HM067684	

		325	A		
		390	C		
		455	T		
		550	C		
2	3	136	A	MUCL52454	1
		142	G	HM067689	
		325	A		
		390	C		
		455	T		
		550	C		
3	6	136	A	MUCL52462	2
		142	G	HM067690	
		325	T		
		390	C		
		455	A		
		550	T		
4	5	136	A	MUCL52445	1
		142	G	HM067686	
		325	A		
		390	A		
		455	T		
		550	C		
5	24	136	A	MUCL52450	1
		142	A	HM067687	
		325	A		
		390	C		
		455	T		
		550	C		

[1]3 isolates were excluded here, due to low coverage at nucleotide position 136, but matched the first genotype at further SNP positions. [2]Nucleotide Positions in 705 bp PCR product after amplification with EF-1αF and EF-1αR, and sequencing with EF-1αF. [3]Selected reference were described by Scauflaire et al., 2011. [4]Phylogenetic groups 1 and 2 were defined according to the phylogenetic tree presented in Figure 2.

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Chapter IV: *Fusarium* ear rot infection in maize in relation to inoculation method, maize variety and *Fusarium* species

Abstract

Fusarium species are common pathogens on maize and can lead to contamination with toxic metabolites which reduces the quality and safety of both animal feed and human food products. *Fusarium* ear rot is caused by a complex of different *Fusarium* species, however *F. graminearum*, *F. verticillioides* and *F. subglutinans* are the prevailing species in Central Europe. Breeding for resistant genotypes is the most promising approach to reduce yield losses and minimize the risk of mycotoxin contamination. In 2018 and 2019, maize hybrids were tested in four locations (Bernburg, Rustenhart, Kuenzing and Liesborn) in Germany and France. In each location, twenty hybrids were inoculated with *F. graminearum*, *F. verticillioides* and *F. temperatum* using two inoculation methods, injection into the silk channel and kernel wounding by kernel stab inoculation. Disease severity was assessed visually as the percentage of plant tissue overgrown with mycelium tissue at harvest time point according to the EPPO guidelines. Our results showed that *F. temperatum* was the most aggressive *Fusarium* species in both years followed by *F. graminearum* and *F. verticillioides,* however, the prevalence differed between locations. Significant differences in genotypic resistance depending on the inoculation method and *Fusarium* species were found in all locations. Silk channel inoculation resulted in higher disease severity of *F. temperatum* and *F. verticillioides* while disease severity following kernel stab inoculation was higher with *F. graminearum*. Correlation between *Fusarium* species was medium to high, however, only low to medium correlation was observed between inoculation method within the *Fusarium* species.

Keywords: *Fusarium* ear rot, ear rot resistance, *F. graminearum, F. verticillioides, F. temperatum,* maize, inoculation method

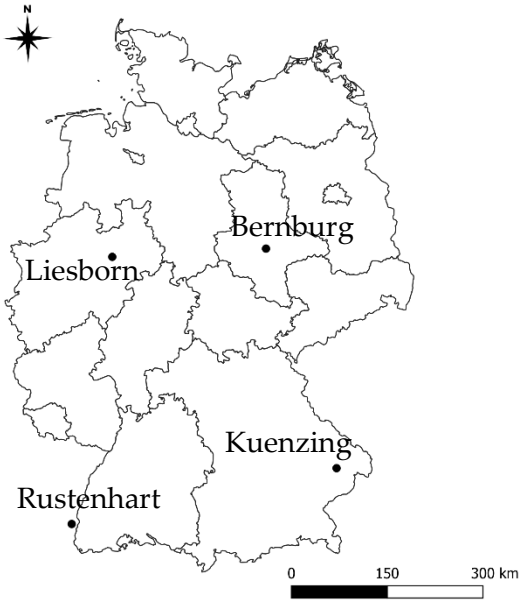
1. Introduction

Fusarium ear rot in maize is caused by a complex of *Fusarium* species with two distinct diseases that differ in their epidemiological characteristics: red ear rot (*Gibberella* ear rot, GER) and pink ear rot (*Fusarium* ear rot, FER) [1,2]. Red ear rot is caused by species of the *Discolor* section, mainly *F. graminearum* and *F. culmorum,* however, pink ear rot is caused by representatives of the *Liseola* section mainly *F. verticillioides*, *F. proliferatum* and *F. subglutinans* sp. [2–4]. Infection with *F. graminearum* is more common in Central to Northern European areas for silage maize production due to frequent rainfall and low temperatures [5,6]. Pink ear rot is commonly observed in Southern to Central European areas associated with grain maize production due to drier and warmer climates [7,8]. *Fusarium* spp. can cause significant yield loss, and downgrade grain quality due to contamination with mycotoxins [4,9–11]. Species

associated with pink ear rot produce various toxins including fumonisin (FB1/ FB2), fusarin and fusaric acid, however, trichothecenes like deoxynivalenol (DON) and nivalenol (NIV) as well as zearalenone (ZEN) are most often detected from ears infected with red ear rot [2,4,12–14], associated with immunosuppression, hypoestrogenism in pigs or esophageal cancer in humans [14–17]. Therefore, it is of major importance to minimize the risk of feed and food contaminations by preventing ear rot disease development. As fungicidal application is not feasible due to plant height during flowering and the need of special machinery, planting resistant maize hybrids is one of the best control strategies [18]. Since the European Union released legal limits for mycotoxin content in maize grain for foodstuff (EC No. 1126/2007) [19,20] and animal feed [21], breeding for resistance to ear rots has become an important criterion for breeders to select less susceptible inbred lines [22]. Disease development and mycotoxin production is affected by several factors like genetic resistance of maize hybrids, weather conditions and agronomical factors. Two types of resistance have so far been identified in maize, silk channel resistance which prevents the fungus from invasion through the silk channel down to the kernel and kernel resistance blocks the spread of the fungus from kernel to kernel [18,23,24]. Different morphological factors such as pericarp thickness [25,26], surface wax layer [27] and husk covering as well as chemical factors, such as phenolic compounds especially ferulic acid [28] have been identified as being resistant mechanisms. Breeding for ear rot resistance in maize is complicated by the fact that it is only quantitative, based on several genes and that there are many different *Fusarium* species which can infect the maize plant [22,29]. Natural infection is usually caused by a mixture of local *Fusarium* species, however, most breeding programs focus either on *F. graminearum* or *F. verticillioides* and reports involving both or other *Fusarium* species are rare [24]. In addition, considering of the two major modes of fungal entry, the inoculation method may have an impact on the response of different genotypes. Therefore, our objective was (i) to investigate the aggressiveness of *F. graminearum*, *F. verticillioides* and *F. temperatum* on twenty maize hybrids in four locations in Germany, (ii) to study the correlation of genotype resistance to different *Fusarium* species and (iii) to determine the aggressiveness and genotype resistance in relation to different inoculation methods (silk channel vs. kernel stab inoculation).

2. Material and Methods

2.1 Field site location, experimental design and plant material



The field trials in 2018 and 2019 were located in Germany and France, in Liesborn (North Rhine-Westphalia), Bernburg (Saxony), Kuenzing (Bavarian) and Rustenhart (Alsace, France) (Figure 1). Plants were grown in a randomized complete block design, with 75 cm between rows and 13.3 cm between plants (9 plants/m²) in two repetitions. Sixteen registered maize hybrids from early to mid-late German grain maize set (FAO 210-270) were selected. Additionally, four highly susceptible hybrids were selected (Variety 1 to Variety 4) . For further experimental details see Table 1.

Figure 1 . Location of field sites

2.2 Inoculum preparation

Spore suspensions were produced according to Reid et al. (1992) [30] in a liquid media culture containing 2 g KH_2PO_4, 2 g KNO_3, 1 g $MgSO_4$, 1 g KCL, 1 g $C_6H_{12}O_6$, 2 mg $FeCl_3$, 0.2 mg $MnSO_4$ and 0.2 mg $ZnSO_4$ diluted in 1 l tap water. A plug of agar medium (PDA or SNA) with a diameter of 1 cm overgrown with a *Fusarium* strain was added to 200 ml of the autoclaved medium in a 500 ml Erlenmeyer flask. The medium was placed on a shaker and shaken slowly for 10 d under Near-UV-light (λ = 440-400 nm). Afterwards, the spore suspension was filtered through gauze and the spore density was assessed with a Thoma haemocytometer. For *F. graminearum*, it was adjusted to $1x10^4$ spores per ml and for *F. temperatum* and *F. verticillioides* to $1x10^6$ spores per ml.

2.3 Inoculation and disease assessment

The primary ear of ten plants per row was inoculated, whereas another ten cobs of the remaining plants per row were inoculated with water (control). The time point of inoculation was determined individually based on the timepoint of female flowering of each row. Silk channel inoculation was performed by a self-refilling syringe (Socorex 173, Ecublens, Swiss) seven days after 50% silk emergence per row. A 2 ml volume of spore suspension was injected

into the silk channel in the area between the cob tip and the point where silks emerge [30]. Kernel stab inoculation was conducted 15 days after silk emergence by stabbing in the center of the ear from the side through the husk leaves. At physiological maturity, husk leaves of ten *Fusarium*-inoculated and ten control ears were removed, and disease severity was rated. The disease severity of the primary ears was assessed visually as the percentage (0-100%) of surface covered with mycelium based on the EPPO Guidelines (PP 1/285) [31]. Ten *Fusarium* inoculated and five water inoculated ears per row were harvested, dried and shelled (Almaco, Iowa, USA). Temperature and rainfall data were obtained during the whole vegetation period from weather stations close each field site (< 5 km).

2.4 Statistical analysis

Statistical analysis was conducted using STATISTICA version 13 (Statistica GmbH, Germany). Disease severity of ears was log (x+1) transformed to normalized data. Analysis of variance (ANOVA) for field experiments were carried out by Tukey-HSD-test at 5% probability. Thousand-kernel-weight (TKW) was analyzed by ANOVA and Tukey-Test at 5% probability.

3. Results

3.1 Field site management and weather conditions

Both years of investigations were characterized by dry conditions compared to the longtime average especially during flowering and grain filling, in all locations. In 2018, two locations and in 2019 three out of four were irrigated (Table 1). Disease assessments took place six to seven weeks after inoculation in all locations, however, sampling of infected cobs was only conducted in Bernburg and Rustenhart.

Table 1: Field site management and weather conditions in Bernburg, Liesborn, Kuenzing and Rustenhart in 2018 and 2019.

Location	**Liesborn**	**Bernburg**	**Kuenzing**	**Rustenhart**
		2018		
Pre-crop	Winter wheat	Maize	Winter wheat	Maize
Tillage	Plough	Plough	Cultivator	Plough
Plot size [cm]	300 x 150	300 x 75	300 x 150	300 x 75
Plants per m²	9	9	9	9
Sowing (Date)	25.04.2019	26.04.2018	08.05.2018	28.04.2018
Sampling (Date)	-	20.09.2018	-	28.09.2018
Disease assessment (Date)	18.09.2018	19.09.2018	05.10.2018	27.09.2018
Irrigation	-	100 mm	-	326 mm
Ø Temperature [°C] *	18	18.5	18.0	18.6
Ø Precipitation [mm]*	145	172	279	208

2019				
Pre-crop	Wheat/greening	Maize	Winter wheat	Maize
Tillage	Cultivator	Plough	Cultivator	Plough
Plot size [cm]	300 x 75	300 x 75	300 x 150	300 x 75
Plants per m²	9	9	9	9
Sowing (Date)	18.04.2019	26.04.2019	15.04.2019	25.04.2019
Sampling (Date)	-	20.09.2019	-	26.09.2019
Disease assessment (Date)	24.09.2019	19.09.2019	16.09.2019	25.09.2019
Irrigation	-	120 mm	80 mm	265 mm
Ø Temperature [°C] *	16.3	16.6	16.7	16.9
Ø Precipitation [mm]*	164	189	280	287

* April – September

3.2 Disease severity

Both years of investigation were conducive for the development of *Fusarium* ear rot infection. Disease severity induced by the *Fusarium* species was significantly affected by the year, inoculation method, location and variety as well as the interactions of these factors. According to the mean squares, ear rot rating was found to vary more among years than among locations. In 2018, disease severity was significantly higher (17.4%) than in 2019 (12.9%). In contrast, infection rates were relatively consistent from location to location and ranged from 12.4% in Kuenzing to 16.7% in Rustenhart (Table 2). Infection of *F. temperatum* was highest in Kuenzing (27.3%) followed by Bernburg (23.7%), Rustenhart (23.1%) and Liesborn (21.4%), however, infection of *F. graminearum* was enhanced in southern field locations (Kuenzing and Rustenhart). In general, mean disease severity was significantly higher after silk channel inoculation (18%) compared to kernel stab inoculation (12.3%). This also applies for *F. temperatum*, however, infection of *F. graminearum* was higher after kernel stab wounding (17.2%) compared with injection into the silk channel (12.3%). In general, disease severity of fungal species was mostly affected by variety and the interaction of variety and inoculation method, according to their mean squares.

Table 2: Diseases severity [%] and standard deviation of maize ears artificially infected with *F. graminearum*, *F. temperatum* and *F. verticillioides* according to year, inoculation method, location, variety and maturity group. Different letters indicate significant differences within the columns (p≤0.05).

Treatment	Disease severity [%]			
	F. graminearum	***F. temperatum***	***F. verticillioides***	***MEAN***
		Year		
2018	18.0 ± 25.9 b	27.0 ± 28.4 b	7.3 ± 10.7 b	17.4 ± 24.4 b
2019	11.5 ± 16 a	20.9 ± 23.3 a	6.2 ± 12.1 a	12.9 ± 18.7 a
		Method		
Kernel stab	17.2 ± 24 b	15.9 ± 13.2 b	3.9 ± 7.2 a	12.3 ± 17.4 a

Fusarium ear rot infection in maize in relation to inoculation method, maize variety and *Fusarium* species

Silk channel	12.3 ± 19.1 a	32.1 ± 32.6 a	9.6 ± 13.9 b	18 ± 25.3 b
		Location		
Bernburg	16.0 ± 21.9 c	23.7 ± 27.4 b	5.8 ± 11.2 a	15.2 ± 22.5 a
Kuenzing	13.6 ± 19.3 b	27.6 ± 26.4 c	7.8 ± 12.4 b	16.4 ± 21.8 ab
Liesborn	9.9 ± 16.3 a	21.4 ± 27.3 a	5.8 ± 11.2 a	12.4 ± 20.5 a
Rustenhart	19.5 ± 27.0 d	23.1 ± 23.0 ab	7.5 ± 10.6 b	16.7 ± 22.4 b
		Variety		
Variety 1	20.0 ± 25.1 de	32.6 ± 29.5 gh	5.6 ± 7.0 cde	19.4 ± 25.3 gh
Variety 2	16.8 ± 21.2 de	20.7 ± 21 bcd	5.1 ± 7.7 bcde	14.0 ± 18.9 de
Variety 3	27.4 ± 28.1 g	45.6 ± 35.6 i	24.7 ± 26.9 j	32.7 ± 31.9 j
Variety 4	26.5 ± 28.7 fg	35.2 ± 30.8 h	10.4 ± 13.6 gh	24.2 ± 27.6 i
Variety 5 (210)[1]	8.2 ± 13.9 a	13.7 ± 14.5 ab	4.0 ± 6.7 abcd	8.7 ± 12.9 bc
Variety 6 (210)	10.0 ± 15.9 ab	24.5 ± 25.9 cdef	6.4 ± 8.8 def	13.7 ± 19.9 de
Variety 7 (220)	15.9 ± 21.3 bcde	28.2 ± 27.5 efg	9.0 ± 11.1 fg	17.7 ± 22.6 fgh
Variety 8 (220)	6.6 ± 11.9 a	9.7 ± 12.2 a	3.3 ± 7.5 abc	6.5 ± 11.1 ab
Variety 9 (220)	19.9 ± 22.9 de	29.7 ± 27 fgh	7.7 ± 11.1 efg	19.0 ± 23.2 gh
Variety 10 (230)	20.6 ± 26.5 ef	27.7 ± 19.4 defg	10.2 ± 11.7 gh	19.5 ± 21.3 gh
Variety 11 (230)	21.7 ± 25.5 efg	30.0 ± 25.8 fgh	6.3 ± 7.7 def	19.3 ± 23.5 gh
Variety 12 (240)	17.8 ± 20.4 de	33.5 ± 21.3 gh	12.1 ± 13.0 hi	21.2 ± 24.5 hi
Variety 13 (240)	20.3 ± 27.9 de	31.1 ± 28.6 fgh	5.6 ± 7.7 cde	18.9 ± 25.7 gh
Variety 14 (240)	7.0 ± 13.5 a	12.2 ± 17.0 a	2.3 ± 4.9 ab	7.1 ± 13.4 ab
Variety 15 (250)	8.0 ± 16.1 a	14.4 ± 16.2 ab	3.3 ± 4.2 abc	8.6 ± 14.1 b
Variety 16 (250)	15.8 ± 19.1 cde	31.6 ± 31.2 fgh	4.0 ± 7.0	17.1 ± 24.4 efg
Variety 17 (260)	4.2 ± 11.1 a	8.7 ± 12.5 a	1.4 ± 2.5 a	4.8 ± 10.2 a
Variety 18 (260)	10.1 ± 16.7 abc	20.5 ± 26.7 bc	5.5 ± 9.4 cde	12.1 ± 20.0 cd
Variety 19 (260)	14.3 ± 21.5 bcd	22.5 ± 18.6 cde	7.2 ± 7.5 ef	14.7 ± 18.1 def
Variety 20 (270)	4.8 ± 9.8 a	7.8 ± 10.2 a	1.7 ± 3.4 a	4.7 ± 8.8 a
		Maturity group		
Early (210-220)	12.1 ± 18.4 b	21.1 ± 23.7 b	6.0 ± 9.5 b	13.1 ± 19.2 b
Mid-early (230-250)	16.0 ± 22.7 c	25.7 ± 26.1 c	6.2 ± 9.2 c	15.9 ± 22.1 c
Mid-late (260-270)	8.3 ± 15.9 a	14.9 ± 19.4 a	3.9 ± 6.8 a	9.1 ± 15.7 a
		Species		
MEAN	14.8 ± 21.8 b	24.0 ± 26.3 c	6.8 ± 11.4 a	12.0 ± 20.0

[1] FAO numbers of grain maize hybrids

The effect of the variety was significant (p≤0,001) and ranged from 6.5% (Variety 8) to 32.7% (Variety 3). Varieties could be grouped in highly susceptible, (Variety 3), susceptible (Variety 4, Variety 12, Variety 10, Variety 1, Variety 11, Variety 13, Variety 9), moderately susceptible (Variety 7, Variety 16, Variety 19, Variety 2, Variety 6, Variety 18,) and moderately resistant (Variety 15, Variety 14, Variety 5, Variety 8, Variety 17, Variety 20). Varieties stayed within these groups in both years (Figure 2). The effect of the maturity group on infection rate was significant over all *Fusarium* species. Mid-late matured plants (FAO 260-270) significantly

lower disease severity (9.1%) followed by early maturity (FAO 210-220) (13.1%) and mid-early maturity (FAO 230-250) (15.9%) groups.

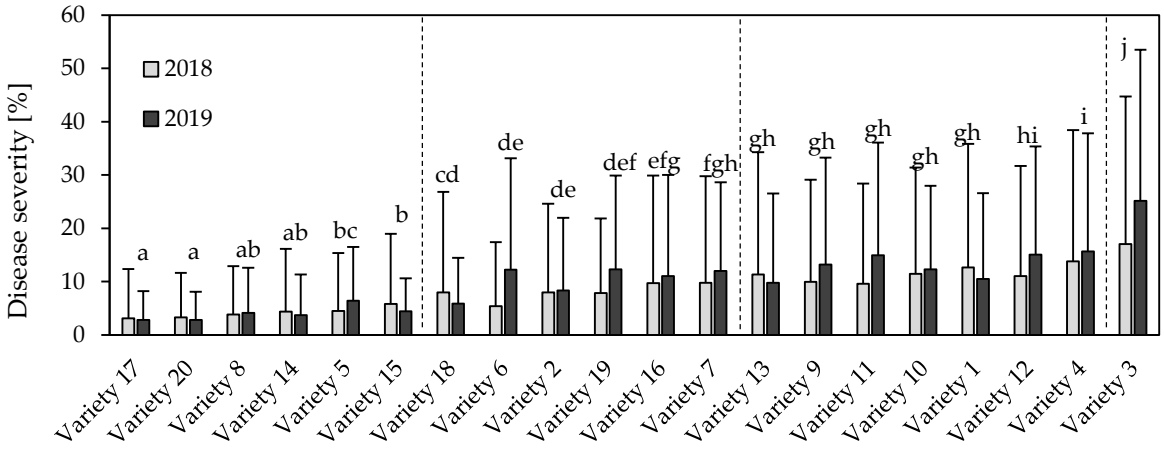


Figure 2: Disease severity [%] of maize varieties tested in 2018 and 2019 (means of four locations and three *Fusarium* species). Vertical bars represent standard deviation. Different letters indicate significant differences (p≤0.05) of mean disease severity. Dashed lines subdivide varieties in moderately resistant, moderately susceptible, susceptible and highly susceptible.

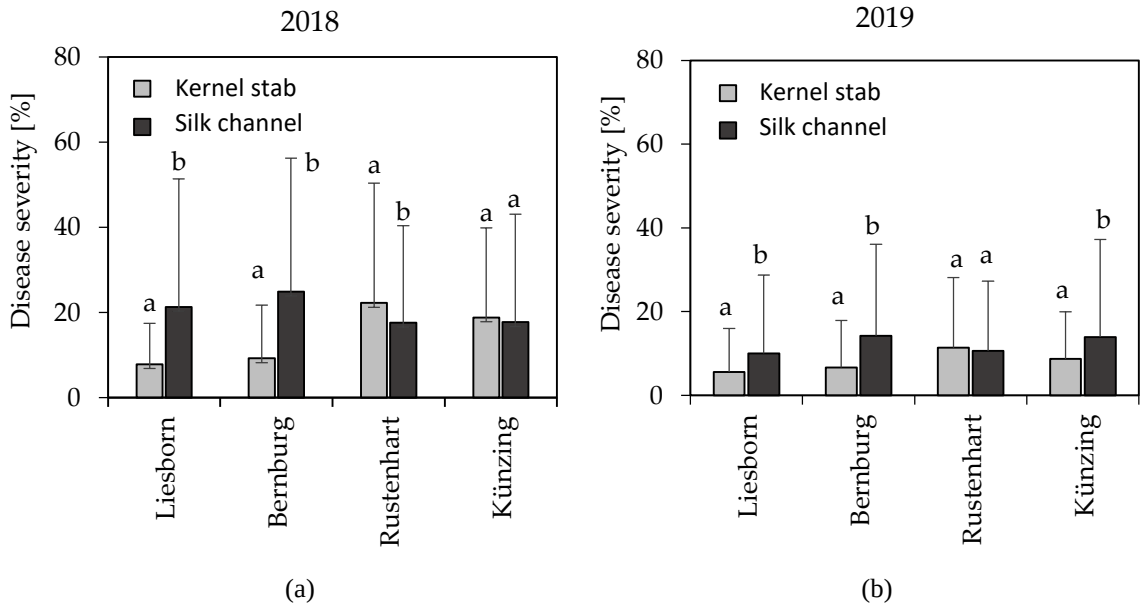


Figure 3. Disease severity [%] on infected cobs in Liesborn, Bernburg, Rustenhart and Kuenzing depending on the inoculation method in 2018 (a) and 2019 (b). Vertical bars represent standard deviations. Different letters indicate significant differences (p≤0.05) within the location.

Disease severity was highest after kernel stab inoculation in most locations, however silk channel inoculation lead to highest infection rate in Rustenhart (Figure 3). Silk channel

inoculation lead to higher infection by *F. temperatum* and *F. verticillioides*. Kernel stab inoculation resulted in higher disease severity of *F. graminearum* (Figure 4).

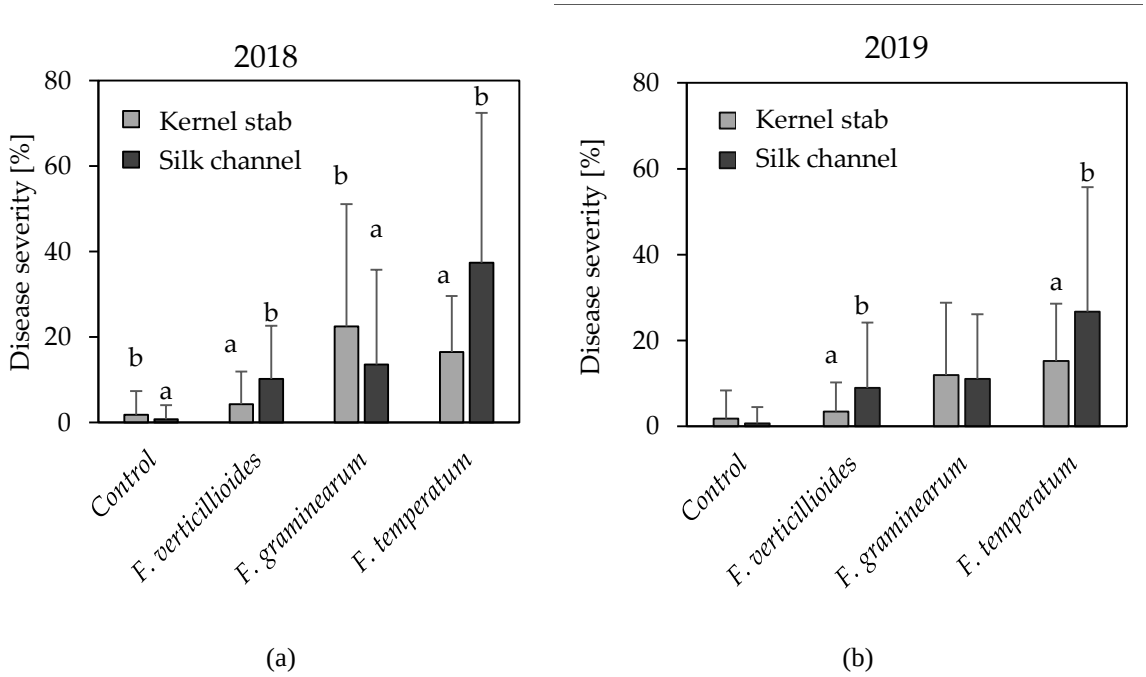


Figure 4 Disease severity [%] of cobs infected with *F. verticillioides, F. graminearum* and *F. temperatum* depending on the inoculation method in 2018 (a) and 2019 (b). Vertical bars represent standard deviations. Different letters indicate significant differences (p≤0.05) within the species.

3.2.1 Responses of maize hybrids to different Fusarium species

In general, responses of maize to inoculation with *F. graminearum*, *F. verticillioides* and *F. temperatum* correlated with each other (Figure 5). Correlation was moderate (r=0.65) between *F. verticillioides* and *F. graminearum*. However, a strong correlation was found between *F. temperatum* and *F. verticillioides* (r=0.73) as well as between *F. temperatum* and *F. graminearum* (r=0.83)

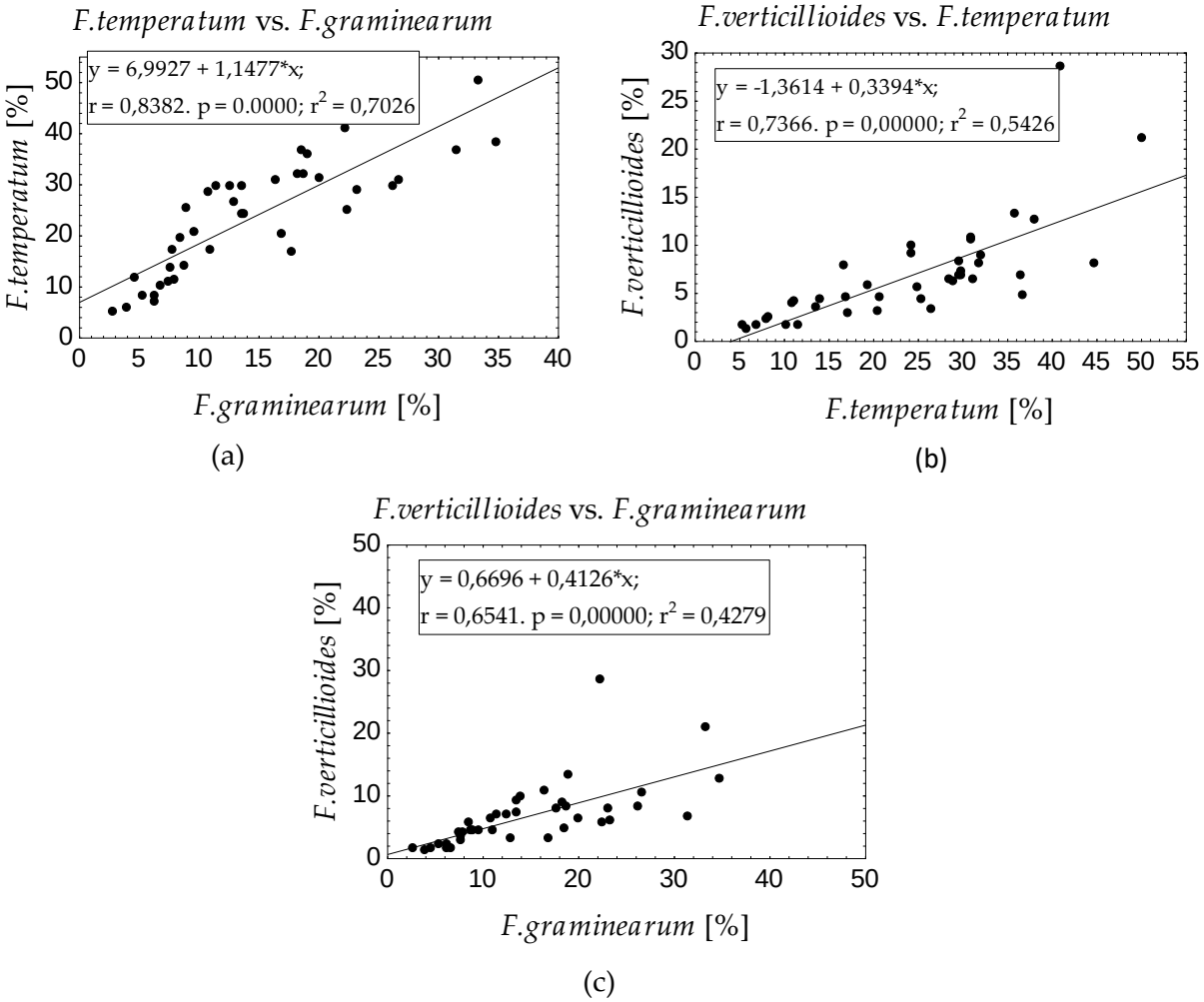


Figure 5. Correlation of disease severity levels of twenty maize hybrids to inoculation with *F. temperatum* and *F. graminearum* (a), *F. verticillioides* and *F. temperatum* (b) as well as *F. verticillioides* and *F. graminearum* (c) on tested in 2018 and 2019.

3.2.2 Effect of inoculation method on disease severity

The correlation between disease severity induced by kernel stab and silk channel inoculation over all *Fusarium* species tested was moderate (r=0.66), however, this correlation was only moderate to low for individual *Fusarium* species (Figure 6). Correlation was moderate between silk channel and kernel stab inoculation for *F. temperatum* (r=0.56) and low after inoculation with *F. graminearum* (r=0.48) and *F. verticillioides* (r=0.44).

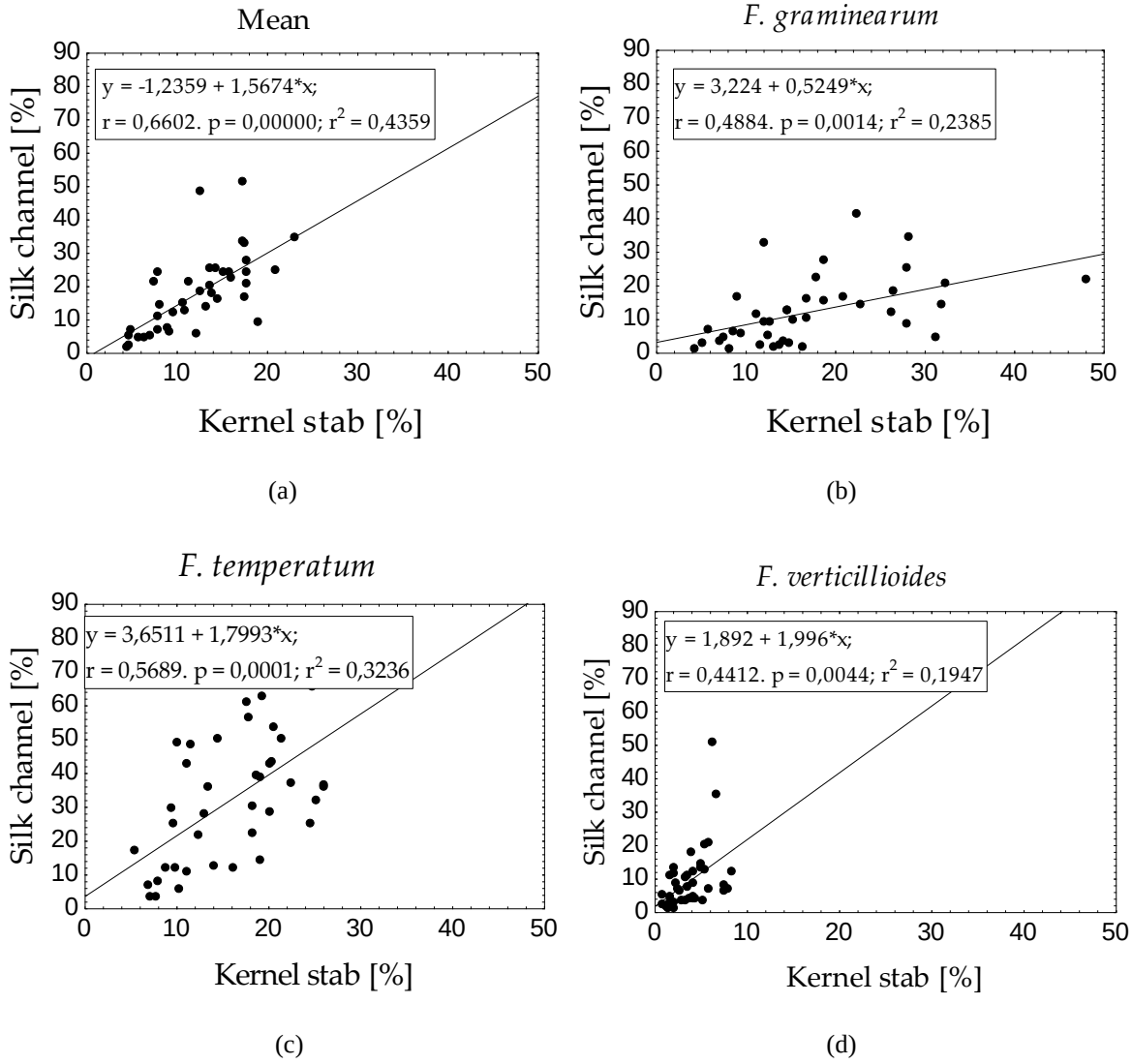


Figure 6: Correlation of disease severity level between silk channel and kernel stab inoculation with the mean of inoculations with all *Fusarium* species (a), with *F. graminearum* (b), *F. temperatum* (c) and *F. verticillioides* (c) on all genotypes tested in 2018 and 2019.

4. Discussion

4.1 Inoculation method

Two inoculation methods were compared according to the two main modes of fungal entry and genotype resistance, silk channel inoculation and kernel stab inoculation. Both inoculation methods were successful in causing significant ear rot infection in all locations and both years. However, natural infection of non-inoculated control plants was very low in most locations. Rustenhart was characterized by high infestation with the European corn borer (*Ostrinia nubilalis*) promoting *Fusarium* infection in control plants and additional infections on inoculated cobs. Therefore, Mesterházy et al. (2011) [22] suggest to carefully

determine which mode of fungal entry is predominant in a particular experimental site and point out that genotypic resistance could be influenced by the mode of fungal entry and the *Fusarium* species prevailing in the area. Hence, numerous studies [18,32–34] suggest the use of both inoculation methods, especially, if varieties are developed for areas in which the European corn borer occurs more frequently.

Silk channel inoculation resulted in higher disease severity compared to kernel stab inoculation especially after inoculation with *F. temperatum* and *F. verticillioides*. Only *F. graminearum* lead to higher infection after kernel stab inoculation. Similar results were obtained by Reid et al (2002) [35] in which *F. subglutinans*, a closely related species of *F. temperatum*, lead to similarly high level of disease severity after silk channel inoculation, however, the infection rate of *F. graminearum* was highest after kernel stab inoculation.. In contrast, infection of *Fusarium* ear rot (FER) (*F. verticillioides* and *F. temperatum*, respectively) is closely related with insect injury such as caused by the European corn borer (*O. nubilalis*) [34,36]. This infection pathway appears the most important one in those areas [37–39]. Comparatively few data are available on the relationship between genotype resistance and inoculation method. Only, one report by Lemmens (1999) [33] provides a low correlation (r = 0.12) in Austrian hybrids and a moderate to high correlation (r= 0.77– 0.89) was found by Chungu et al. (1996) [34] after inoculation with *F. graminearum*. In the later study, a moderate correlation (r=0.66) between both inoculation methods was found for the mean of all *Fusarium* species, however, a lower correlation was estimated for individual *Fusarium* species especially for *F. verticillioides* and *F. graminearum*. Löffler et al. (2010) [32] reported a similar relationship of silk channel and kernel stab inoculation (r=0.66) indicating that at least some resistance QTL are acting against both modes of fungal entry. Therefore, we also recommend to use both inoculation methods, especially for genotypes grown in regions with high European corn borer infections.

4.2 Location and environmental conditions

Both years of investigations were characterized by very dry conditions at flowering and during maturity stages. In 2018, two locations and in 2019, three out of four locations had to be additionally irrigated. Monthly mean temperature did not differ much between locations, however, water availability estimated from precipitation and irrigation varied between locations. *F. verticillioides* and *F. temperatum*, members of the FER complex are favored by distinctly different conditions. FER is more common in warmer areas with dry conditions during grain-filling [1,8,40], however, GER, especially *F. graminearium,* is favored by high moisture level around silking, followed by moderate temperature and high precipitation during maturation [10]. Highest precipitation (>500 mm, from May to October) around silking and grain filling and additional irrigation was recorded in Rustenhart which may explain the high infection rate with *F. graminearum*. In contrast, *F. temperatum* was favored in Kuenzing and Bernburg due to dryer conditions (~300 mm). Lowest disease severity and fungal growth of all *Fusarium* species was observed in Liesborn. In both years of

investigations, no additional irrigation was applied in Kuenzing and low precipitation (~150 mm) resulted in limited fungal growth and disease development of all species.

Additional irrigation is necessary to ensure plant growth and reliable disease development for hybrids assessment, however, it distorted the effect of weather conditions on disease severity in different locations in Germany. Therefore, further studies are needed to gain a better understanding of the effect of changing temperature and precipitation on disease severity and mycotoxin concentration induced by individual *Fusarium* species in maize cobs.

4.3 Maize hybrids

Disease severity in all locations ranged from 0% to 86% between the hybrids resulting in a sufficient level of infection for differentiation. Seven varieties ranged as moderately resistant seemed to have multiple resistance against several *Fusarium* species, whereas other varieties reacted differently to individual species. Several quantitative mechanisms of resistance are considered to play a role for initial kernel penetration and spread between infected kernels acting against multiple species and both modes of fungal entry [41,42]. Husk covering [43] plays an important role for epidemiology and disease development. Kernel penetration and spread can be affected by morphological traits like pericarp thickness and pericarp wax layers [25] as well as biochemical traits like kernel water content, content of (E)-ferulic acid and dehydrodimers of ferulic acid [28,44,45]. In addition, certain traits are known to implicate the resistance of maize silks such as long-chain alkanes and silk flavonoids [23,46,47]. Therefore, Ali et al (2005) [48] found eleven active QTLs after silk channel inoculation and 18 QTLs after kernel inoculation for GER resistance. However, only two QTLs across environment could be detected for silk resistance and only one for kernel resistance, indicating a strong influence of the environment. FER resistance is determined polygenically [44] with at least 15 QTLs identified for FER and 17 for fumonisin B1 production [49]. However, QTLs have relatively small effects and are not consistent between populations [50,51]

Overall *Fusarium* species, mid-late maturity varieties (FAO 260-270) lead to the significantly lower disease severity compared to early (FAO 210-230) and mid-early (230-250) varieties. All hybrids were inoculated individually according to their silking date within ten days but harvested and scored at the same time point, seven to eight weeks later. Hybrids of the early maturity group reached already physiological maturity six to seven weeks after flowering, however, late maturing hybrids were still at late dent stage. Actually, we expected that late maturity varieties are stronger infected than early varieties due to slower maturity and longer period from inoculation to physiological maturity which would imply that the fungus would have more time to infect and grow, develop symptoms and higher DON levels. However, the opposite results were obtained in our study. One reason for lower disease levels in later maturing hybrids might be, that environmental conditions at the later flowering timepoint and during maturity stage in September were not favorable for fungal growth. Reid and Sinha (1998) [52] observed no significant differences of disease development and DON concentration between late and early maturity hybrids inoculated with *F. graminearum.*

For further assessments on genotypic resistance, maturity groups should by studied separately or disease ratings should be adjusted by the flowering time point.

4.4 Fusarium species

Inoculation with *F. temperatum* resulted in the highest severity of ear rot infection in both years of investigation, followed by *F. graminearum* and *F. verticillioides*. The lower visual infection rate by *F. verticillioides* might be caused from symptomless endophytic infection and does not reflect the absolute infestation of the cob and mycotoxin production [53–55].

Significant correlation between the three *Fusarium* species indicate a medium to high relationship of hybrid resistance towards *F. graminearum*, *F. temperatum* and *F. verticillioides*. Lowest but moderate correlation was found between *F. graminearum* and *F. verticillioides,* however, correlation between *F. temperatum* and *F. verticillioides* as well as between *F. temperatum* and *F. graminearum* were stronger. Löffler et al. (2010) [32] and Presello et al. (2004) [56] reported also a moderate correlation between *F. graminearum* and *F. verticillioides* in flint and dent pools. Several studies of Mesterházy on maize hybrids inoculated with *F. graminearum*, *F. culmorum*, *F. verticillioides* and *F. avenaceum* indicated a close correlation between the members of GER (*F. graminearum* and *F. culmorum*, respectively), however, no correlation was observed towards the species causing FER (*F. verticillioides* and *F. avenaceum)* [18,22,57]. In addition, moderate to strong correlation was observed for the disease severity of *Fusarium* infection and common smut (*Ustilago zeae*) [58] as well as *Aspergillus* ear rot (*Aspergillus flavus*) [59,60]. This moderate correlation between different *Fusarium* species and other ear rot pathogens might be explained by some general unspecific resistance mechanism such as surface wax layers and pericarp thickness [25,27]. However, it is not known whether the same resistance genes in maize genotypes or the interaction between the environment are responsible for this [18]. In addition, there is no clear relation between visible infection and mycotoxin concentration for all *Fusarium* species, which makes breeding for reduced mycotoxin contamination more difficult. Several studies indicated a high correlation of disease severity and toxin concentration for GER which suggests that visual scoring can be sufficient in resistance selection [42,52,61]. In contrast, low or moderate correlations were found between fumonisin production and infection rate of *F. verticillioides* in maize [62,63]. Breeding programs mostly rely on visible symptom evaluation because it is rapid and can be applied to hundreds of genotypes, while toxin analysis is time consuming and cost intensive [18]. Depending on the strength of these correlations, phenotypic selection maybe conducted either directly by mycotoxin analysis or indirectly by ear rot evaluations.

Our results indicate that breeding programs should focus on different *Fusarium* species to enhance genotype resistance and be aware of new emerging species like *F. temperatum*.

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Chapter V: *Trichoderma afroharzianum* ear rot– a new disease on maize in Europe

Trichoderma afroharzianum ear rot– a new disease on maize in Europe

Annette Pfordt *[1], Simon Schiwek[2], Petr Karlovsky[2] and Andreas von Tiedemann[1]

[1]Plant Pathology and Crop Protection, University of Goettingen, Goettingen, Germany
[2]Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Goettingen, Germany

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Abstract

Trichoderma species are widespread filamentous fungi in soils, on plant roots and decaying plant residues. Due to their strong competitiveness and mycoparasitic activity against other fungi, particular strains of *Trichoderma* sp. are used in agriculture as biocontrol agents against plant pathogens. Commercial products based on strains of *T. harzianum* or *T. afroharzianum* have been applied to control *Rhizoctonia* spp., *Fusarium* spp. and *Phytophthora* spp. in various crops. In 2018, however, severe infections of *Trichoderma* on maize ears were recorded for the first time in a field in Southern Germany. Infected maize cobs were sampled, the fungus was isolated in pure culture and preliminarily identified microscopically as *Trichoderma harzianum*. After silk channel inoculation in the greenhouse, ear rot disease of high severity was observed. In addition to fungal colonization, the dry matter content in cobs was significantly reduced compared to water inoculated cobs. In 2018 and 2019, a total of 13 *T. harzianum* isolates from maize cobs and maize stalks were isolated and tested, for pathogenicity on maize plants in the greenhouse, compared to several reference isolates. Four isolates proved to be highly aggressive, two biocontrol isolates, Trichodex (T39) and strain T12, induced slight infection and eleven isolates were non-pathogenic. After sequencing of the translation elongation factor-1α (*tef-1α*) and internal transcribes spacers (*ITS*), the four highly aggressive isolates were reassigned to *T. afroharzianum*, while the commercial biocontrol isolates Trichodex (T39) and T12, as well as the other non-pathogenic strains belonged to *T. harzianum*, *T. atroviride* or *T. tomentosum*. This, to our knowledge, is the first report on *Trichoderma* sp. as a pathogen causing ear rot disease in maize in Europe with the potential to incite significant yield losses. We therefore propose to name this disease as ‚*Trichoderma* ear rot on maize‘.

Keywords: *Trichoderma harzianum*, *Trichoderma afroharzianum*, pathogenicity, *Trichoderma* ear rot on maize

1 Introduction

Members of the genus *Trichoderma* are classified as imperfect fungi in the division Ascomycota and are ubiquitous in various types of soil. Some species of *Trichoderma* have biocontrol potential and can suppress pathogen growth by direct and indirect mechanisms including mycoparasitism, antibiosis, induction of host resistance and competition for nutrients and space (Ghazanfar et al.,2018; Kubicek et al., 2008; Jaklitsch & Voglmayr, 2015; Samuels and Hebbar, 2015). Especially, the ability to detoxify zearalenone protects *Trichoderma* spp. from the chemical defense of *Fusarium* spp. (Popiel et al., 2014). They can thereby control and antagonize a broad range of economically important plant parasitic pathogens (Harmann, 2005; Gupta et al., 2014; Ferrigo et al., 2014). In addition, they may increase plant resilience against drought conditions and promote shoot and root growth (Arora et al., 2003). Harman et al. (2004) reported a significant yield increase in maize due to *Trichoderma* treatments. Apart from the control of root and foliar pathogens, *Trichoderma* spp. enhance nutrient solubilization and uptake as well as enhanced root and root hair development (Herrera-Estrella & Chet, 2004; Schuster & Schmoll, 2010).

Trichoderma species have been described as opportunistic, basically avirulent plant symbionts in soil (Harman et al., 2004); however, a few reports have mentioned *Trichoderma* as ear rot pathogen on maize in the US (Iowa State University; Munkvold & White, 2016; Wise et al., 2016). *Trichoderma* ear rot infection has been characterized by the occurrence of dark, blue-green layers of conidia on and between the kernels of infected ears causing premature germination of the kernels (Wise et al., 2016). In addition, the dry matter content of ears infected with *Trichoderma* was strongly reduced compared to uninfected ears. The occurrence of *Trichoderma* ear rot was associated with injuries caused by feeding birds or other mechanical damage in Kentucky and Ohio (Vincelli, 2014).

Surprisingly, in 2018 a severe occurrence of *Trichoderma* on the maize cobs was recorded at a field site in Southern Germany. Cobs sampled from 20 maize varieties were overgrown with mycelium producing green layers of conidia between the kernels and on the outside of the husk leaves. Similar disease symptoms have been previously observed in Southern Bavaria, after warm and dry summers.

The aim of the present study was to identify and verify *Trichoderma* as a new pathogen causing ear rot disease on maize. Therefore, *Trichoderma*-infected cobs from four locations in Germany and France were sampled, cultured and microscopically examined as well as analyzed by sequencing the gene for translation elongation factor-1α (*tef-1α*) and internal transcribes spacers (*ITS*). Furthermore, pathogenicity of *Trichoderma* isolates and the impact of infection on dry matter content of maize cobs were tested after artificial inoculation at flowering in the greenhouse.

2 Materials and Methods

2.1 Fungal isolation and cultivation

Maize cobs and stalks were collected from naturally infected silage and grain maize in Germany in 2018 and 2019. Thirty randomly chosen kernels from each cob were surface sterilized for 10 min with 0.1% silver nitrate and placed on potato dextrose agar (PDA). The stalk samples were cut in nine slices, surface sterilized as described above and placed on PDA

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plates. After two days, mycelium outgrown from the sample was transferred to PDA plates. Single conidia cultures were produced, and isolates were stored on synthetic low nutrition agar (SNA) plates at 4°C.

2.2 Inoculation procedure

Spores from single spore cultures were transferred to PDA plates containing antibiotics (400 µg/ml streptomycin, Duchefa Biochemie, Haarlem, The Netherlands;30 µg/ml rifampicin, AppliChem, Darmstadt, Germany) and incubated under Near-UV-light (λ = 340-400 nm) at 23°C in a growth chamber. After two weeks, sterile water was added to plates and conidia were scraped off with a microscope slide. The conidia suspension was then filtered through gauze and cell density was measured with a Thoma haemocytometer and adjusted to $1x10^6$ conidia per ml. Primary ears of maize plants were inoculated seven days after silk channel emergence (BBCH 65). For this purpose, one ml of conidia suspension was injected with a syringe (Braun, Melsungen, Germany) into the silk channel between the cob tip and the point where silks emerge from the husk.

2.3 Plant cultivation and pathogenicity assessment on maize ears

Maize seeds of two varieties were sown in a mixture of soil (potting soil/compost/ sand mixture of 1:2:1) in 20 cm diameter pots. Pots were placed in the greenhouse at 23°C under a seasonal day-/night light cycle. Five plants per isolate were inoculated by silk channel injection and five additional plants were inoculated with water, as control. Thirteen *Trichoderma* isolates originally isolated from maize cobs in the field were compared to four reference strains of *T. harzianum (*IPP0318, IPP0319, IPP0320, T12), one type strain of *T. afroharzianum* (CBS 124620) and one strain of *T. atroviride* (IPP0316) obtained from different fungal collections (see Table 1). In addition, one commercial biocontrol isolate, T39 (Trichodex), and one isolate, T12, with potential biocontrol activity (provided by the Department for Phytopathology of Justus-Liebig-University, Gießen, Germany) were tested. Four weeks (28 dpi) after inoculation, husk leaves of inoculated and control ears were removed, and disease severity was assessed visually as the percentage (0-100%) of the cob surface covered with fungal mycelium (Fig. 1). Finally, cobs were weighed and dried for five days at 60°C to assess the dry matter content. In addition, isolates were re-isolated and cultured on PDA to confirm the Koch´s Postulate.

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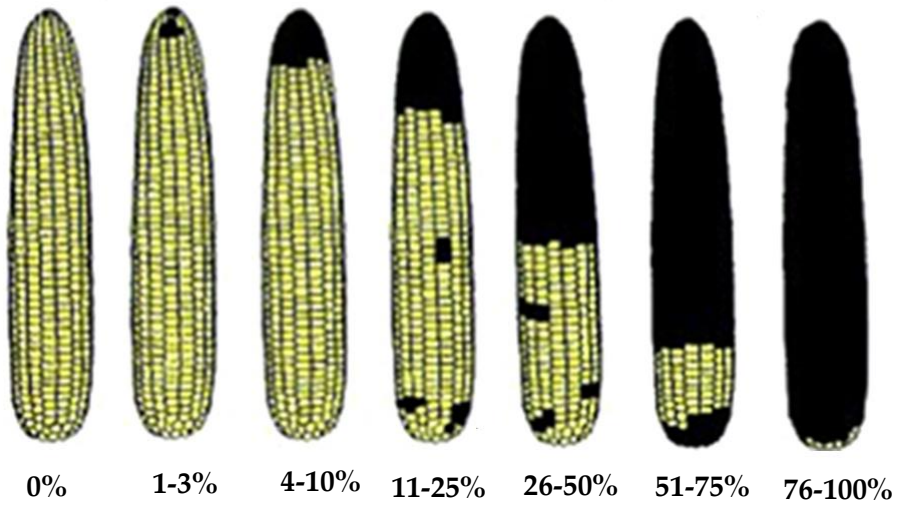

Figure 3. Disease rating scale for cob infection according to EPPO Guidelines (PP 1/285).

Table 1. Host, geographic locations and year of isolation of *Trichoderma* isolates used in this study.

Isolates	Species	Year	Location	Host
Tri1	*Trichoderma afroharzianum*	2018	Croix de Pardies (F)	*Zea mays* (cob)!
Tri2	*Trichoderma afroharzianum*	2018	Kuenzing (D)	*Z. mays* (cob) !
Tri3	*Trichoderma afroharzianum*	2018	Pocking (D)	*Z. mays* (cob) !
Tri4	*Trichoderma tomentosum*	2018	Altoetting (D)	*Z. mays* (cob) !
Tri5	*Trichoderma afroharzianum*	2019	Bernburg (D)	*Z. mays* (cob) !
IPP0316	*Trichoderma atroviride*	1976		Baby food
IPP0318	*Trichoderma harzianum*	1992	Mae Hia (T)	Soil
IPP0319	*Trichoderma harzianum*	1992	Chiang Mai (T)	Soil
IPP0320	*Trichoderma harzianum*	1992	Mae Hia (T)	Soil
Tri6	*Trichoderma harzianum*	2019	Kleinwanzleben (D)	*Z. mays* (cob)
Tri7	*Trichoderma harzianum*	2019	Grucking (D)	*Z. mays* (cob)
Tri8	*Trichoderma harzianum*	2019	Loeningen (D)	*Z. mays* (cob)
Tri9	*Trichoderma harzianum*	2019	Loeningen (D)	*Z. mays* (cob)
Tri10	*Trichoderma harzianum*	2019	Großumstadt (D)	*Z. mays* (cob)
Tri11	*Trichoderma harzianum*	2019	Pfaffenhofen (D)	*Z. mays* (cob)
Tri12	*Trichoderma harzianum*	2019	Pfaffenhofen (D)	*Z. mays* (stalk)
Tri14	*Trichoderma harzianum*	2019	Pfaffenhofen (D)	*Z. mays* (stalk)
T12*	*Trichoderma harzianum*	-	-	-
T39 (Trichodex)	*Trichoderma harzianum*	-	Tel Aviv (ISR)	-
CBS 124620	*Trichoderma afroharzianum*	-	Peru	*T. cacao*

F=France; D=Germany; T=Thailand; ISR=Israel; IPP-fungal collection of the Plant Pathology Division, University of Goettingen; CBS-fungal collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, NL; * provided by the Department of Phytopathology of Justus-Liebig-University, Gießen, Germany; ! (exclamation mark) indicating samples with *Trichoderma* disease symptoms

2.4 DNA extraction and phylogenetic analysis

Total DNA was extracted from lyophilized mycelium of single spore cultures by using a CTAB-based method described by Karlovsky and Brandfass (2008). Quality and quantity of the extracted DNA was assessed by agarose gel electrophoresis (60 min at 4.6V/cm) and stained with ethidium bromide. Partial translation elongation factor-*1α* (*tef-1α*) and internal transcribed spacer (ITS) were used to differentiate within the *Trichoderma harzianum* complex. Amplification was performed in a peqSTAR96 universal gradient thermocycler (PEQLAB, Erlangen, Germany) using 1:100 dilution of DNA extract in a total reaction volume of 25 µl. Marker genes *tef-1α* and *ITS* were amplified with the primers EF1 (ATGGGTAAGGARGACAAGAC) and EF2 (GGARGTACCAGTSATCATGTT) (O´Donnell et al., 1998) and ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990), respectively. Reactions were carried out in a mixture of standard Taq reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 at 25°C; NEB), 100 µM of each deoxyribonucleoside triphosphate, 0.3 µM of each primer, 0.62 U HotStart-polymerase (New England Biolabs) and 1 µL template DNA solution. The final $MgCl_2$ concentration was adjusted to 2 mM. The PCR cycling conditions for amplification of *tef-1α* included an initial denaturation for 30 s at 95°C; 30 cycles consisting of 30 s at 94°C, 30 s at 58°C, and 1 min at 68°C; and final extension for 5 min at 68°C. The PCR cycling conditions for amplification of *ITS* included an initial denaturation for 30 s at 95°C; 10 cycles consisting of 30 s at 94°C, a gradual decrease from 62°C to 53°C (-1°C/cycle) for 40 s, and 1 min at 68°C; 30 cycles of 30 s at 94°C, 40 s at 56°C, and 1 min at 68°C; and final extension for 5 min at 68°C. Species were identified by multiple alignment of each sequence with reference sequences using ClustalW (Thompson et al., 1994) in MEGA Version 7.0.2 (Kumar et al., 2016).

2.5 Statistical analysis

Statistical analysis was conducted with STATISTICA version 13 (Statistica GmbH, Germany). Differences between means of disease severity was analysed using the non-parametric Kruskal-Wallis ANOVA by 5% probability. Analysis of variance (ANOVA) for dry matter content was carried out, followed by Tukey's-HSD-test at the 5% probability level.

3 Results

3.1 Geographic origin of samples

In 2018, four isolates from four locations (Altoetting, Pocking, Kuenzing and Croix de Pardies) were obtained from maize cobs in Germany and France and subjected to further identification. In 2019, ten cobs from six locations, mainly from southern Germany, especially Bavaria and along the Rhine valley (Bernburg, Kleinwanzleben, Grucking, Loeningen, Großumstadt and Pfaffenhofen), were examined. In addition, two isolates from maize stalks from a single location (Pfaffenhofen) were obtained. Isolates Tri1, Tri2, Tri3 and Tri5 were isolated from maize cobs displaying strong *Trichoderma* infection, whereas the other *Trichoderma* isolates isolated from cobs or stalks did not induce any visual disease symptoms.

3.2 Species identification

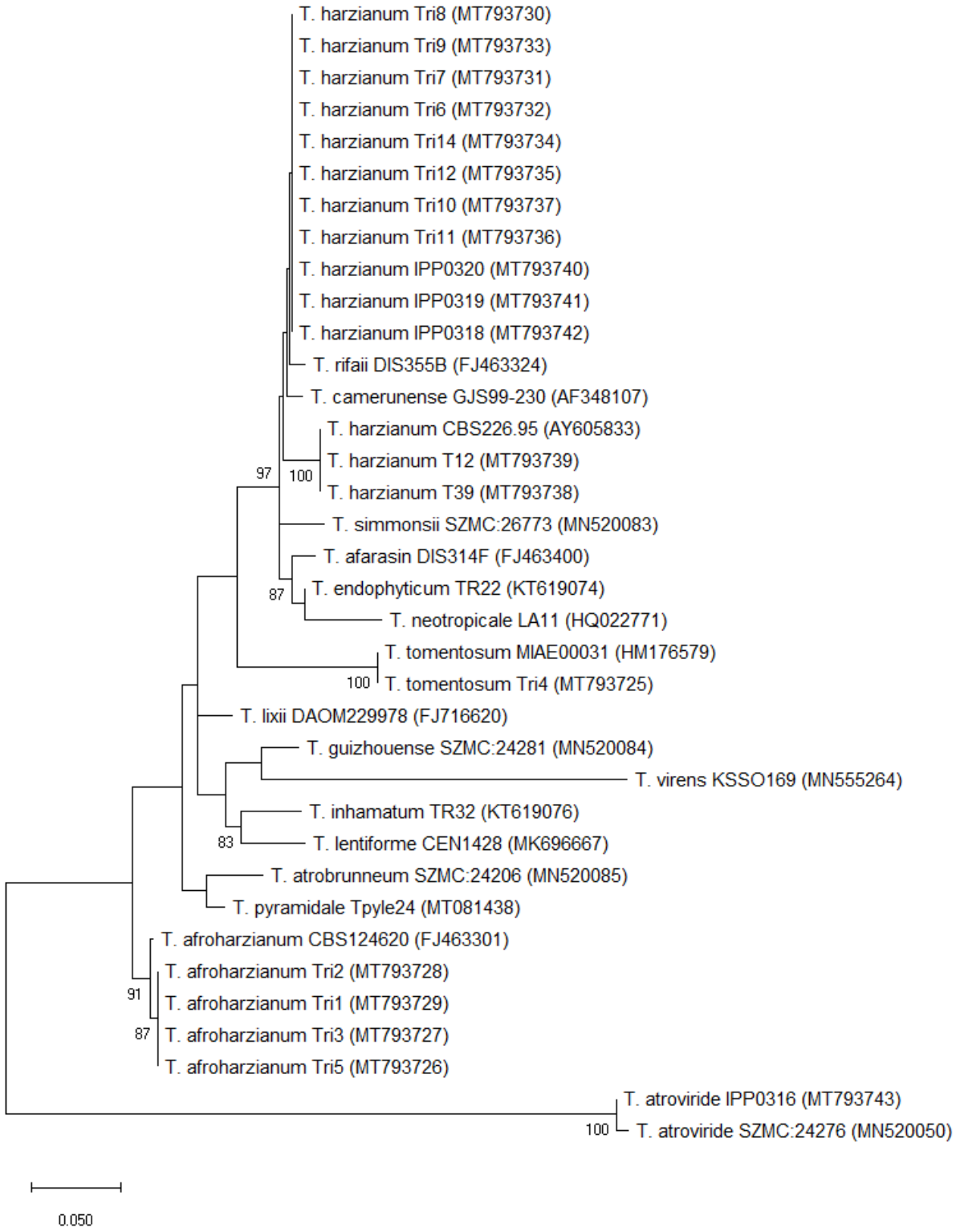

Figure 2. Molecular phylogenetic analysis of translation elongation factor 1 alpha (*tef-1α*) by maximum likelihood method (1000 bootstrap replicates) (Tamura & Nei, 1993).

The analysis was performed with MEGA version 7.0.26 (Kumar et al., 2016) with partial *tef-1α* sequences. The strains are listed in Table 1; GeneBank accession numbers of the sequences are shown in brackets. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are presented next to the nodes. Individual accession numbers are presented in Table S1. Sequence alignment is available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S26786).

A molecular phylogenetic analysis was performed with thirteen *Trichoderma* isolates from maize cobs and six isolates obtained from fungal culture collections, based on partial *tef1α* (Figure 2) and *ITS* (Figure S1). Sequences of 17 species in the *T. harzianum* complex Chaverri et al. (2015) were selected from GenBank. Multiple gene alignments assigned eight isolates (Tri6-Tri12, Tri14) to *T. harzianum*, four isolates (Tri1-Tri3, Tri5) to the clade *T. afroharzianum*, one isolate (Tri4) to *T. tomentosum.* Strains IPP0318-0320 were verified as *T. harzianum* and IPP0316 as *T. atroviride.* Isolates designated as *T. harzianum* clustered in two separate groups highly supported by bootstrap values. This result was largely confirmed by analysis of ITS sequences. Isolates Tri6-Tri12 an Tri14 showed high similarity to the selected references of *T. harzianum*, however, two isolates (T12 and T39) formed a separate clade, together with reference sequence of *T. harzianum* CBS 226.95 (AY605833). All obtained sequences of *T. afroharzianum* clustered in one phylogenetic group highly supported by bootstrapping and showed 99.4% (523 out of 526) nucleotide similarity to the *tef-1α* sequence of *T. afroharzianum* CBS124620 (FJ463301). Newly obtained sequences were deposited at Genbank under the accession numbers MT793725 to MT793743 for *tef-1α* and MT793744 to MT793762 for *ITS*, and sequence alignments were lodged at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S26786).

3.3 Disease symptoms and severity on maize ears

Trichoderma strains obtained from maize cobs displayed typical characteristics of this genus on PDA plates, such as initial growth of white mycelium, soon turning into green and grey-green colonies, while the reverse side of the Petri plates stayed uncoloured or light yellow. *Trichoderma* ear rot infection is characterized by white mycelium growing between the kernels and on the husk leaves with massive production of green to grey-green conidia. Under natural infection in the field, symptoms occurred from the base to the middle part of the cob, covered all kernels and all layers of husk leaves (Fig. 3 A-C). No mechanical damage or injuries by birds or insects were observed on infected cobs. After inoculation of ears in the greenhouse, the whole cob, inside and outside of the husk leaves was covered with mycelium and a green layer of conidia (Fig.3. D-F). Some infected cobs in the field as well as in the greenhouse showed premature ripening of the kernels (Fig.3. C, E).

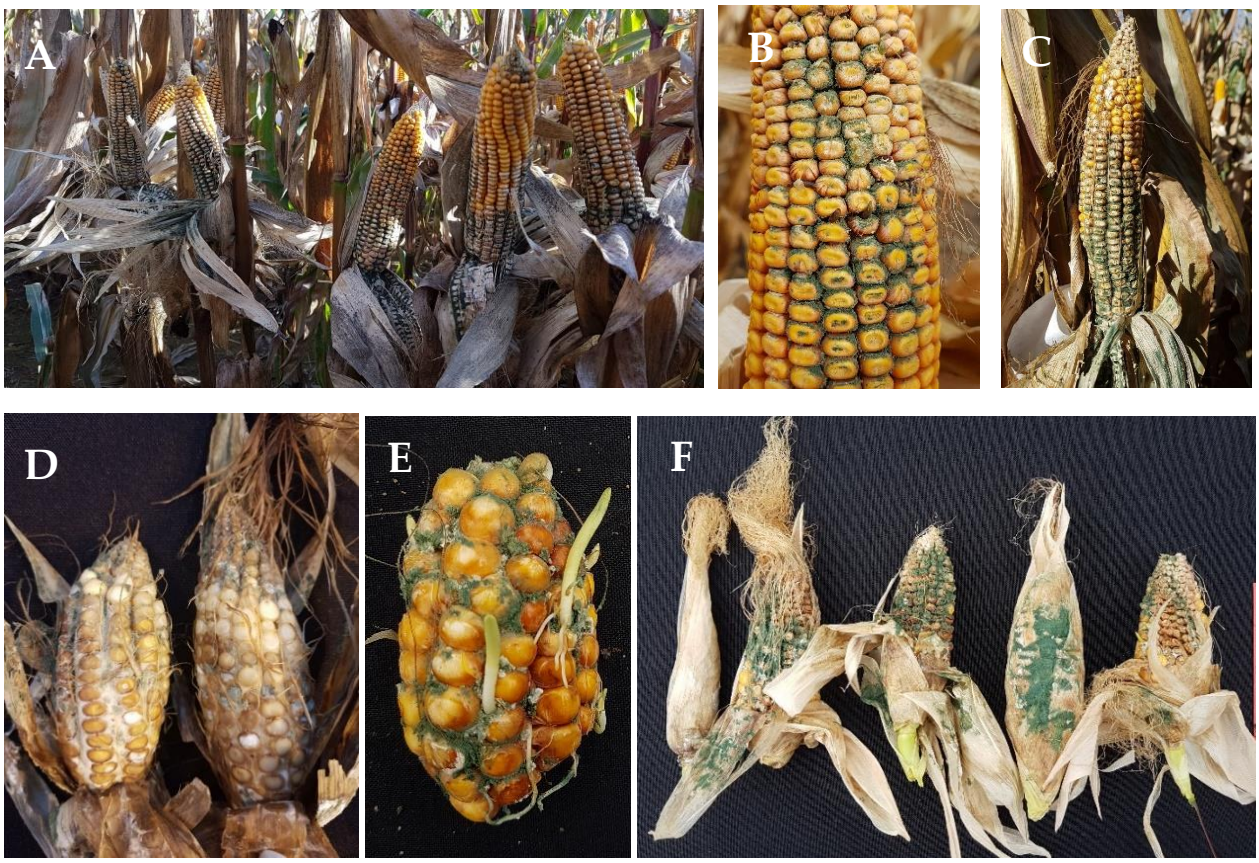

Figure 3. Disease symptoms of natural infection with *Trichoderma* sp. on maize cobs in the field, (A) at Künzing, (B) at Bernburg, (C) and at Altoetting; (A-C); and after silk channel inoculation in the greenhouse (D-F).

After artificial inoculation of the cob in the greenhouse, six isolates were pathogenic on maize ears and ten isolates did not induce any infection. The highest disease severity was observed with *T. afroharzianum* isolates Tri5 (96.0%), Tri1 (94.0%), Tri2 (91.1%) and Tri3 (78.0%), which overgrew the kernels and husk leaves (Table 2). Isolates T12 and T39 (Trichodex) lead to greenish discoloration only at the tip of the cob. The reference strains of *T. afroharzianum* (CBS 124620), *T. harzianum* (IPP0320, IPP0318) and the two remaining strains (IPP0316 and Tri4) as well as the *T. harzianum* isolates from maize cobs (Tri6, Tri7, Tri8, Tri10 and Tri11) were non-pathogenic on maize. Non-pathogenic strains did not cause any significant reduction in dry matter content compared to water treated, non-infected control cobs. However, the three most aggressive strains Tri2, Tri1 and Tri5 of *T. afroharzianum* caused significant losses in dry matter content of cobs 28 days after inoculation (Table 1).

Table 2. Disease severity (%) and dry matter content (%) of maize cobs, 28 days after inoculation. Means are given with standard deviation. Letters indicate significant differences between the isolates ($\alpha \leq 0.05$).

Isolate	Disease severity [%]	Dry matter content [%]
	Water	
Control	0.0 ± 0.0 a	47.9 ± 4.6 a
	T. harzianum	
IPP0320	0.0 ± 0.0 a	48.5 ± 2.5 a
Tri6	0.0 ± 0.0 a	46.5 ± 13.5 a
IPP0318	0.0 ± 0.0 a	46.2 ± 6.7 a

Tri7	0.0 ± 0.0 a	41.6 ± 4.7 a
T39	11.0 ± 6.5 b	40.4 ± 12.4 a
Tri11	0.0 ± 0.0 a	39.1 ± 12.9 a
Tri8	0.0 ± 0.0 a	37.2 ± 6.1 a
Tri10	0.0 ± 0.0 a	36.7 ± 7.9 a
T12	14.0 ± 10.8 b	36.7 ± 5.8 a
	T. afroharzianum	
CBS 124620	0.0 ± 0.0 a	39.7 ± 6.0 a
Tri1	94.0 ± 5.4 d	26.6 ± 4.1 b
Tri2	91.1 ± 10.5 d	20.9 ±7.7 b
Tri3	78.0 ± 22.8 c	34.3 ± 6.8 ab
Tri5	96.0 ± 8.9 d	20.3 ± 6.0 b
	others	
IPP0316 (*T. atroviride*)	0.0 ± 0.0 a	49.9 ± 15.0 a
Tri4 (*T. tomentosum*)	0.0 ± 0.0 a	45.7 ± 13.2 a

4 Discussion

Although *Trichoderma* is known as a plant symbiont or antagonist of fungal phytopathogens, our findings support previous observations in the US (Munkvold and White, 2016; N.N, 2018; Wise et al., 2016) that *Trichoderma* can infect maize cobs and cause ear rot diseases. Molecular-phylogenetic analysis of partial translation elongation factor-1α (*tef-1α*) and internal transcribes spacer (*ITS*) genes revealed pathogenic isolates as *T. afroharzianum*. To the best of our knowledge this is the first report on *T. afroharzianum* as an ear rot pathogen in Europe. The disease symptoms described in this study are in agreement with the observations by Wise et al. (2016) indicating that infection of *Trichoderma* appeared as white mold associated with massive production of green or grey-green conidia mass between the kernels and husk leaves, often involving the entire ear and causing premature ripening of the kernels. In contrast to the report by Munkvold and White (2016), *T. afroharzianum* infection, in the present study, did not require any previous damage on husk leaves and was not associated with injuries from birds or insects. Furthermore, artificial inoculation studies in the greenhouse confirmed that high disease severity occurred without any mechanical wounding after silk channel injection. Therefore, we conclude that *Trichoderma* ear rot infection is not a result of or promoted by mechanical injuries. This new pathogen cannot be regarded as a wound or opportunistic pathogen, as it is clearly able to infect kernel and husk leaf tissue and cause disease without any previous damages. We therefore propose to use the name ‚*Trichoderma* ear rot on maize' for this disease.

Highest disease severity and highest losses of dry matter content were observed after inoculation with three *T. afroharzianum* (Tri1, Tri2, Tri3 and Tri5) strains; however, the reference type strain of *T. afroharzianum* CBS 124620 did not induce any disease symptoms on maize but may have lost its pathogenicity during cultivation for over 15 years. Otherwise, this may indicate a phylogenetic separation on the species or subspecies level of pathogenic and non-pathogenic strains. Further research is required to clarify the genetic differences between these strains within the *T. afroharzianum* cluster. Surprisingly, a low-level pathogenicity of the two *T. harzianum* strains which were considered for use as biocontrol agents was recorded. Although these strains are usually applied to the soil for control of soilborne diseases, the pathogenicity found in our greenhouse experiment with cob inoculation

cannot be ignored with regard to risk analysis in the registration of *Trichoderma* strains as biocontrol agents. The question whether beneficial *Trichoderma* strains could mutate into aggressive plant pathogens is difficult to determine at this stage and requires further research.

Besides these findings, several epidemiological aspects about *Trichoderma* ear rot remain to be elucidated. Firstly, it is not known how the conidia of *Trichoderma* reach and infect the maize ears under natural conditions in the field, which sources of inoculum exist, and whether there are any alternative hosts. Secondly, there are no reports so far about the effect of weather conditions or agronomic practices possibly favoring infection with *Trichoderma*. The aggressive strains of *T. afroharzianum* were restricted to warmer regions with enhanced maize production in Southern Germany and along the Rhine valley. This is consistent with our previous observations indicating enhanced *Trichoderma* infection in the field in years with high mean temperatures and low precipitation such as in 2018 and 2019 in Germany. Furthermore, the potential production of mycotoxins by aggressive strains of *Trichoderma* awaits examination. Finally, field monitoring to explore the spread of the disease in Europe and yield loss analyses under field conditions are required to assess the economic significance of *Trichoderma* ear rot disease in maize production.

5 Conflict of Interest

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

6 Authors Contributions

Conceptualization. A.P.; methodology. A.P., S.S; validation. A.P.; S.S.; investigation. A.P.; S.S; resources. A.P.; data curation. A.P., S.S.; writing—original draft preparation A.P.; writing—review and editing. A.vT, P.K., S.S.; visualization. A.P., S.S.; supervision. A.vT.; project administration. A.vT., P.K.; funding acquisition. A.vT., P.K.; All authors have read and agreed to the published version of the manuscript.

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Chapter VI: Co-authored publication as part of the present investigations

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High-Resolution Melting (HRM) Curve Assay for the Identification of Eight *Fusarium* Species Causing Ear Rot in Maize

Simon Schiwek [1,*], Lukas Beule [1], Maria Vinas [2], Annette Pfordt [3], Andreas von Tiedemann [3] and Petr Karlovsky [1,*]

[1] Molecular Phytopathology and Mycotoxin Research, University of Goettingen, 37077 Goettingen, Germany; lukas.beule@agr.uni-goettingen.de (L.B.)
[2] Centro para Investigaciones en Granos y Semillas, University of Costa Rica, 2060 San Jose, Costa Rica; maria.vinasmeneses@ucr.ac.cr
[3] Plant Pathology and Crop Protection, University of Goettingen, 37077 Goettingen, Germany; annette.pfordt@uni-goettingen.de (A.P.); atiedem@gwdg.de (A.v.T.)
* Correspondence: simon.schiwek@uni-goettingen.de (S.S.); pkarlov@gwdg.de (P.K.)

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Abstract: Maize plants are often infected with fungal pathogens of the genus *Fusarium*. Taxonomic characterization of these species by microscopic examination of pure cultures or assignment to mating populations is time-consuming and requires specific expertise. Reliable taxonomic assignment may be strengthened by the analysis of DNA sequences. Species-specific PCR assays are available for most *Fusarium* pathogens, but the number of species that infect maize increases the labor and costs required for analysis. In this work, a diagnostic assay for major *Fusarium* pathogens of maize based on the analysis of melting curves of PCR amplicons was established. Short segments of genes *RPB2* and *TEF-1α*, which have been widely used in molecular taxonomy of *Fusarium*, were amplified with universal primers in a real-time thermocycler and high-resolution melting (HRM) curves of the products were recorded. Among major *Fusarium* pathogens of maize ears, *F. cerealis, F. culmorum, F. graminearum, F. equiseti, F. poae, F. temperatum, F. tricinctum*, and *F. verticillioides*, all species except for the pair *F. culmorum*/*F. graminearum* could be distinguished by HRM analysis of a 304 bp segment of the *RPB2* gene. The latter two species could be differentiated by HRM analysis of a 247 bp segment of the *TEF-1α* gene. The assay was validated with DNA extracted from pure cultures of fungal strains, successfully applied to total DNA extracted from infected maize ears and also to fungal mycelium that was added directly to the PCR master mix ("colony PCR"). HRM analysis thus offers a cost-efficient method suitable for the diagnosis of multiple fungal pathogens.

Keywords: *Fusarium*; high-resolution melting (HRM) curves, HRM analysis; maize ear rot; fungal colony PCR; *RPB2*; *TEF-1α*

1. Introduction

Infection of crop plants with *Fusarium* spp. causes yield losses and leads to contamination of grains with mycotoxins [1]. *Fusarium* ear rot and ear mold are cosmopolitan diseases of maize, caused by *Fusarium* species producing secondary metabolites toxic to mammals, which are called mycotoxins. The most important mycotoxins found in maize grains are trichothecenes, zearalenone, and fumonisins. These contaminants impair grain quality and pose a risk to food safety [2,3]. Pre-harvest ear rot disease of maize is characterized by the appearance of white or reddish fungal mycelium with rotting symptoms on the cob. The disease is classified into two groups: *Gibberella* ear rot, also known as red ear rot, which is caused predominantly by *F. graminearum*, and *Fusarium* ear rot, also known as *Fusarium* ear mold and pink ear rot, which is caused by *F. verticillioides.* The most important infection route of maize cobs for both pathogens is colonization of silks [4,5], while *F. verticillioides* can also systemically colonize plants [6–9]. Other *Fusarium* species such as *F. temperatum*, *F. subglutinans*, *F. poae*, *F. cerealis*, *F. tricinctum*, and *F. culmorum* have also been reported to infect maize plants [10–15]. In the past decade, mycotoxins primarily known from maize and their producers have been reported also in other crops. The infection of wheat [16,17] and asparagus [18] with fumonisin-producing species of the *Gibberella fujikuroi* species complex is well established. Other species, not previously known to be infected by *F. verticillioides* in the field, such as rice and sugar beet, were shown to be susceptible to the pathogen when artificially infected [19]. In addition, weeds in maize fields were found to be heavily colonized by *Fusarium* spp., which are pathogenic on maize [20]. Residues of these plants might be the source of infection of maize in the next season. Infestation of the ears [21] and roots [22] of maize by herbivores facilitates infection by breaking mechanical barriers and disseminating inoculum. Due to the multiplicity of sources of inoculum and the complexity of factors affecting infection, the contamination of maize with *Fusarium* toxins is highly variable and difficult to predict [11,14].

Identification of *Fusarium* species can be achieved via a combination of phenotypic characterization (micro-/macromorphology) [23], assignment to mating populations [24,25], and analysis of selected gene loci [26–28]. Furthermore, production of specific secondary metabolites can support taxonomic assignments of *Fusarium* spp. [29–31]. Phenotypic traits alone are often not sufficient for taxonomical classification at the species level, especially regarding members of species complexes such as the *F. fujikuroi* species complex (FFSC) [13] or the *F. oxysporum* species complex (FOSC) [32]. Molecular tools are, therefore, widely used. Species-specific PCR primers [33–35] and real-time PCR assays [31,36–39] have been developed for all economically important *Fusarium* species. Previous studies have reported that sequencing of several marker genes, such as the RNA polymerase II second largest subunit (*RPB2*), translation elongation factor 1 alpha (*TEF-1α*), and beta-tubulin (*β-TUB*) [26,28,40,41], enables reliable distinction at the species level. However, only minor nucleotide variations or single nucleotide polymorphisms (SNPs) may distinguish between closely related species, as was observed for the differentiation of *F. temperatum* from *F. subglutinans* [13]. Therefore, for robust taxonomical classification, the use of additional marker genes is recommended.

Species-specific PCR assays are available for all economically relevant *Fusarium* species, but carrying out numerous assays for each sample multiplies the costs. Multiplexing reduces the costs of polymerase and nucleotides (not the costs of primers), but it adds the need to separate the signals. Electrophoretic separation of PCR products is not scalable; therefore, fluorescence-based species-specific detection is used, but minisequencing [27] and double-labeled hybridization probes [36] significantly increase the costs of such assays.

Melting curve analysis is a closed-tube technique for the characterization of genetic variation in DNA amplicons based on the dissociation of double-stranded DNA with increasing temperature [42]. The amount of double-stranded DNA in each step is determined by the fluorescence of DNA-intercalating dye. High-resolution melting (HRM) curves generated with small temperature increments (commonly 0.1–0.2 °C) allow DNA fragments differing by as little as a single nucleotide to be distinguished. The entire analysis is carried out in the real-time PCR thermocycler that was used for the amplification. SYBR Green® is the standard dye used in real-time PCR, but EvaGreen® is used

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instead in melting curve analysis because it binds to all DNA base pairs [43]. Melting curves reflect not just GC composition, but also the sequence of the amplicon, and can therefore differentiate among amplicons with identical GC content. The analysis of DNA melting curves has successfully been applied in clinical medicine [44], virology [45], and in the identification of plants [46], insects [47], and phytopathogenic fungi [48,49].

In the present study, the suitability of melting curve analysis of short variable subsections of *RPB2* and *TEF-1α* genes for the differentiation of eight major *Fusarium* pathogens infecting maize ears in Germany [50] was established.

2. Results

2.1. HRM Analysis of sRPB2 *and* sTEF-1α *for the Identification of Fusarium Species*

Primers commonly used for the amplification of *RPB2* and *TEF-1α* in taxonomy amplify fragments that are too long for HRM analysis. Therefore, new primers were developed for the identification of eight major *Fusarium* species that cause ear rot in maize, namely *F. cerealis, F. culmorum, F. graminearum, F. equiseti*, *F. poae*, *F. temperatum*, *F. tricinctum*, and *F. verticillioides*, via HRM analysis (Table 1, Figure S1). Taxonomically characterized reference strains were used as standards (Table S1). Short variable segments of *RPB2* and *TEF-1α* genes, which we refer to herein as s*RPB2* (short*RPB2*, 304 bp) and s*TEF-1α* (short*TEF-1α*, 247 bp), respectively (Table 1), were used. s*RPB2* reliably distinguished *F. cerealis, F. equiseti*, *F. poae*, *F. temperatum*, *F. tricinctum*, and *F. verticillioides* (Figure 1, Figure 2C,E). The melting curves allowed secure discrimination of DNA of these pathogens extracted from pure culture as well as from naturally infected maize ears. s*RPB2* amplicons of *F. culmorum* and *F. equiseti* had similar melting temperatures but could be distinguished with an additional melting domain of the amplicon of *F. equiseti*, which caused a shoulder in the melting curve (Figure 2C). The differentiation between *F. culmorum* and *F. graminearum* was more difficult, due to highly similar melting curves. However, the melting curves of the s*TEF-1α* fragment allowed reliable differentiation between *F. culmorum* and *F. graminearum* DNA extracted from pure cultures as well as from infected maize cobs (Figure 2D,F). Therefore, HRM for the s*TEF-1α* fragment was included in the assay. Both s*RPB2* and s*TEF-1α* were amplified using identical PCR conditions. The simultaneous amplification within the same PCR run enabled the identification of all eight tested *Fusarium* species in a single HRM analysis (Figure 2C–F).

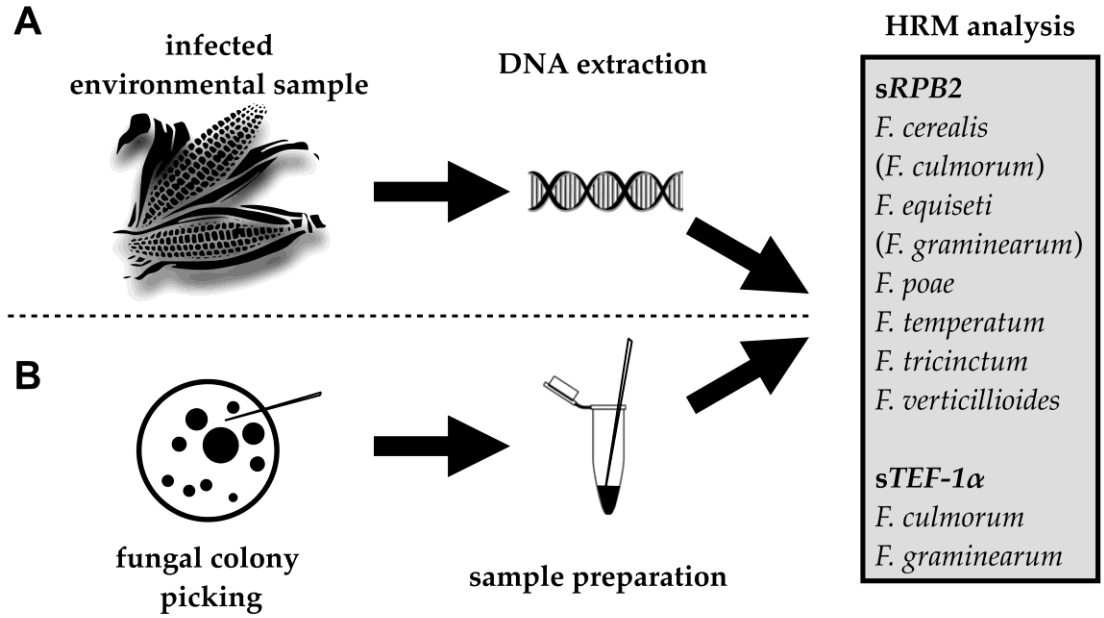

Figure 1. Workflow of high-resolution melting (HRM) curve analysis of eight major *Fusarium* pathogens of maize ears. (A) Identification of *Fusarium* species in infected maize ear samples; (B) identification or pure cultures using fungal colony PCR. s*RPB2* and s*TEF-1α* are short and variable subsections of *RPB2*

and *TEF-1α*, suitable for HRM analysis (Table 1). *Fusarium* species in brackets were not well distinguishable using the s*RPB2* assay.

Melting temperatures of PCR products of the reference strains are listed in Table S2. The GC content ranged from 49% to 54% for s*RPB2* and 49% to 50% for s*TEF-1α* (Table S2, Figure S1). No primer dimers or unspecific products were observed for the reference strains. Nucleotide sequences obtained for both s*RPB2* and s*TEF-1α* showed high similarity (≥ 85%) across the reference strains (Figure 2A,B, Figure S1). In total, 73 SNPs were found in s*RPB2* and 4 SNPs in s*TEF-1α* (Figure S1). Nucleotide polymorphisms were relatively evenly distributed across the length of s*RPB2* (Figure S1A), but clustered in s*TEF-1α* (Figure S1B). Comparing the number of DNA polymorphisms with the melting curves, we concluded that the minimum number of nucleotide differences sufficient for differentiation between two *Fusarium* species by melting curve analysis was four. In the reference strains of *F. graminearum* and *F. culmorum*, these differences occurred at nucleotide positions 102, 158, 188, and 190 of s*TEF-1α* (Figure S1B). In s*RPB2*, only two distant nucleotide positions differed between the reference strains of these species (nucleotide positions 104 and 275). Based on s*RPB2*, no reliable separation of *F. graminearum* and *F. culmorum* by HRM was possible. A maximum number of 46 SNPs were observed in s*RPB2* sequences of the reference strains for *F. temperatum* and *F. poae* (Figure S1A).

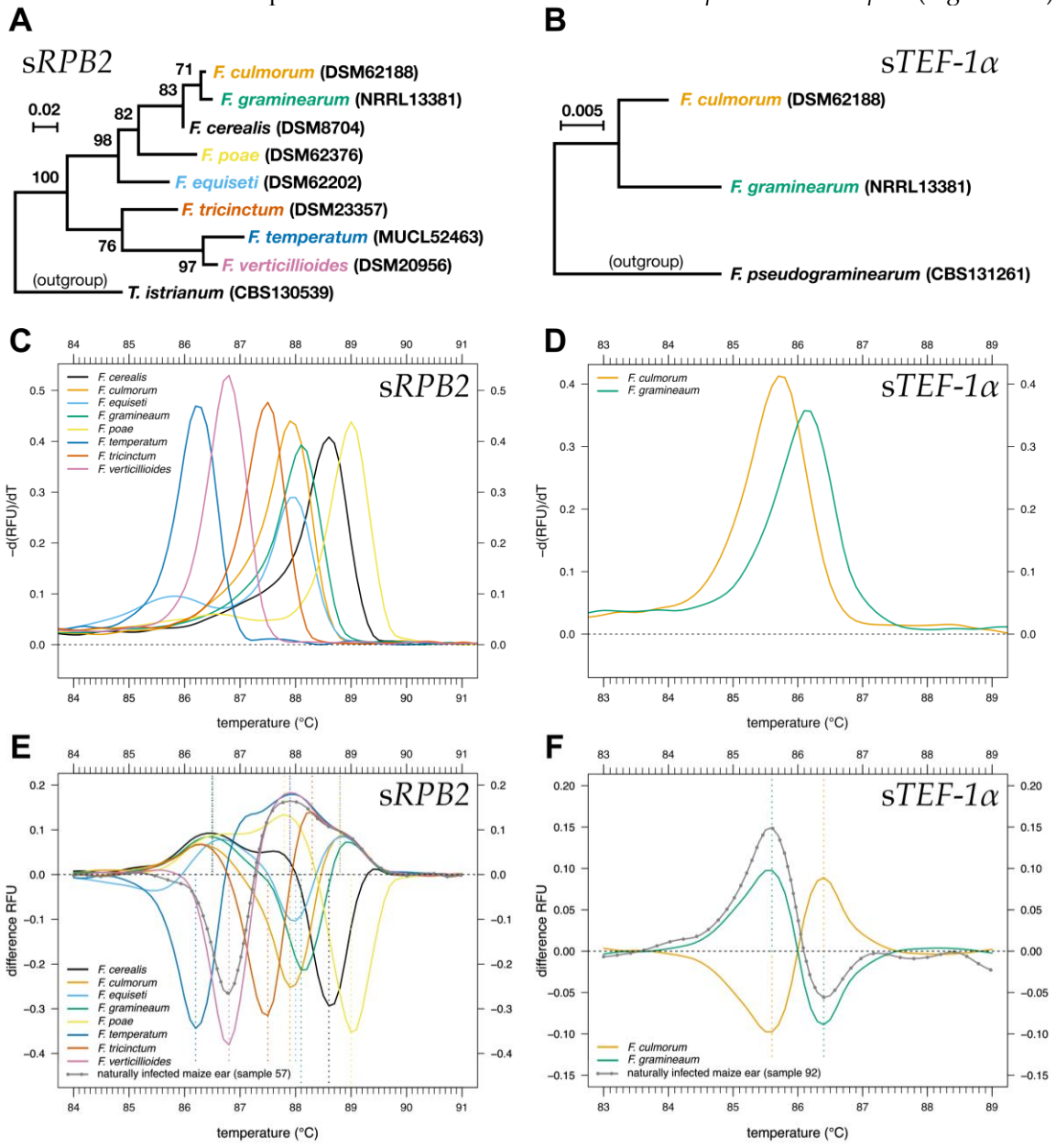

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Figure 2. Maximum likelihood analysis of the DNA sequences used for HRM analysis. (A) s*RPB2* sequences (1000 bootstrap replications) (B) s*TEF-1α* (without bootstrapping due to a low number of sequences (n = 3)). We included partial sequences of *RPB2* of *Trichoderma istrianum* CBS130539 (Accession KJ665281.1) and *TEF-1α* of *F. pseudograminearum* CBS131261 (Accession JX118971.1) as outgroup references. Melting curves using normalized relative fluorescence unit (RFU) data of s*RPB2* (C) and s*TEF-1α* (D). Melting curves were generated as negative first derivative (-d(RFU)/d(T)) of relative fluorescence. Difference curves of the reference strains and a naturally infected maize ear for s*RPB2* (E) and s*TEF-1α* (F) are shown. The difference curves were obtained by subtracting melting curve data of each reference strain or environmental sample from the mean melting curves of all reference strains (dashed horizontal line). Vertical dashed lines indicate the maximum and minimum for each reference strain. s*RPB2* and s*TEF-1α* are short subsections of *RPB2* and *TEF-1α* (Table 1).

The specificity of the assay was assessed by determining the melting temperatures of s*RPB2* and s*TEF-1α* amplicons for an additional 12 *Fusarium* species (Table S4). Except for four species, melting temperatures of s*RPB2* amplicons differed from the melting temperatures of s*RPB2* of all target species (Table S2) by more than 0.15 °C. Only *F. redolens*, *F. proliferatum*, *F. fujikuroi*, and *F. avenaceum* could not be differentiated from some of the target species by the melting temperature of s*RPB2*. The amplification of s*TEF-1α* failed for *F. redolens* and *F. avenaceum* (Table S4), distinguishing them from the target species. *F. proliferatum* and *F. fujikuroi* could not be distinguised from *F. temperatum* by melting temperatures of s*RPB2* or s*TEF-1α* amplicons; it has to be noted that *F. fujikuroi* does not infect maize.

Both amplicons, used to generate melting curves, were generated with primer pairs consisting of a well-established primer (RPB2-5F2 and EF1αR) and a new primer designed for this study (RPB2-5R1s and TEF-1aFs2). The presence of binding sites for the established primers in all *Fusarium* spp. has been documented in numerous studies but the robustness of the newly designed primers was unknown. Nucleotide variation in binding sites might lead to a failure of the method with field isolates. To assess primer binding to DNA from other strains, 64 sequences of *RPB2* or *TEF-1α* from isolates of target species were retrieved and aligned with the sequences of newly designed primers. Not a single mismatch was found; the list of the sequences is provided in Table S5. Regarding *F. graminearum* Schwabe, many genetic lineages of this traditional species have been defined as species, although the boundaries of the new species are incongruent with the biological species concept and remain controversial [23]. To check for binding of newly designed primers to target sequences from these lineages, we retrieved 28 sequences of *RPB2* and *TEF-1α* from members of seven phylogenetic lineages of *F. graminearum* sensu lato. Aligning the sequences with primers RPB2-5R1s or TEF-1aFs2 did not reveal any mismatch (Table S6).

2.2. Identification of Fusarium Species in Naturally Infected Maize Ears

The HRM assay was evaluated by screening DNA samples extracted from 100 maize ears naturally infected with *Fusarium* spp. (Figure 1A), which were sampled from across Germany (Figure S2). Morphological examination of fungal strains isolated from these ears prior to DNA extraction revealed that most ears were infected with several *Fusarium* species. HRM analysis successfully identified the most abundant *Fusarium* species in 80% of the ears (Table S3). The majority of maize ears (62%) were infected with *F. graminearum*, followed by *F. verticillioides* (10%), *F. temperatum* (6%), and *F. poae* (2%).

2.3. Fungal Colony PCR

All eight reference strains of *Fusarium* were successfully identified via HRM analysis after colony PCR (Figure 3). Boiling a small piece of mycelium picked from an agar plate for 10 min in 100 µL H_2O released sufficient amounts of DNA for amplification. The use of larger amounts of mycelium for DNA preparation by boiling occasionally led to the inhibition of PCR; thus, a very small piece of mycelium (just visible by the naked eye) was sufficient. The inhibition of PCR by mycelial extracts was particularly pronounced for *F. poae*: Extracts of 100 µg mycelium (dry weight) boiled in 100 µl water always inhibited PCR, while extracts of 10 µg mycelium reliably generated the desired amplicons.

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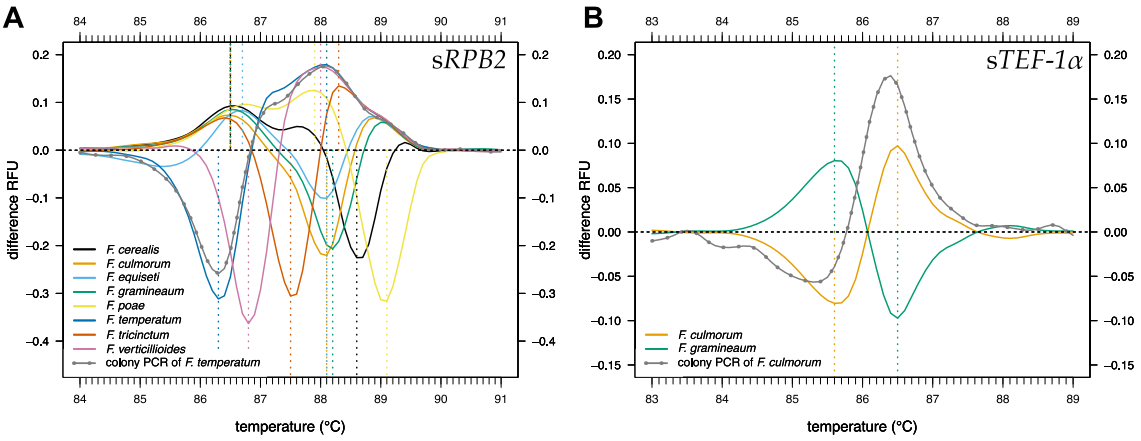

Figure 3. Fungal colony PCR followed by high-resolution melting (HRM) curve analysis of (A) s*RPB2* and (B) s*TEF-1α*. Difference curves were obtained by subtracting melting curves of each reference strain from the mean melting curve data of all reference strains (dashed horizontal line). Vertical dashed lines indicate the maximum and minimum of the curve for each reference strain. s*RPB2* and s*TEF-1α* are short subsections of *RPB2* and *TEF-1α* (Table 1).

3. Discussion

Analysis of melting curves of PCR products has been used previously to diagnose pathogens [51,52], including a duplex assay for two *Fusarium* species [37] and intraspecific differentiation within a *Fusarium* species complex [49]. In this work, the analysis of melting curves of PCR amplicons was exploited for the development of a multiplex diagnostic assay. *Fusarium* species commonly infecting maize ears in Germany [50] were selected for the implementation of the concept, but disease diagnosis in many crops faces the same challenge: numerous pathogens can infect the crop, though only a single pathogen or a few pathogens are typically found in each sample. For instance, many *Fusarium* pathogens can be isolated from ears of small grain cereals afflicted with *Fusarium* Head Blight [53]. To overcome the limit of multiplexing species-specific PCR, detection of PCR products by hybridization to an array of DNA targets has been suggested. The concept has been successfully implemented for several systems, including the differentiation among members of the *Fusarium solani* species complex of pathogens of solanaceous plants [54] and identification of numerous *Pythium* spp. [55]. Detection by hybridization allows high-level multiplexing, but the specificity of hybridization is lower than the specificity of PCR or melting curve analysis; careful optimization of hybridization conditions is required to prevent the hybridization of DNA of a single pathogen with several targets. A powerful PCR-based diagnostic system with high-level multiplexing and quantitative detection has been developed by BioTrove [56]. The method requires a complex proprietary instrumentation, which seems to no longer be available since the acquisition of BioTrove by Life Technologies in 2009 and the acquisition of Life Technologies by Thermo Fisher in 2014.

Among the molecular sequences used in *Fusarium* taxonomy, *RPB2* and *TEF-1α* have been used most frequently [57,58]. The distinction between similar sequences by HRM relies on differences in GC content, amplicon length, and the sequence [44]. In order to maximize the specificity of HRM analysis, short and highly polymorphic regions are used as amplicons [45,49,50]. The sequences of *RPB2* and *TEF-1α* genes used in the molecular taxonomy of *Fusarium* are too long for HRM; therefore, segments of the genes flanked by an established primer on one end and a new primer on the other end were amplified (Table 1). The location of both primers in highly conserved regions reduces the chance that the assay may fail for new isolates because of the lack of primer binding.

The assay fulfilled the purpose of detecting and distinguishing all eight major *Fusarium* pathogens infecting maize ears in Germany. DNA from some minor pathogens or saprophytes might generate indistinguishable melting curves, causing false positive signals. Due to their low abundance, however, the impact of these false positives on decisions about crop protection is expected to be negligible. To

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assess the specificity of the assay, melting temperatures of s*RPB2* and s*TEF-1α* amplicons were determined for an additional 12 *Fusarium* species (Table S4). Only *F. fujikuroi* and *F. proliferatum* could not be differentiated from the target species. *F. fujikuroi* does not colonize maize. *F. proliferatum* infects maize in some growing areas [5,11,12,15], but recent studies reported the species to be at a low abundance on maize in Poland [14], and essentially missing from maize in Germany [50]. Because the melting curves of s*RPB2* and s*TEF-1α* cannot distinguish between *F. proliferatum* and *F. temperatum*, an additional amplicon would be needed for the extension of the assay to *F. proliferatum*.

In addition to the identification of eight *Fusarium* species by HRM analysis of s*RPB2* and s*TEF-1α* amplicons, the assay proved suitable for the identification of dominant pathogens in DNA extracted from naturally infected maize ears and for the identification of fungal colonies without DNA extraction by HRM followed by colony PCR. This shows that the technique is sufficiently robust to be used in routine diagnosis. Two of the eight species could not be discriminated by HRM analysis of a single amplicon, but were reliably distinguished by the melting profiles of another amplicon. An extension of the assay to further *Fusarium* species may require the integration of further amplicons, which could originate from the same gene or from other genes. The relatively high level of multiplexing and the simplicity of HRM assays, which work with universal primers and consist of a single run on a real-time thermocycler without further sample processing, truly compensates for the need to integrate additional amplicons with growing numbers of target species. The costs of HRM assays are lower than the costs of multiplex PCR with species-specific primers, TaqMan probes, hybridization of PCR products to immobilized species-specific targets, or DNA sequencing, let alone advanced technologies such as BioTrove's OpenArray.

Field samples are often infected with multiple pathogens. The fact that our HRM assay is based on PCR primers that amplify DNA from multiple *Fusarium* species leads to two potential problems. First, amplicons in DNA extracted from samples infected with multiple pathogens will compete for primers, nucleotides, and DNA polymerase. The amplification of abundant pathogens may thus suppress the amplification of minor pathogens, preventing their detection. For the use of the assay in crop production, this does not pose a problem, because plant protection focuses on major pathogens. The second issue is that co-amplification of multiple amplicons may lead to the formation of hybrids, which melt at lower temperatures than the parent molecules. The presence of hybrids in amplification products will complicate the HRM patterns. Whether, and to what extent these hybrids may interfere with the assignment of amplicons/curves to taxa has to be further investigated. In the analysis of 100 naturally infected maize ears reported here, melting curves of hybrid amplicons were not detected. We suggest that this can be accounted for by the unequal abundance of pathogens in ears with mixed infection. Extrapolating the frequency of detection of dominant pathogens (see Section 2.2) to mixed infections, 6% of ears were likely infected concomitantly with the two most dominant pathogens, *F. graminearum* and *F. verticillioides*. If the abundance of pathogens in the infected ears was unequal, the melting curve of the less abundant pathogen and the melting curves of hybrid amplicons likely escaped detection. This will not pose a problem when the assay is used to guide crop protection against major pathogens.

In monitoring programs that include isolation of fungal strains, melting curve analysis of amplicons generated by colony PCR might be used to identify minor pathogens or resolve ambiguous results of melting curve analysis of samples with mixed infection. The advantage of this approach is that the same technique is used for both the original analysis and the follow-up analysis of problematic samples. If melting curves are used in a routine diagnostic pipeline, the products of colony PCR can simply be inserted into the pipeline to be analyzed with the next sample batch.

4. Material and Methods

4.1. Reference Strains, Sample Collection, and DNA Extraction

We selected eight *Fusarium* species (*F. cerealis, F. culmorum, F. equiseti, F. graminearum, F. poae, F. temperatum, F. tricinctum,* and *F. verticillioides*) for identification via HRM analysis. For each species,

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a reference strain (Table S1) was cultivated on potato dextrose agar plates for 5 to 7 days at 25 °C in the dark. For the comparison of melting temperature of amplicons, strains of 12 additional *Fusarium* spp. (Table S4) were grown in the same way. Mycelium was carefully scrubbed from the surface of the plates and lyophilized. In addition to reference strains, 100 naturally *Fusarium*-infected ears were collected from silage and grain maize, harvested at 30 field sites (one to nine ears per site) in seven federal states across Germany in 2017 (Figure S2). Ears were crushed, lyophilized, and finely ground to 1 mm using an ultra-centrifugal mill (ZM 200, Retsch, Haan, Germany). DNA from 30 mg naturally infected maize ears, as well as lyophilized mycelium of the reference strains, was extracted using a cetyltrimethylammonium bromide (CTAB)-based protocol [37]. Quality and quantity of the extracted DNA were assessed on agarose gels (0.8% (w/v) in 1 × Tris-acetate-EDTA buffer) stained with ethidium bromide. Gel electrophoresis was carried out for 60 min at 4.6 V/cm.

4.2. Fungal Colony PCR

Reference strains of *Fusarium* were cultivated as described above (see Section 4.1.), and aerial mycelium was carefully scrubbed from the surface of the plates using sterilized toothpicks and placed into a 1.5 mL tube containing 100 µL double-distilled water (ddH_2O). For *F. cerealis, F. culmorum, F. graminearum, F. equiseti, F. temperatum, F. tricinctum*, and *F. verticillioides*, the mixture was incubated at 100 °C for 10 min and subsequently centrifuged at 16,000 × *g* for 30 s to pellet the mycelium. The obtained supernatant was transferred into a new 1.5 mL tube and directly used for PCR. For *F. poae*, the mycelium was briefly (approximately 10 s) introduced to the ddH_2O. The mycelium was largely removed using the toothpick and colony PCR was performed from the remaining ddH_2O without any further processing.

4.3. Primer Design and Maximum Likelihood Tree Analysis

We selected the *RPB2* and *TEF-1α* region for HRM analysis. In order to design primers flanking short and variable subsections of *RPB2* and *TEF-1α*, we first amplified both regions for the eight reference strains of *Fusarium* (Table S1), as described by Lofgren *et al.* [59] for *RPB2* and O'Donnell *et al.* [60] for *TEF-1α*. PCR products were purified and sent to Macrogen Europe for Sanger sequencing (Macrogen Europe B.V., Amsterdam, the Netherlands). The results were evaluated with Chromas version 2.6.6 (Technelysium Pty Ltd, South Brisbane, Australia). Multiple sequence alignment was then performed using ClustalW [61] in MEGA version 7.0.26 [62]. Alignments were processed in T-Coffee version 11.00 [63] and ESPript version 3.0 [64] (Figure S1). Two new primers suitable for HRM analysis were designed based on multiple gene alignments using the sequences of our eight reference strains (Table 1). Primer binding sites were conserved among species. We hereinafter refer to the selected subsections as s*RPB2* and s*TEF-1α*. The amplicon length was 304 bp for s*RPB2* and 247 bp for s*TEF-1α* (Table 1). Finally, a maximum likelihood analysis was conducted for s*RPB2* (1000 bootstrap replications) and s*TEF-1α* (without bootstrapping due to low sample size (n = 3)) using MEGA 7.0.26.

Table 1. Primers used in this study.

Name	Sequence (5'–3')	Gene	Amplicon Length (bp)	Reference
RPB2-5F2	GGGGWGAYCAGAAGAAGGC	*RPB2*	1200	[59]
RPB2-7CR	CCCATRGCTTGYTTRCCCAT			
EF1αF	ATGGGTAAGGARGACAAGAC	*TEF-1α*	694	[60]
EF1αR	GGARGTACCAGTRATCATGTT			
RPB2-5R1s	TCAACVACTTCCATACCTC	s*RPB2**	304 (with RPB2-5F2)	This study
TEF-1aFs2	CAATAGGAAGCCGCYGAG	s*TEF-1α**	247 (with EF1αR)	This study

* Short and variable subsections of *RPB2* and *TEF-1α*, which were selected for high-resolution melting (HRM) curve analysis.

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4.4. HRM Analysis

PCR conditions were optimized using gradients of annealing temperature and final $MgCl_2$ concentration. Amplification was performed in a CFX384 Thermocycler (Biorad, Rüdigheim, Germany) in 384 well microplates (SARSTEDT AG & Co. KG, Nümbrecht, Germany) with a total reaction volume of 4 µL. Reaction mixtures were composed of 1 µL template DNA or ddH_2O for negative controls and 3 µL of reaction mixture (reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8 at 25 °C); 0.5 mM $MgCl_2$, 200 µM of each deoxyribonucleoside triphosphate (Bioline, Luckenwalde, Germany), 0.3 µM of each forward and reverse primer (Table 1); 3.3-time diluted EvaGreen® solution (Jena Bioscience, Jena, Germany); 1 mg/mL bovine serum albumin, and 0.03 U Taq DNA Polymerase (New England Biolabs, Beverly, MA, USA)). Template DNA of the reference strains was adjusted to 100 pg μL^{-1} in ddH_2O using gel densitometry [31]. DNA from naturally infected maize ears was diluted 1:100 in ddH_2O before use in PCR. For fungal colony PCR, DNA was obtained as described in Section 4.2. Thermocycling conditions were as follows: 95 °C for 2 min (initial denaturation), 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 68 °C for 30 s. Final elongation was performed at 68 °C for 5 min. Following this, samples were heated to 95 °C for 30 s and cooled to 55 °C for 60 s. For fungal colony PCR, 35 cycles were performed. Fluorescence data for HRM analysis was obtained by increasing the temperature step-wise from 65 °C to 95 °C at 0.1 °C for 5 s per step with continuous fluorescence measurement. Reference strains of *Fusarium* species and negative controls (ddH_2O) were amplified in triplicate.

4.5. Fluorescence Data Processing and Taxonomic Assignment

Relative fluorescence unit (RFU) data were obtained from CFX Maestro™ Software (Bio-Rad CFX Maestro 1.1 version 4.1.2433.1219) Biorad, Rüdigheim, Germany and analyzed in the R environment (version 3.6.1) [65]. RFU data were normalized by scaling all RFU values of each sample between 0 and 1. The negative first derivative (-d(RFU)/d(T)) was calculated employing the "diffQ2"-function in the R-package "MBmca" (version 0.0.3-5) and plotted to obtain normalized melting curves. The melting curves of the reference strains were generated from the mean fluorescence of the three technical replicates. Difference curves were obtained by subtracting the melting curve data of each reference strain or environmental sample from the mean melting curve data of all reference strains. Taxonomic identification was performed manually by carefully comparing the difference curves of the environmental samples against the reference strains.

5. Conclusions

Analysis of high-resolution melting (HRM) curves for the identification of *Fusarium* pathogens in plant material is an attractive technique for routine diagnostics in plant protection because it is cost-efficient, does not require any post-thermocycle sample processing, and allows multiplexing.

Supplementary Materials: The following materials are available online at www.mdpi.com: Figure S1: Multiple sequence alignment of s*RPB2* and s*TEF-1α*, Figure S2: Sampling sites of naturally *Fusarium*-infected maize ears across Germany, Table S1: Reference strains of *Fusarium spp.* used in this study, Table S2: Melting temperature and GC-content of s*RPB2* and s*TEF-1α* amplicons of reference strains, Table S3: Species identification in naturally infected maize ears, Table S4: Melting temperature of s*RPB2* and s*TEF-1α* amplicons of additional *Fusarium* species, Table S5 and Table S6: Target sequences checked for binding of newly designed primers.

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Chapter VII: General discussion

Maize is one of the most important crops in Europe, covering a production area of approximately 17 M hectares in 2018 [1]. Maize is cultivated for different purposes, such as grain production for food and feed, and as green maize for silage feed or as substrate for biogas production. Grain maize production dominates in Central and Southern Europe, while maize in Northern Europe is typically grown to produce maize silage [2].

Fungal ear rot diseases of most concern are caused by species of the genera *Aspergillus*, *Fusarium* and *Penicillium* producing various mycotoxins like aflatoxin, fumonisin and ochratoxin [3–6]. *Fusarium* spp. causing ear, stalk and root rot were rated as the economically most significant diseases worldwide [7]. Savary et al. (2019) [8] estimated the future global yield loss in maize caused by *Fusarium* stalk rot to be approximately 5% and *Fusarium* ear rot at around 2.5%. The primary mycotoxins produced by these *Fusarium* spp. are fumonisin, trichothecenes (e.g., deoxynivalenol, nivalenol, T-2, HT-2), and zearalenone, which lead to the contamination of both, human food and animal feed [9–11].

Main conclusions

I Continuous research on the range of *Fusarium* species occurring on maize and on the impact of agronomical practices and changing weather conditions is important to prevent mycotoxin contamination in food and feed products.

Agronomical practices such as crop rotation and tillage as well as other practices of integrated pest managemen, can affect survival and dispersal of the above mentioned pathogens and potentially minimize the risk of mycotoxin contamination [12–14].

The results of our study indicate that the incidence of local *Fusarium* species on ears and stalks is highly affected by conventional ploughing compared to chisel ploughing or no tillage. In particular, most *Fusarium* species causing ear and stalk rot were reduced through conventional ploughing. These findings have been confirmed by several previous reports [12,15], which demonstrated that most *Fusarium* species were reduced after moldboard ploughing as compared to reduced tillage. In contrast, we observed no or only little impact of

crop rotation on the frequency of *Fusarium* species on ears and stalks. *Fusarium* spp. can survive as mycelium and clamydospores on residues of these crops as well as on senescent tissue of other crop or weed species, which may later serve as primary inoculum for infection [16]. The most important source of inoculum for *Fusarium* spp. is plant debris, especially maize stalks. Covering crop residues with soil enhances their decomposition by promoting microbial activity which reduces inoculum density [17,18]. Therefore, management of surface residues of previous crops by tillage and wide crop rotations with non-host crops are suggested as important strategies to control ear and stalk rot diseases [19]. Although integrated pest management strategies are important for disease control, the current maize cultivation practices in Europe shows that the opposite is the case. Intensification of agricultural production as well as expansion of maize acreage in Europe lead to short crop rotations with maize and wheat dominated rotation systems [2,20]. The current status of maize production in Europe and the effect on pests and diseases was evaluated by Meissle et al. (2010) [20]. They collected data from eleven European maize growing regions on the use of (i) integrated pest management methods (IPM), (ii) crop rotation range and (iii) soil tillage. Guidelines for IPM exist in all considered regions, however, the fulfillment of guidelines varied strongly between countries. Denmark reported to conduct 100% IPM whereas other countries like Germany, Spain and Italy implement IPM strategies on less than half of their maize production area and even no IPM was reported from Poland. Crop rotation ranged from continuous growth of maize (80%) in southwest Poland, Spain and Hungary, to well-planned rotation systems. Short crop rotation with winter wheat and even mono-maize systems were also reported from Vasileiadis et al. (2011) in Europe [19]. Ploughing was the most often reported soil tillage method. More than 80% of all maize production areas were cultivated by conventional tillage.

Fusarium ear and stalk rot were evaluated as the economically most important diseases in maize in Europe with particular importance in Italy, Spain, Germany, Netherlands and Poland [20]. While *Fusarium* severity remains unchanged in France, Denmark and Hungary, disease severity and significance of *Fusarium* infections increased in Germany and Southwest Poland [20]. However, ear rot infection in Spain decreased in recent years due to the fact that almost 30-35% acreage (115.246 ha in 2018) is cultivated with Bt-maize [21–23]. This prevents plants from feeding of the European corn borer wich is often associated with secondary

infection with *Fusarium* spp. The increase of *Fusarium* infection in Germany could result from an increase of silage maize production of about 70% (2000 to 2014) in the last two decades due to the renewable energy law (EEG) [24,25]. This lead to shorter maize and wheat-dominated rotation systems, which enhance the risk of infection with *Fusarium* spp. in wheat and maize as well as infestation with the European corn borer [21,26,27].

In addition to agronomical practices, weather conditions, particularly precipitation and temperature have a major impact on disease severity and the incidence of *Fusarium* spp. High incidence of *F. graminearum* and *F. culmorum* was observed in 2016 and 2017 due to moderate average temperatures (18.8°C) and high precipitation in July (110 mm). In contrast, *F. verticillioides* was the prevailing species in 2018 and 2019 (unpublished data), most likely due to higher mean temperatures (20.6°C) and dry conditions (40 mm in July). We assume that increasing temperatures and dry periods in summer can shift the prevalence of *Fusarium* species and increase the risk of contamination with fumonisin-producing species such as *F. verticillioides* and *F. temperatum* [28]. In addition, feeding by the European corn borer (*Ostrinia nubilalis*) and the Western corn rootworm (*Diabrotica virgifera*) in Germany will likely further enhance disease incidence and mycotoxin contamination of ears and stalks as well as root rots in maize [26,29,30].

The occurrence and significance of pests and diseases are affected by the shift of warming and other climatic conditions resulting in (i) range expansion or retreat, (ii) coincidence of pathogen life cycle stages with host plant stages and (iii) changes in population dynamics such as over-wintering or the number of generations per year [3,31–34]. The two most important factors which affect the life cycle of all microorganisms including ear rot pathogens are water availability and temperature [35,36]. Both factors influence the infection and colonization in different ways, which could lead to a shift in the comparative abundance of the species [31] and appearance of new species with higher levels of aggressiveness and altered mycotoxin profiles [37].

Therefore, knowledge about the impact of environmental factors on the ability of fungi to grow, survive and interact with plants is important in order to better understand the variation in the population dynamic of *Fusarium* species and their ability to produce mycotoxins [33]. Ear rot of maize can be caused by several different species with different environmental optima. Considering this, individual species can be easily replaced by others,

which are better adapted to the certain environment [3,4]. Thereby, no change of the overall disease symptoms are observed, however, it results in a dramatic shift towards more harmful mycotoxins in maize grain [38–40]. Europe is characterized by a wide range of diverse climatic conditions leading to differences in the occurrence of *Fusarium* species associated with *Fusarium* head blight (FBH) of cereals [41,42]. During the last decades, *F. culmorum* was the dominant *Fusarium* species in FHB complex in cooler temperate climates like Finland, UK, Germany and Poland. However, *F. graminearum* occurred more often in Central and Southern Europe, especially Northern Italy, Spain, Portugal and Southern France [41,43,44]. This situation changed significantly in the early 2000s, when *F. graminearum* became the most abundant species on wheat and oats in the Netherlands and UK [37,45] as well as Poland [28,46] due to slightly increasing temperatures in Northern Europe [42,47]. Such overall shift in the *Fusarium* species complex has not yet been observed in maize from Europe, however, the present study indicates a high year to year variability of the individual species and a recent shift towards *F. verticillioides* and *F. temperatum*.

The present results emphasize the importance of further studies on the impact of changing climatic conditions and their interplay with cultural practices on the development of *Fusarium* populations and the mycotoxin contamination of maize. This becomes even more important as the maize acreage is further expanded, often in combination with shorter crop rotations with other small grain cereals. The key factor to reach this goal is the implementation of integrated pest management (IPM) schemes, including the choice of varieties and the development of more specific pesticides treatments [2,19]. Another strategy to lower the risk of *Fusarium* infection is to prevent feeding damage by the European corn borer through the cultivation of Bt maize, use of chemical insecticides or biological control with *Trichogramma* spp. [48–53]. In addition, the exposure of maize cobs to humid conditions in autumn can be reduced by early planting and harvesting or the use of early maturing varieties [54,55]. Balanced fertilizing (200 kg/ha) resulted in lower mycotoxin contamination in an Italian study [56]. However, the most important measure remains reducing the amount of initial inoculum by wide crop rotations with non-host crops and deep ploughing of infected residues [12,54,57].

II Reliable taxonomic assignments are fundamental for differentiatiing *Fusarium* species on the species level to better estimate virulence and toxicological risks of individual species.

Results obtained in this study indicate a higher toxigenic risk by an infection with *F. temperatum* compared to *F. subglutinans* based on the production of beauvericin, fumonisin, fusaric acid and fusaproliferin by the former species. Considering this, infection with *F. temperatum* is particularly important for growers, processing industries and consumers due to its prevalence in Germany, its higher aggressiveness on maize cobs and the ability to cause FHB in wheat. The correct identification of the *Fusarium* species contaminating maize in different areas in Germany is important, not only to be able to study the interaction between *Fusarium* pathogens and maize, but also to evaluate the toxicological risks for maize consumption by humans and animals. The taxonomic characterization and identification of *Fusarium* species by microscopic or species-specific PCR is expensive, time-consuming and requires specific expertise as the number of described species increases continuously. Therefore, thorough taxonomic identification and assignment of the major *Fusarium* pathogens on maize was conducted in the present study. This assignment was based on the analysis of melting curves of PCR amplicons to develop modern, easy and fast tools for correct identification of toxigenic *Fusarium* species in relation to different environmental conditions in Europe.

The *Fusarium* taxonomy concept has been changed several times by taxonomists during the past 100 years with more than 1,000 species being described [58]. In general, three different species concepts have been employed: the morphological, biological and phylogenetic concept [59]. The morphological species concept is based on primary characters like spore size and shape of micro- and macroconidia as well as secondary characters like pigmentation, presence or absence of sporodochia [60]. The biological concept requires that species are sexually crossing, and progenies are viable and fertile. The phylogenetic species concept indicates that species with same DNA sequences belong to the same monophyletic group [59]. The most common errors resulting from morphological species description is to group isolates that should be separated into different species by their phylogenetic characters and mycotoxin

production. However, the most common error with classification due to phylogenetic criteria is splitting species into more groups than biologically meaningful [58].

F. subglutinans belongs to the *Gibberella fujikuroi* species complex (GFSC) and is descripted mainly on morphological and biological species recognition by diagnostic sexual crosses and DNA sequence polymorphism. Within this species complex, *F. subglutinans* is included in the American clade, according to phylogeographic studies by O´Donnel et al. (1998) [61]. Later in 2002, Steenkamp et al. [62] mentioned for the first time a cryptic speciation within the species by showing distinct PCR-RFLP patterns of six nuclear regions of *F. subglutinans* isolates from South Africa, North America and Mexico. The phylogenetic separation was further strengthened by the production of beauvericin (BEA), exclusively in group 1 (later named *F. temperatum*) present in European isolates [63]. In 2011, those cryptic subgroups were classified as individual species, establishing *F. temperatum* as a new species, formerly known as group 1, and separating it from *F. subglutinans sensu stricto* (*F. subglutinans*), formerly known as group 2, due to different phylogenetic positions and distinct mycotoxin profiles.

Although, BEA is a well-known mycotoxin causing apoptosis in cell lines, no risk assessment strategies or limits for daily intake are established for food and animal feed worldwide. BEA belongs to the cyclic hexapeptide inducing reactive oxygen species (ROS). Additionally, it is responsible for cytotoxicity to mammalian cells causing apoptosis [64]. BEA has been frequently reported almost worldwide, contaminating especially wheat, rye, oat, barley and rice [65,66]. Contamination with BEA is a serious problem in grains and wheat-based products like pasta, infant food, breakfast cereals, and biscuits, especially in Southern Europe [67–70]. However, BEA contamination is less frequently reported in grains from cooler climates, higher contamination levels are usually observed in Italy and Morocco [67]. Although BEA is regularly found in these products, especially in cereal-based food and feed [71–74], the EFSA Panel (CONTAM Panel, 2014) concluded that acute exposure to BEA does not indicate concern for human health [75]. Therefore, one approach to prevent mycotoxin contamination in grain is to screen fungal species for their abilities to produce beauvericin.

While *F. subglutinans* is mostly associated with ear rot in maize, results of our study indicate that *F. temperatum* and *F. subglutinans* were both able to infect winter wheat and cause symptoms of *Fusarium* head blight at anthesis. *Fusarium* infected maize crop residues are considered as the primary inoculum source for the most important *Fusarium* species causing

different diseases in small grain cereals in Europe [76,77]. They survive in stalk and cob residues of maize and can cause several diseases like seedling and root rot at the seedling stage as well as *Fusarium* head blight during anthesis [78]. However, *F. subglutinans* sp. which is not commonly found as an FHB causing species [79–81], is responsible for moniliformin and beauvericin contamination in small grain cereals from central to north-east European countries [80,82]. Because of further expansion of maize production in short crop rotations with wheat and other small grain cereals, we expect that FHB in wheat following infection with *F. temperatum* will increase and lead to increasing BEA contamination in feed and food products.

III Understanding host-pathogen interactions and resistance modes is essential for breeding maize genotypes improved in resistance to *Fusarium* infection and mycotoxin contamination.

Several strategie are suggested to reduce ear rot infection and mycotoxin contaminations in food and feed. Among other agronomical control mechanisms, the use of resistant cultivars is one of the most important control strategies to prevent *Fusarium* ear rot infection on maize. Ear rot resistance and host-pathogen interaction are influenced by several contrasting factors. The findings of our study confirm the results of other authors that genotype resistance is affected by environmental conditions, the mode of fungal entry, the susceptibility level of the genotype and the aggressiveness of the isolate.

There are three main modes of fungal entry by which *Fusarium* may enter the ear; (i) by silk channel, (ii) after wounding of insects and/or birds or (iii) through systemic growth from the stalk into the ear [78,83–85]. The first two infection pathways, via the silk channel and via infection through wounds, seem to be the most important pathways for infection with most *Fusarium* species [86,87]. Results of the present study indicate a moderate correlation (r=0.66) between both inoculation methods with highest disease severity of *F. temperatum* and *F. verticillioides* after silk channel inoculation and highest severity for *F. graminearum* after kernel stab inoculation. This indicates, that genotype resistance is dependent on the inoculation method simulating different infection modes, in particular, silk channel resistance prevents the penetration along the silk channel and kernel resistance inhibits the spread from

kernel to kernel.In addition, *Fusarium* species appear to differ in their preferred mode of penetration in to the cob [88–90]. The third infection pathway, systematic growth from the stalk, often remains unnoticed, however, systemic transmission from infected stalks and seeds has been reported for *F. verticillioides* and *F. subglutinans* in maize [91–94]. Therefore, breeding for ear rot resistance should not only focus on resistance mechanisms on the cob itself, but also consider stalk resistance to prevent systemic infection of the cob from the stalk. Which infection pathway is more important depends on the prevalence of *Fusarium* spp. and the presence of insect pests in the particular location [7]. The inoculation technique should therefore be selected based on the major mode of fungal entry in a given geographical area.

Inoculation with *F. temperatum* in both years of investigation resulted in stronger ear rot infection than with *F. graminearum* and *F. verticillioides*. Respective isolates were previously selected in the greenhouse based on similar disease severity and high mycotoxin production. For artificial inoculation, concentration of inoculum was adjusted considering spore production and aggressiveness of isolates to obtain isolates with similar pathogenicity. The high levels of disease severity due to *F. temperatum* in the field is most likely resulted from weather conditions favoring the infection with this species. The lower visual infection rate of *F. verticillioides* may be caused by a symptomless endophytic infection and may not reflect the real colonization of the cob [26,92,95]. This implies that disease assessment of *F. verticillioides* infection should not only rely on symptom expression but should also assess endophytic colonization by quantitative molecular methods. In addition, there is no clear relation between visible infection and mycotoxin concentration for all *Fusarium* species, which makes breeding for low mycotoxin levels more difficult. Several studies indicate a high correlation of GER resistance and toxin concentration, which suggests that simultaneous selection for both traits may be possible [96–98]. In contrast, low or moderate correlation was found between fumonisin production and visible infection with *F. verticillioides* in maize, due to symptomless and endophytic colonization [99,100]. Regarding these findings, phenotypic selection for resistance to *F. verticillioides* should be conducted directly by mycotoxin analysis even though toxin analysis is more time consuming and cost intensive.

Results of our study also indicate a medium to strong relationship of genotype resistance to *F. graminearum*, *F. temperatum* and *F. verticillioides*. This supports the idea of multiple resistance for several species, however, it is not clear whether individual QTL are effective to

all species, or if they secure the broad sense resistance [7,88]. Many breeders inoculate with either a single isolate of *F. graminearum* or *F. verticillioides* to evaluate more precisely the resistance in a single epidemic situation [7]. Considering the high correlation between resistance found *Fusarium* species in our study, the assessment of genotype resistance could also be done by inoculation with a mixture of all three *Fusarium* species. Resistance level of the genotype reflects the mean susceptibility to different species and lower additionally the effect of the environment to individual species. Unfortunately, mixed infection leads to interactions between the species effecting fungal development and mycotoxin accumulation [98,101,102]. In general, Gaikpa et al. (2019) [103] point out that phenotyping maize for ear rot resistances is influenced by numerous factors. Firstly, field trials have to be conducted in several environments and years due to the large genotype x environment interactions of the disease resistances. High variation in flowering time among genotypes makes it very time demanding to inoculate at the right time point. Secondly, relationships among different species occurring on a particular field may compromise the accuracy of phenotypic results. Thirdly, it is difficult to identify and score accurately the particular symptom on the ear [103]. Therefore, the use of modern and fast-track phenotyping technologies like thermal imaging and hyperspectral reflectance by drones or structural and chlorophyll vegetation indices could be a future perspective for field evaluation [104,105]. Breeding for ear rot resistance is difficult because resistance in maize is only quantitatively inherited, based on several genes and many different *Fusarium* species can infect the maize plant at highly different environmentally conditions [7,28]. So far, only a few putative genes have been discovered across environments which are directly linked to ear rot resistance in maize [106–108]. It is certain that *Fusarium* spp. have virulence factors, but it is not known whether the different *Fusarium* spp. possess common and/or different virulence factors [7]. For example, *F. graminearum* possesses different pathogenesis related (PR) genes but these are not necessarily virulence genes and they cannot be used in breeding programs [109]. Phenotypic analyses from conventional breeding methods contributing immensely to the understanding of ER resistances in maize but selection of resistant genotypes, are slow, time-consuming and labor intensive [103]. To improve precise assessments of ear rot resistances in maize, data from new breeding methods such as genetic mapping, genomic profiling and bioinformatic should

be transferred into a simpler but robust strategy to improve maize and reduce mycotoxin contamination.

IV Besides changing environmental conditions, increasing global trade and traffic are likely to cause more problems in the future through introduction of new diseases like *Trichoderma* ear rot.

Although *Trichoderma* species have been described as opportunistic, basically avirulent plant symbionts in soil [110], our findings support previous observations from the US, where *Trichoderma* spp. has been reported to cause ear rot symptoms in maize [111–113].

The incidence of pathogens and pests has noticeably increased in recent years [114]. With the continuously high or even increasing global traffic of humans and commodities, frequency of introduction events of new hosts and pathogens and the spread of diseases and vectors has increased [115]. Anderson et al. (2004) [115] described emerging infectious diseases which either (i) increased in incidence, geographical or host range; (ii) changed in pathogenesis; (iii) or have newly evolved, discovered or newly recognized. These changes are driven by anthropogenic introduction of pathogens and changing climate conditions [116, 132]. Espacially plant diseases, are affected by global trade, land use and severe weather events [117]. Climate change is most likely a strong driver of evolutionary changes in plant and pathogen population by interfering with the host-pathogen interactions, gene expression and population dynamics [118]. Changing weather conditions can lead to disease through altering the distribution of pest and diseases or increasing water or temperature stresses on plants and a greater frequency of unusual weather events [119]. Harvell et al. (2002) [118] suggested that milder winters and higher overall temperatures will increase winter survival of plant pathogens, accelerate life cycles as well as increase sporulation and aggressiveness of foliar fungi. Another important driver for disease emergence is simplification of agricultural ecosystems in which biological diversity has been reduced due to socioeconomic development and technical advances. Intensification of agricultural practices such as irrigation leads to the increases of the plant pathogen populations and has replaced diverse agroecosystems with increased vulnerability to pest attacks [120,121]. In addition, genetically uniform host plants,

often lacking in natural defense to their wild reletives, promoting population growth and rapid establishment of new pests and diseases [132].

Hence, several factors could have caused *Trichoderma* species to emerge in Europe inducing *Trichoderma* ear rot in maize. Previously, *Trichoderma* was only described as an ear rot pathogen with minor importance in the United States occurring predominantly in the "corn belt" regions (Kentucky, Ohio and Iowa), which are characterized by intensive maize production, largely planted in short corn-soybean rotations [111,112,122–124]. The present study reports the occurrence of *Trichoderma* ear rot for the first time in Southern Germany in 2018 and 2019. However, *Trichoderma* ear rot was observed to a lower extend already since several years in Germany, France, Italy and Poland (personal communication, Silvia Mueller 04th October 2018). Noticeably, its incidence increased in recent years which were associated with higher temperatures and longer drought periods.

One factor potentially driving the emergence of *Trichoderma* ear rot in Europe could be antropogenic movement by international trade of plant products especially infected maize seeds. For example, it has been estimated that at least 2,400 different plant pathogens were contained in the seeds of 380 plant genera [125], and that up to one third of the plant pathogenic viruses are transmissible through seeds [126,127]. Several maize leaf pathogens such as *Bipolaris zeicola* and *Colletotrichum graminicola* have been confirmed also to be seedborne [128,129]. This mechanism of transmission could also explain the arrival and distribution of the maize leaf pathogen *Kabatiella zeae* to areas like New Zealand [130]. Therefore, seedborne dispersion through commercial seed production in the United States could have contributed to the dispersal of *Trichoderma* ear rot. In addition, environmental conditions may have been favorable to cause ear rot diseases in maize in Central to Southern regions of Europe. Therefore, each step of the infection cycle such as inoculum sources and survival, infection, latency period, production and release of new spores needs specific environmental requirements. As the *Trichoderma* ear rot incidence in Germany appears to be higher in years with temperatures above average and precipitation below average, years with higher temperature und lower humidity may favor infection and spread of the disease. However, the impact of weather conditions is difficult to determine because it is not yet known whether higher temperature and drought are favorable weather conditions for *Trichoderma* infection or whether drought and temperature stress enhance the susceptibility

of maize plants. Future research is needed to examine the complete life cycle of *Trichoderma* ear rot and the impact of temperature and humidity on inoculum survival, infection pathway and time point of infection.

Although *T. harzianum* and *T. afroharzianum* are known as plant symbionts or antagonists of fungal phytopathogens, we discovered pathogenic strains of both species being able to infect maize cobs. This led to the question whether beneficial *Trichoderma* strains could mutate and become aggressive plant pathogens or whether aggressive strains, presumably from the US, were introduced. Five evolutionary forces were described by McDonald and Linde (2002) [131] which can change the pathogenicity of microorganisms, (i) mutation, (ii) population size and genetic drift, (iii) gene and genotype flow, (iv) reproduction and mating system as well as (v) selection imposed by resistance genes. It remains unclear, which of those factors may have led to the observed changes in pathogenicity of *T. harzianum* and *T. afroharzianum* and this question requires further research. A key aspect is, whether pathogenic species have been introduced from the US or whether aggressive strains in Europe developed independently. This requires an in-depth population genetic analysis based on a sufficiently large number of isolates from different regions. In such a study, pathogenic *Trichoderma* isolates from US should be compared with strains from Europe in order to explore phylogenetic differences between populationsfrom different geographic origin. Such studies on this relatively novel pathogen on maize should also expand on potential impact of Trichoderma ear rot on food and feed quality associated with the production of secondary metabolities with antifungal activity like ergokonin A, trichodermin, pyrone, harzianopyridone, viridin and β-1,6-Glucanases. Finally, yield loss analyses under field conditions are required to assess the economic significance of *Trichoderma* ear rot disease in European maize production.

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Summary

Ear rots, caused by fungi, are among the most important maize diseases worldwide causing severe yield losses and a reduction of grain quality. Several toxigenic *Fusarium* species are known to cause yield losses and reduce grain quality, thus endangering the safety of both animal feed and human food products.

For this purpose, we investigated the occurrence and significance of *Fusarium* and *Trichoderma* ear rot on maize in Germany. Within this framework, we evaluated the impact of environmental conditions and agronomic practices on the prevalence of *Fusarium* species associated with ear and stalk rot. Therefore, we conducted a monitoring of maize ears and stalks in Germany infected with *Fusarium* to determine the range of *Fusarium* species present in the field and to assess the impact of tillage, crop rotation and weather conditions. From 2016 to 2018, a total of 387 infected ears and 190 stalk segments were collected from 58 locations in Germany. For each sampling location, site-specific agronomic data on tillage and previous crops as well as meteorological data on precipitation, air temperature and relative humidity during the vegetation period were recorded. The most frequent *Fusarium* species detected in maize ears were *Fusarium graminearum*, *F. verticillioides* and *F. temperatum,* while *F. graminearum*, *F. equiseti*, *F. culmorum* and *F. temperatum* were the species prevailing on maize stalks. Differences in the local species composition were found to be primarily associated with weather variations between the years and the microclimate at the different locations. The results indicate that mean temperature and precipitation in July, during flowering, has the strongest impact on the local range of *Fusarium* species on ears, whereas the incidence of *Fusarium* species on stalks is mostly affected by weather conditions during September. Ploughing significantly reduced the infection with *F. graminearum* and *F. temperatum*, while crop rotation exerted only minor effects.

Another aim of the present study was to determine the occurrence, mycotoxin production and pathogenicity of *Fusarium temperatum* from maize in Germany. For this purpose, a Germany-wide monitoring of maize ears and stalks was carried out in 2017 and 2018. Within this monitoring, 79 isolates of *F. temperatum* and seven isolates of *F. subglutinans* were obtained. Inoculation of maize ears revealed the highest aggressiveness of *F. temperatum*, followed by *F. graminearum,* and *F. verticillioides and F. subglutinans.* On maize stalks, *F. graminearum* was the most aggressive species while *F. temperatum* and *F. subglutinans* caused only small lesions. The temperature optima for infection of maize ears with *F. temperatum* and *F. subglutinans* were 24 °C and 21 °C, respectively. Artificially induced infection of wheat ears with all strains of *F. temperatum* and *F. subglutinans* caused head blight symptoms, thus indicating wheat as an alternative host. In rice cultures, 60 strains of *F. temperatum* produced beauvericin, moniliformin, fusaric acid, fusaproliferin, and one strain also produced fumonisins B1 and B2. The results demonstrate the increasing importance of *F. temperatum* in German maize cultivation areas.

Thirdly, we investigated the aggressiveness of several *Fusarium* species in maize in relation to inoculation method, maize variety and location. Therefore, in 2018 and 2019, maize hybrids were tested in four locations (Bernburg, Rustenhart, Kuenzing and Liesborn) in Germany and France. In each location, twenty hybrids were inoculated with *F. graminearum*, *F. verticillioides* and *F. temperatum* using two inoculation methods, injection

into the silk channel and kernel wounding by kernel stab inoculation. Disease severity was assessed visually as the percentage of plant tissue colonized with mycelium at harvest time according to the EPPO guidelines. Our results showed that *F. temperatum* was the most aggressive *Fusarium* species in both years followed by *F. graminearum* and *F. verticillioides,* however, the prevalence differed between locations. Significant differences in genotypic resistance depending on the inoculation method and *Fusarium* species were found in all locations. Silk channel inoculation resulted in higher disease severity of *F. temperatum* and *F. verticillioides* while disease severity following kernel stab inoculation was higher with *F. graminearum*. Correlation between *Fusarium* species concerning disease severity induced on different maize cultivars was medium to high, however, only low to medium correlation was observed between inoculation methods within the *Fusarium* species.

In 2018, massive infections with *Trichoderma* on maize ears were recorded for the first time in a field in Southern Germany. Within this study, first investigations were conducted to identify and verify *Trichoderma* as a new pathogen causing ear rot disease on maize in Europe. For this purpose, *Trichoderma*-infected cobs from four locations in Germany and France were sampled, cultured and isolates microscopically examined as well as analyzed by sequencing the gene for translation elongation factor *tef-1α*. Furthermore, the pathogenicity of *Trichoderma* isolates and the impact of infection on dry matter content of maize cobs were tested after artificial inoculation at flowering stages in the greenhouse. In 2018 and 2019, a total of 13 *T. harzianum* isolates from maize cobs and maize stalks were isolated and tested, compared to several reference isolates. Four isolates proved to be highly aggressive, two biocontrol isolates, Trichodex (T39) and strain T12, induced slight infection and eleven isolates were non-pathogenic. After sequencing of the pathogeneic *Trichoderma* isolates based on the translation elongation factor 1α (*tef-1α*), the four highly aggressive isolates were assigned to *T. afroharzianum*, while the commercial biocontrol isolates Trichodex (T39) and T12, as well as the other non-pathogenic strains belonged to *T. harzianum, T. atroviride* or *T. tomentosum*. This, to our knowledge, is the first report on *Trichoderma* sp. as a pathogen causing ear rot disease in maize in Europe with the potential to incite significant yield losses. We therefore propose to name this disease as ‚*Trichoderma* ear rot on maize'.

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LEBENSLAUF

Name	Annette Pfordt
E-Mail	annettepfordt@web.de
Anschrift	Beethovenstrasse 2 37308 Heilbad Heiligenstadt
Geburtsdatum/-ort	07. Januar 1992 in Heilbad Heiligenstadt

Ausbildung

2017 Mai - 2020 Mai	Ph. D. Agrarwissenschaften, Georg-August-Universität Göttingen Im Bereich Phytopathologie und Pflanzenschutz
2015 Okt. - 2017 Feb.	M.Sc Agrarwissenschaften, Georg-August-Universität Göttingen Schwerpunkt: Nutzpflanzenwissenschaften; Masterarbeit im
2010 Okt. - 2014 März	B.Sc. Agrarwissenschaften, Universität Hohenheim, Schwerpunkt: Nutzpflanzenwissenschaften;
2002 - Mai 2010	Abitur, Johann-Georg Lingemann Gymnasium, Heilbad Heiligenstadt

Berufliche Erfahrung

April 2019 – heute	Sitz im Fakultätsrat, Fakultät für Agrarwissenschaften
Mai 2018 - Mai 2020	VDL Landesverband Niedersachsen, Vorsitzende VDL Studierendengruppe Göttingen
September 2016 – heute	DPG- Deutsche Phytomedizinische Gesellschaft
Juli 2016 – Sep. 2016	VR- Bank Südniedersachsen eG, Getreideannahme und Analyse von Qualitätsparametern
März 2016 - Juli 2016	Ingenieurgemeinschaft für Landwirtschaft und Umwelt (IGLU),
Feb. 2016 – März 2016	Landwirtschaftlicher Betrieb, Tauweru, Neuseeland Mitarbeit auf Rinder- und Schaffarm
Okt. 2015 - Januar 2016	Gemüsebaubetrieb Scottfresh (New Zealand), Christchurch, Neuseeland Produktionsleitung und Qualitätsmanagement

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Statutory declaration

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