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ORIGINAL PAPER

Impact of aggressiveness of *Fusarium graminearum* and *F. culmorum* isolates on yield parameters and mycotoxin production in wheat

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Abstract Plant-associated isolates from *Fusarium graminearum* and *F. culmorum* were inoculated on wheat in field experiments in 2007 and 2008 to ascertain their influence on fungal colonization of the ears, as well as mycotoxin contamination (deoxynivalenol, DON; nivalenol, NIV; zearalenone, ZEA) and yield parameters in the mature crop after inoculation with or without irrigation. The isolates were assigned to four different groups of aggressiveness on the basis of pathogenic symptom development and mycotoxin production in vitro. Increased levels of trichothecene-producing *Fusarium* DNA in the ears indicated a successful inoculation of the plants, which resulted in increased DON content in the wheat kernels in 2007. Dry conditions at anthesis markedly suppressed fungal colonization as well as mycotoxin accumulation. However, due to precipitation during the ripening period, yield and thousand-kernel weight were similar whether or not irrigation was applied at the time of inoculation. The level of aggressiveness among the isolates as determined in vitro was not reflected in the field experiment. The activity of the extracellular invertase in developing ears increased as a plant response to pathogen infection, especially when the plants were irrigated at the time of inoculation. In 2008, the *Fusarium* inoculation of wheat heads did not cause fungal growth and mycotoxin contamination in the grain, because of the dry weather conditions that occurred over the entire period of anthesis and ripening. The risk of future mycotoxin contamination in grains was discussed based on climate change prognosis.

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Introduction

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* [Schwein] Petch) and anamorphic *F. culmorum* (W.G. Sm.) Sacc. are widespread and destructive pathogens causing Fusarium head blight (FHB) worldwide. Fusarium head blight is an epidemic disease of cereals that reduces grain yield as well as quality, and causes contamination of grain with the mycotoxins DON and/or NIV and ZEA. *F. graminearum* and *F. culmorum* are necrotrophic *Fusarium* species, which colonize and persist on dead plant material and have a short pathogenic phase in the FHB disease cycle. Sexual recombination occurs in *F. graminearum*, and the high gene flow across large distances explains the genetic similarity among populations in diverse geographical regions (for review, see Miedaner et al. 2008). Initial *F. graminearum* infection is usually caused by ascospores from infected wheat or maize stubble, while conidia produced on flowering spikes may cause secondary infections (Khonga and Sutton 1988). No sexual stage is known for *F. culmorum*, but population analysis suggests a high level of genetic recombination (Mishra et al. 2003; Zeller et al. 2004) probably caused by frequent alternation between saprophytic and parasitic life cycles commensurate with a sexual mode of reproduction. Conidia are the principal or only source of inoculum in *F. culmorum* (Schlüter et al. 2006). Despite their different modes of reproduction, both species are characterized by high levels of genetic diversity among isolates sampled within geographically defined large regions as well as within individual field populations (Naef and Défago 2006;

Schmale et al. 2006). The genetic diversity manifests itself in different phenotypic traits such as pathogenicity, aggressiveness and mycotoxin production. Populations of *F. graminearum* and *F. culmorum* are not only highly diverse but, as a consequence, also highly flexible in adapting to their environment. Crosses between and among *F. graminearum* lineages have shown a rather simple, additive inheritance of aggressiveness (Miedaner et al. 2008).

Warm and humid weather at flowering predisposes wheat to FHB infection (Sutton 1982). At least 1 day with a minimum of 4 mm rain and a daily average temperature of more than 18°C between completed inflorescence (Zadoks scale Z59) and anthesis (Z65) (Zadoks et al. 1974) guarantee the dissemination of ascospores and conidia and subsequently enable the infection of ears (Obst et al. 2000). However, with the forecast of climatic change, higher temperatures and periods of drought are considered to be key stress factors with high potential impact on crop yield in northeast Germany (Wiggering et al. 2008). Therefore, the effects of dry weather conditions following successful infection of cereal plants by *Fusarium* spores of differently aggressive isolates should be addressed to predict yield and quality of the grain under these circumstances.

Isolates of various *Fusarium* species have often been tested in inoculation experiments for their influence on FHB disease development, yield and/or mycotoxin contamination in wheat under controlled environmental conditions (e.g., Del Ponte et al. 2007; Gilbert et al. 2001; Vogelgsang et al. 2008; Xu et al. 2007). Under controlled environments in climate chambers, the effects of environmental conditions and individual isolates on disease and mycotoxin variables can be investigated undisturbed. However, field experiments (Culler et al. 2007; Cumagun et al. 2004; Vogelgsang et al. 2008) realistically reflect agricultural practice with changing environmental conditions and the presence of other competing fungi, which may suppress or enhance the activity of the inoculated isolates.

One of the plant's responses to pathogen infection may be an alteration in carbohydrate partitioning between source and sink tissues. A complex defence response is induced, which is usually accompanied by a fast reduction of sink metabolism, possibly to satisfy the increased demand for carbohydrate as an energy source (Roitsch 2004). This mechanism requires the interruption of sugar export via the phloem and an increase in supply of hexoses replacing the transport sugar sucrose, which cannot be used directly for metabolic processes. Therefore, sucrose-cleaving enzymes, especially extracellular invertase, become upregulated as key enzymes in these processes (Roitsch et al. 2003). Invertases irreversibly hydrolyze the sucrose into its monomers glucose and fructose. They are present in most plant organs in multiple forms (Roitsch and González 2004). An extracellular invertase is ionically bound to the cell wall. Additionally, there are two classes of intracellular invertase isoenzymes in higher plants: an invertase with an acidic pH optimum in the vacuole and another with neutral pH optimum in the cytoplasm. Another enzyme catalyzing the same reaction is the sucrose synthase. While invertases are active in the regulation of sucrose partitioning, osmoregulation, cell enlargement and responses to abiotic stress and pathogen infection (Roitsch and González 2004), the sucrose synthase is mainly active in the storage and maturation processes of plants, and its cleaving process is reversible (Koch 2004). Sucrose cleavage is crucial not only for the allocation of carbon resources but also for the initiation of hexose-based sugar signals, which activate different pathways that regulate gene expressions for inducing stress responses (Koch 2004). The extent of the pathogenic activity of isolates differing in aggressiveness is possibly reflected in the activity of sucrose-cleaving enzymes in plant tissues.

Isolates of *F. graminearum* and *F. culmorum* were obtained from plant material from different geographical regions in the State of Brandenburg (Germany). Their aggressiveness was quantified on the basis of symptom development and mycotoxin production in vitro in this study. The isolates were inoculated onto wheat plants during the flowering stage in the field in two consecutive years. The aim of the study was to determine fungal colonization of the ears, and mycotoxin contamination and yield parameters in mature wheat grain after inoculation with and without irrigation.

Materials and methods

Fungal isolates used as inoculum

The *Fusarium* isolates used as inoculum in this study originated from wheat plants (spike or root; $n=12$) and forage grass (leaf; $n=1$) and were obtained from different regions in the State of Brandenburg (Havelland, Uckermark, Märkisch-Oderland) between 2001 and 2006. They are stored in the collection of cultures of microorganisms of the Institute of Landscape Matter Dynamics at the Leibniz-Centre for Agricultural Landscape Research Müncheberg. Single-spore stock cultures of these isolates are maintained in sterile soil mixtures at 8°C. Their taxonomic identification was performed after cultivation on Potato Dextrose Agar (PDA) (VWR International, Darmstadt, Germany) and Synthetic Nutrient Agar (SNA) (Nirenberg 1976) for 7 days, followed by incubation under mixed black light (near UV, emission ca. 310–360 nm) and artificial daylight with a 12 h:12 h light:dark cycle for 5 days. Identification of species was based on the morphological structures (phialides, macro- and microconidia) described by Nelson et al. (1983), Nirenberg (1990) and Samson et al. (2002).

Inoculum preparation

Single conidia of the isolates were grown in Petri dishes containing SNA medium at 25°C for 10 days with a 12 h:12 h light:dark cycle for 5 days. Macroconidia were harvested by washing the culture surface with sterile quarter-strength Ringer's solution (VWR International) supplemented with 0.01 % Tween 80 (VWR International). After the determination of the spore titres in a Thoma chamber (Roth, Karlsruhe, Germany), the resulting inoculum suspensions were adjusted to 2 x 10^5 conidia/ml for necrotic lesion tests and 5×10^4 conidia/ml for field trials with quarter-strength Ringer's solution.

Necrotic lesion test with fungal isolates

The ability of the *Fusarium* isolates to cause necrosis was examined in vitro using a detached leaf assay according to Browne and Cooke (2004). Winter wheat cv. Tuareg was grown in sterile silica sand using 3 seeds/9-cm pot in a climate chamber with a 14 h:10 h light:dark cycle and day/night temperature of 20°C/16°C. The susceptibility of the cultivar Tuareg to an infection by *Fusarium* spp. (expressed as a rating of 2–7; Anonymous 2008) is 6, a relatively high susceptibility. After 14 days, primary leaves were harvested and the mid-section was cut into 4-cm leaf segments and injured (1 mm) at the middle of the adaxial surface. Leaf segments were plated on 0.5% water agar containing 10 mg/l kinetin as a senescence retarder. The inflicted injury on the adaxial surface was inoculated with 5 µl inoculum suspension diluted to 2 x 10^5 conidia/ml. Sterile distilled water was applied on the control leaves. Petri dishes were incubated at 25°C with a 12 h:12 h light:dark cycle. After 5 days, the length of the necrotic lesions was measured. The test included six repetitions for each isolate and was conducted twice.

Mycotoxin analyses in laboratory cultures

The toxicity of the fungal isolates was determined by analysing the DON, NIV and ZEA concentrations in laboratory cultures. Rice medium (5 g commercially available long-grain rice and 8 ml of water was sterilized three times at 100°C for 30 min) was inoculated with single spore cultures and incubated at 25°C in darkness for 3 weeks. The rice-fungi mixtures were ground in a mortar and then dispensed in an Erlenmeyer flask with 75 ml of acetonitrile:methanol:water (80:5:15, v/v). The further extraction steps and the HPLC analyses of the mycotoxins were carried out as previously described in detail (Müller et al. 2010a). The limits of detection (LOD) in wheat grains were 20 µg/kg for DON, 50 µg/kg for NIV and 2 µg/kg for ZEA. Each analysis was performed in duplicate.

Rating the aggressiveness of fungal isolates

Based on the results of the tests measuring necrotic region length and in vitro mycotoxin analyses (Table 1), a cluster analysis was performed in order to assign the fungal isolates into groups of comparable aggressiveness. Values were adjusted to normality by $\log_{10}$ transformation, and the dendrogram was calculated with the unweighted pair-group method using arithmetic averages of all four test parameters (Table 1). SPSS software v.15.0 (SPSS, Chicago, Ill, USA) was employed.

Table 1 Mean lesion length on detached wheat leaves inoculated with *Fusarium* isolates and mycotoxin production in vitro (production in rice medium ±standard deviation)

Isolates	Lesion length (mm) n=12	DON (µg/kg) n=4	NIV (µg/kg) n=4	ZEA (µg/kg) n=4
F. culmorum 1	139 (± 4.2)	520 (± 9.6)	27,600 (± 20.8)	254,000 (± 31.0)
F. culmorum 13	175 (± 4.5)	122,000 (± 25.0)	8,380 (± 5.0)	235,000 (± 55.6)
F. culmorum 32	38 (± 3.4)	12,000 (± 29.3)	12,700 (± 17.1)	40 (± 5.0)
F. culmorum 51	43 (± 2.6)	870 (± 8.2)	3,590 (± 12.6)	180 (± 8.2)
F. culmorum 53	28 (± 2.9)	270 (± 12.9)	31,600 (± 17.3)	178,000 (± 41.9)
F. culmorum 64	63 (± 3.7)	5,220 (± 21.6)	22,000 (± 68.6)	385,000 (± 97.4)
F. culmorum 65	60 (± 3.5)	1,310 (± 18.3)	53,100 (± 94.2)	3,530 (± 17.1)
F. culmorum 67	43 (± 3.9)	1,190 (± 9.6)	7,650 (± 9.6)	<LOD[a]
F. graminearum 18	28 (± 3.4)	1,960 (± 17.1)	550 (± 8.2)	224,000 (± 49.7)
F. graminearum 20	51 (± 3.6)	1,380 (± 8.2)	580 (± 9.6)	61,600 (± 12.6)
F. graminearum 21	52 (± 4.0)	13,600 (± 26.5)	2,670 (± 12.9)	185,000 (± 9.6)
F. graminearum 23	97 (± 4.7)	25,300 (± 12.9)	1,380 (± 17.3)	110,000 (± 69.9)
F. graminearum 24	44 (± 4.5)	5,080 (± 17.1)	630 (± 5.0)	248,000 (± 41.1)

[a] *LOD* Limit of detection (µg/kg): DON: 20, NIV: 50, ZEA: 2

Design of the field experiments

The experiments were carried out at the experimental farm of the Leibniz-Centre for Agricultural Landscape Research Müncheberg in 2007 and 2008. The soil type was a sandy loam (albeluvisol, according to the World Reference Base for Soil Resources).

The field trial of 2007 was conducted as a screening experiment with all *Fusarium* isolates, which are listed in Table 1. Thirteen field plots for inoculation and two untreated plots as control were laid out in 2007. In the following year, the *F. culmorum* isolates Fc 13 (very highly aggressive, as determined in vitro) and Fc 53 (moderately aggressive) as well as the *F. graminearum* isolates Fg 18 and Fg 23 (both highly aggressive) were chosen for inoculation in the field. In 2008, a randomized block design with 6 repetitions for each isolate and untreated control, respectively, was established. In both years, the plots, each 3 m × 2 m in size, were surrounded by buffering plots of 2 m in width to each adjacent plot. Half of the plots were irrigated from the onset of anthesis (Z60) until the late milk state (Z77), while the other half was exposed to natural weather conditions. Precipitation was interpolated from data measured at a weather station 50 m from the study site (Fig. 1). The irrigation regime was calculated with the software BEREST 90 (Wenkel and Mirschel 1991) in consideration of soil type, air temperature, air humidity and rainfall. All plots were irrigated using a driving sprinkler irrigation system with spray-type nozzles. In both years, irrigation was already started before inoculation (in 2007, 50 mm; in 2008, 82 mm) (Fig. 1). A moderate irrigation of 4 mm over 3 days after inoculation (DAI) was applied to encourage ear infection in the irrigated block. The total amount of irrigation until the end of June was 57 mm in 2007, and 111 mm in 2008, as indicated in Fig. 1. Winter wheat cv. Tuareg was sown at 330 seeds/m^2 in the same field in autumn of each previous year. The crops were maintained according to standard procedures of farms without fungicide applications.

Plant heads were inoculated with spore suspensions (see "Inoculum preparation") of the 13 *Fusarium* isolates (in 2007) or the 4 isolates (in 2008) using a high-pressure sprayer at the beginning of anthesis (Z63: anthesis one-third complete). The inoculum was sprayed (100 ml/m^2) on wheat heads within 2 h of preparation. All plants of the plots were combine-harvested and threshed when fully mature (Z92). Grain yield and 1,000-kernel weight (TKW) were determined. In tables and figures, grain yield and TKW is expressed at a dry matter content of 85%.

Mycological analyses of wheat kernels

Fungi were isolated from infected wheat plants at the early ripening stage (21 DAI, Z75) and before harvest (42 DAI, Z87). At each time, ten ears were randomly removed from each plot. The wheat kernels were surface disinfected for 90 s in a 0.35% solution of sodium hypochlorite, followed by washing four times with sterile water. The kernels were incubated on PDA at 25°C. The *Fusarium* colonies were identified by colony and conidial morphology as described above in "Fungal isolates used as inoculum".

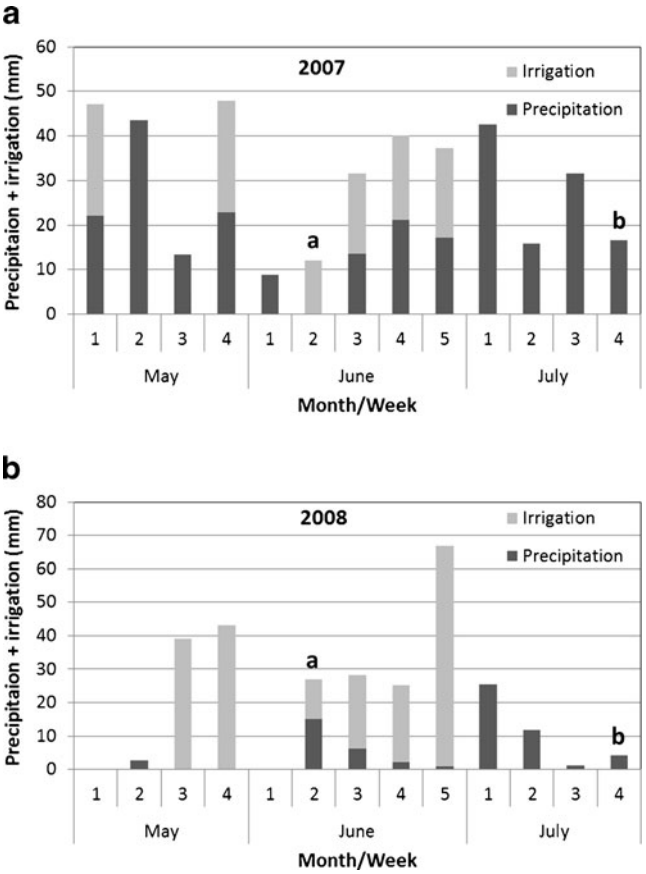

Fig. 1 Amount of precipitation (*black bars*) and irrigation (*grey bars*) (mm) for winter wheat plots during the ripening of grain in **a** 2007 and **b** 2008. *Columns* represent the sum of the weeks. *a* and *b* indicate the time of inoculation and harvest, respectively

DNA extraction and real time PCR

Wheat heads for real-time PCR were taken on two assessment dates, 21 (Z75) and 42 (Z87) days after inoculation. Ten ears per plot at each assessment date were dried at 60°C and ground to fine flour for DNA extraction as described below in "Mycotoxin analyses in wheat kernels".

Duplicate DNA extracts of each of the plant samples were prepared following a modification of the CTAB-based method by Lipp et al. (1999). In addition to the original protocol, the sample (100 mg) was suspended in 400 µl 100 mM potassium phosphate buffer, pH 8.0, two steel balls were added and the suspension was processed for 40 s in a FastPrep Instrument (MP Biomedicals, Eschwege, Ger-

many). The resulting suspension was mixed with 400 µl twofold-concentrated CTAB buffer and kept at 65°C for 1 h. After purification, DNA yield and quality was visually evaluated by comparison with a DNA standard after electrophoresis on 0.8% agarose gel. The fungal strain *F. graminearum* M22 (collection of cultures of microorganisms of the Institute of Landscape Matter Dynamics) used to construct the standard amplification curves was grown on Sabouraud broth (Merck, Darmstadt, Germany) and harvested by filtration. Genomic DNA from mycelium was extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions but with a modified lysing step: the lysing matrix was supplemented with a ceramic sphere (Sarlin et al. 2006). DNA quality was checked by electrophoresis and the DNA was quantified with a NanoDrop ND-1000 spectrophotometer.

The primer and probes used to detect the members of the genus *Fusarium* (ITS assay) as well as the trichothecene producing group of *Fusarium* (TRI 6 assay) have been reported previously (Bluhm et al. 2004). Using the TRI 6 assay, the concentration of DNA encoding trichothecenes (TRI-DNA) was determined and expressed in ng TRI-DNA per g dry matter of wheat heads. The probes were labeled with the reporter dyes 6-FAM and VIC, respectively, and with the quencher dye TAMRA. All reactions were performed in duplicate using a 1:5 dilution of the sample DNA. The standard amplification curves were generated using serial dilutions (in triplicate) of purified DNA from *F. graminearum* strain M22. Multiplex reactions were performed using a TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) on a 7500 Fast Sequence Detection System (Applied Biosystems). The concentration of primers and probes, as well as the cycling conditions, have been described earlier (Bluhm et al. 2004).

Mycotoxin analyses in wheat kernels

All harvested and threshed samples were dried at 60°C for 48 h for the determination of DON, NIV and ZEA concentrations in the wheat grain. The kernels were ground to a fine flour in an ultra centrifugal mill with a vibratory feeder (ZM 200; Retsch, Haan, Germany) to pass a 0.75-mm sieve for the following toxin extractions. Aliquots of 15 g were mixed with 3 g silica gel 60 (0.063–0.2 mm particle size or 70–230 mesh; Merck) and extracted by shaking with 75 ml of organic solution. Purification and quantification of DON, NIV and ZEA were conducted as described by Müller et al. (2010a).

Invertase and sucrose synthase activity analysis

Three wheat heads from each of the plots inoculated with and without (control) *Fusarium* isolates were randomly harvested seven DAI, transported on ice and stored at −80°C within 1 h after harvest. These plots were chosen as examples for inoculation with isolates of low, moderate and high aggressiveness as determined in vitro. Plant material from the top of each ear was homogenized with liquid nitrogen in a mortar for protein preparation. Fresh matter of 0.5 g homogenized plant material was used to extract the enzymes. The extraction, partial purification of the extract by dialysis, the enzyme activity analyses and the determination of protein concentration were carried out as previously described (Müller et al. 2010b).

Statistical analyses

Data from the in vitro and the field studies were tested for normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test). Values of all parameters analysed in vitro (except ZEA production) failed to follow a normal distribution, and thus the rank correlation Kendall´s-Tau was conducted to find any relationship between these parameters. For the field experiment, differences between inoculated and non-inoculated plots were calculated with the *t* test including Bonferroni correction or the non-parametric Mann–Whitney test. The influence of irrigation and the level of aggressiveness (excluding group 4) on the analyzed parameters were examined using ANOVA or the Mann–Whitney test. All statistical analyses were computed with the SPSS statistical package (v.15.0; SPSS).

Results

In vitro studies

The in vitro tests revealed considerable differences in disease symptoms and the ability to produce mycotoxins among the *Fusarium* isolates (Table 1), which caused the clusters of four different groups of aggressiveness (Fig. 2). Isolates of low aggressiveness (group 1) were weak in both traits, necrotic lesion length as well as mycotoxin production, whereas those of high or very high aggressiveness (groups 3 and 4) showed a strong potential at least in one of these traits or in both. Variations in the parameters tested were high in each of these groups. However, no correlations were found between the mycotoxins analyzed or between the necrotic lesion length and the several toxins.

Effects of inoculation in field experiments

The level of trichothecene-producing *Fusarium* DNA in the ears was determined with the TRI-DNA assay and analyzed to check whether or not the inoculation was successful. In 2007, colonization of the wheat plants by trichothecene-

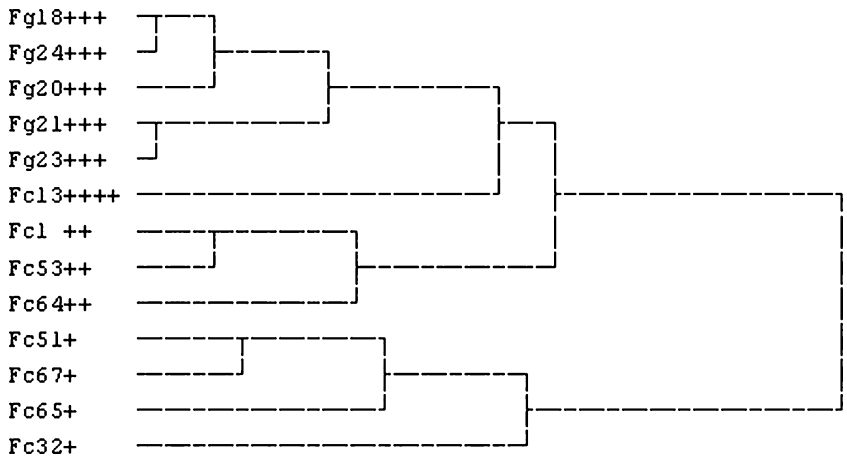

Fig. 2 Groups of aggressiveness based on necrotic lesion length and in vitro production of DON, ZEA and NIV. + group 1, low aggressiveness; ++ group 2, moderate aggressiveness; +++ group 3, high aggressiveness; ++++ group 4, very high aggressiveness

producing *Fusarium* species increased from the first (21 DAI) to the second (42 DAI) assessment date, independent of the inoculated isolates used (Fig. 3a, b). Simultaneously, the content of the natural *Fusarium* species increased from the first to the second assessment date as seen in the non-inoculated control plots. At 42 DAI, the inoculated wheat ears (ranging from 2,300 to 9,020 ng/g) showed significantly ($P<0.05$) higher amounts of TRI-DNA than the wheat kernels from the non-inoculated control plots (1,095–3,000 ng/g) (Fig. 3b).

The examination of the surface-sterilized wheat head material revealed that *F. cerealis*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. avenaceum, F. sambucinum* and *F. sporotrichioides* occurred in the florets before inoculation (Z63), in immature kernels at 21 DAI (Z75), and in mature kernels at 42 DAI (Z87) from all plots. However, the *Fusarium* isolates inoculated prevailed over this natural *Fusarium* flora and dominated in the corresponding plots. In contrast, neither naturally occurring *Fusarium* species nor the four inoculated isolates could be detected in the wheat ears on the two assessment dates in 2008. On the 42nd DAI (Z87), the TRI-DNA content of most of the samples persisted close to the detection limit (0.3 ng/g) of this method (Table 2) and therefore considerably lower compared to 2007 (Fig. 3b).

In contrast to the ZEA contamination, the DON levels of the control and inoculated wheat kernels differed significantly ($P<0.05$) in the year 2007: a mean value of 2,020 μg/kg was measured in samples of inoculated plots compared with 606 μg/kg for the untreated control plots (Fig. 3c, d). In 2008, the mycotoxin concentrations in the grain of all plots were considerably depressed (57–128 μg/kg DON; <2–16 μg/kg ZEA) independently of the inoculated isolate (Table 2). Although most of the *F.* isolates inoculated were able to produce NIV in vitro, this mycotoxin was not found in any of the field plots in 2007 and 2008.

Although the *Fusarium* inoculation was observed to have a significant influence on the TRI-DNA content and the DON concentration in the wheat kernels in 2007, the grain yields and the 1,000-kernel weights in samples of the non-inoculated and inoculated plots were similar (Fig. 3e, f).

The dry conditions at anthesis in non-irrigated plots markedly suppressed fungal colonization as well as mycotoxin accumulation markedly in 2007. Irrigation resulted in significantly ($P<0.05$) higher TRI-DNA, DON and ZEA contents in the wheat kernels (Fig. 3a-d). Due to high rainfall during the ripening period, additional irrigation did not affect yield or 1,000-kernel weight significantly (Fig. 3e, f). In 2008, plot irrigation did not lead to an increase of TRI-DNA and mycotoxin content. It seems that the extremely dry conditions throughout the entire period of anthesis and ripening (Fig. 1) during the summer negated the intended effects of irrigation.

Impact of isolate aggressiveness on fungal growth, mycotoxin production and yield parameters

The different levels of aggressiveness led to some significant differences in the TRI-DNA concentrations at the second assessment date in 2007 only (Fig. 3b). Control plots showed significantly lower TRI-DNA concentrations than the inoculated plots ($P<0.05$) and group 2 had the highest ($P<0.05$) TRI-DNA concentrations within the non-irrigated plots.

The contamination of the wheat kernels with DON was highest in the plots inoculated with isolates of group 2 (Fig. 3c). Within the samples of the non-irrigated plots, treatments with isolates of group 2 caused the significantly highest ($P<0.05$) DON contamination. The group 2 isolates also led to significantly higher ($P<0.05$) DON concentrations for irrigated inoculated plots compared to the non-inoculated control plots (Fig. 3c). In contrast to the field experiment results, in vitro tests revealed the great ability of the group 2 isolates in producing NIV and ZEA, but only low abilities in terms of DON. The ZEA production did not differ among the groups (Fig. 3d).

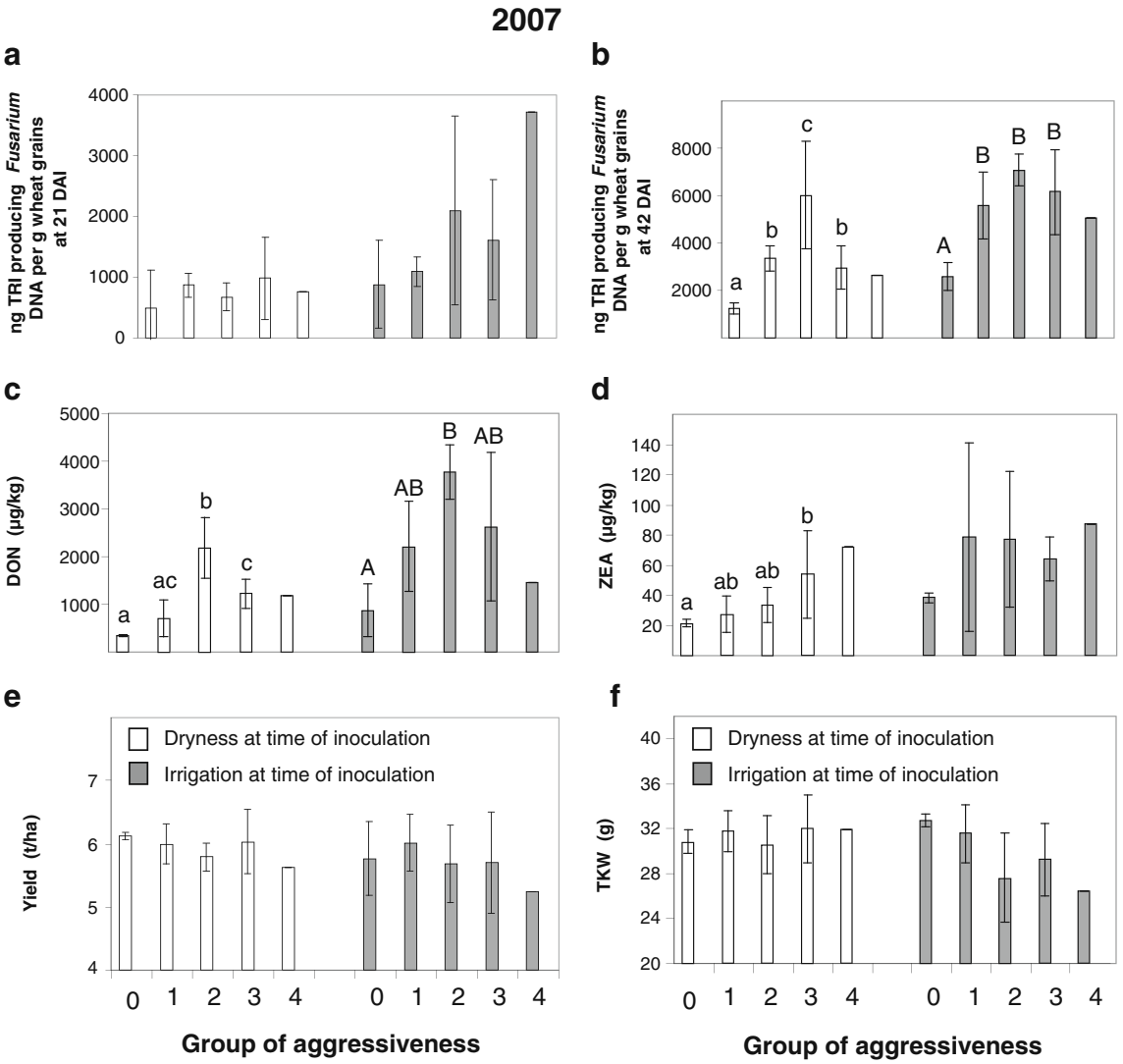

Fig. 3 **a** TRI-DNA concentration in wheat grains at 21 DAI and **b** at 42 DAI, **c** DON and **d** ZEA content, and **e** yield, and **f** 1,000-kernel weight of wheat grains in 2007 Means and standard deviations of the aggressiveness groups as characterized by the in vitro studies are displayed (*0* control group, *1* low aggressiveness, *2* moderate aggressiveness, *3* high aggressiveness, *4* very high aggressiveness). Columns with different letters (*lowercase letters*, plots under dryness at time of inoculation; *uppercase letters*, plots irrigated at time of inoculation) indicate significant differences ($P<0.05$)

None of the aggressiveness groups had an effect on yield or TKW (Fig. 3e-f). The grain yields and the 1,000-kernel weights in samples of the non-inoculated and inoculated plots were similar.

In 2008, the effect of inoculation was strongly depressed. Differences in aggressiveness between the selected isolates that were inoculated were not observed.

Activities of sucrose cleaving enzymes

The activity of sucrose cleaving enzymes was measured to check the influences of pathogen attack and irrigation on the plant's carbohydrate metabolism. The results of the investigations in 2007 are recorded in Fig. 4, which demonstrates the effect of *Fusarium* isolates arranged according to increasing aggressiveness as determined in vitro (see "Materials and methods"). The extracellular, cell wall-bound invertase was found to be the sucrose-cleaving enzyme with the most activity followed by sucrose synthase. However, sucrose synthase was neither influenced by the aggressiveness of the isolates inoculated nor by irrigation (Fig. 4d). The activity of the invertase isoenzymes was generally increased by irrigation. Aggressiveness differently affected the enzyme activities. The extracellular invertase activity seemed to increase with rising fungal aggressiveness, but only when the plants

Table 2 Mean DON and ZEA content (at harvest) and TRI-DNA concentration (at 42 DAI) in the wheat kernels as well as the activity of the extracellular invertase (at 7 DAI) in the wheat heads (± standard deviation) in 2008

	DON (µg/kg)	ZEA (µg/kg)	TRI-DNA (ng/g DM)	extract. invertase (µg glucose/ min mg protein)
Non-irrigated plots n=6				
Control plots	77.5 (± 62.1)	0.0 (± 0.0)	0.9 (± 1.2)	17.6 (± 9.4)
Fc53[a]	68.2 (± 90.5)	16.3 (± 38.0)	12.1 (± 15.5)	21.1 (± 6.9)
Fg18	56.8 (± 45.6)	0.0 (± 0.0)	2.8 (± 4.0)	13.4 (± 5.5)
Fg23	65.3 (± 79.2)	0.0 (± 0.0)	0.6 (± 0.5)	15.6 (± 4.5)
Fc13	69.3 (± 67.7)	0.0 (± 0.0)	9.0 (± 13.6)	13.2 (± 3.0)
Irrigated plots n=6				
Control plots	66.6 (± 56.4)	14.4 (± 35.1)	3.0 (± 3.3)	22.7 (± 13.6)
Fc53	69.9 (± 67.6)	8.1 (± 12.1)	35.0 (± 27.8)	23.4 (± 15.3)
Fg18	128.1 (± 107.4)	13.9 (± 31.7)	237.9 (± 377.9)	17.7 (± 7.2)
Fg23	77.0 (± 55.1)	10.1 (± 14.9)	2.7 (± 1.8)	14.2 (± 2.7)
Fc13	106.1 (± 31.3)	4.3 (± 5.6)	11.5 (± 15.5)	19.5 (± 2.9)

[a] *Fc53 F. culmorum* 53, moderate aggressiveness; *Fg18 F. graminearum* 18, high aggressiveness; *Fg 23 F. graminearum* 23, high aggressiveness; *Fc 13 F. culmorum* 13, very high aggressiveness

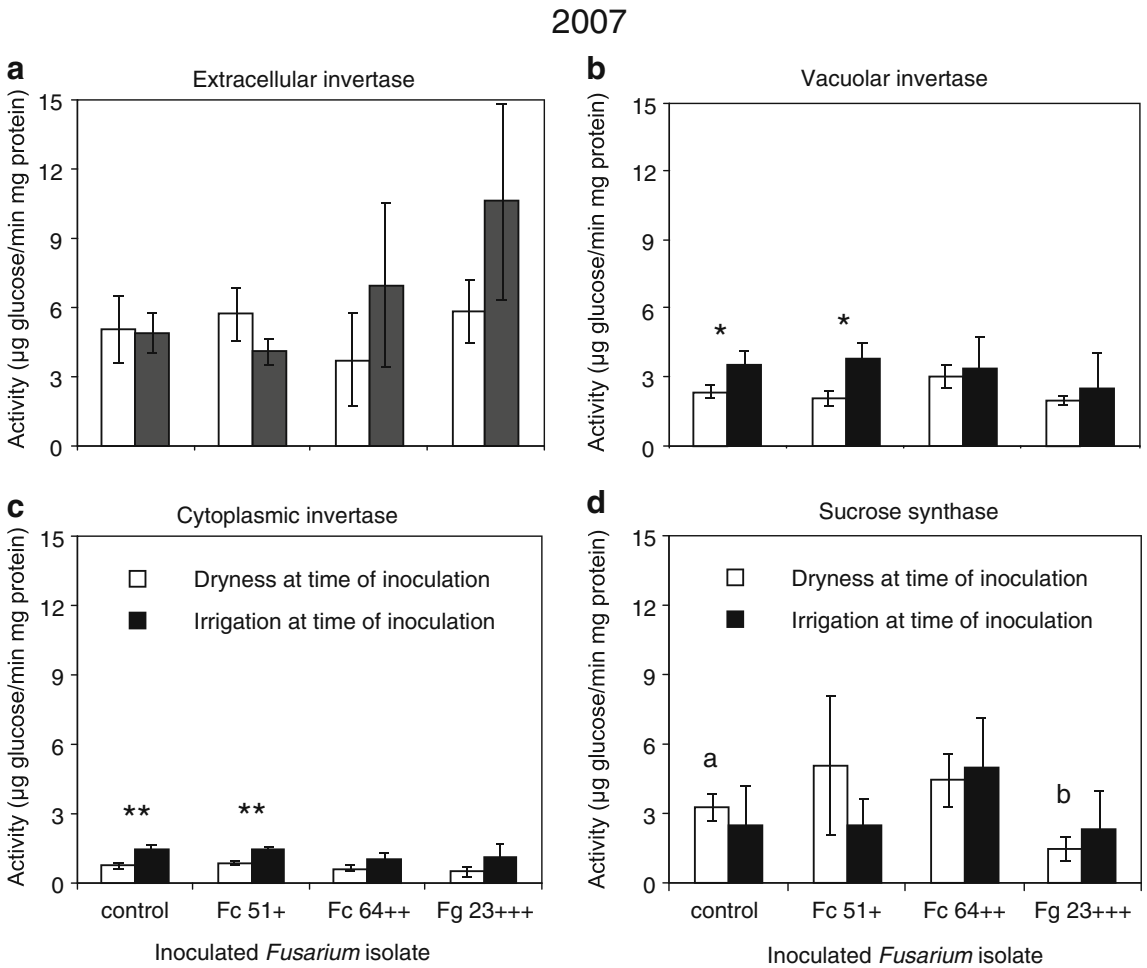

Fig. 4 Activities of sucrose cleaving enzymes in wheat heads harvested 7 DAI in 2007 dependent on the aggressiveness of the isolates and irrigation. *Fc 51 F. culmorum* 51, low aggressiveness; *Fc 64 F. culmorum* 64, moderate aggressiveness; *Fg 23 F. graminearum* 23, high aggressiveness. Means and standard deviations (n=3). Significant differences between mean values from non-irrigated and irrigated wheat heads with the same inoculum: *P<0.05, **P<0.01, both t test). Different *letters* indicate significant differences (P<0.05, t test) between mean values from heads with different inoculation

were irrigated (Fig. 4a). The activity of the vacuolar invertase (Fig. 4b) was significantly enhanced in part by irrigation, but was not affected by the fungal infection. A similar effect was observed with the cytoplasmic invertase, but on a negligible level (Fig. 4c). On the other hand, the infection of the highly aggressive isolate *F. graminearum* 23 repressed the sucrose synthase activity significantly under the condition of dryness at the time of inoculation (Fig. 4d).

In 2008, the activities of all sucrose cleaving enzymes were in the same proportion to one another, but approximately two times greater than the year before (data not shown). Neither irrigation nor fungal infection significantly enhanced any of these enzyme activities. The extracellular invertase data demonstrate that *Fusarium* inoculation had no effect on the plant's carbohydrate metabolism during this year (Table 2).

Discussion

Aggressiveness and mycotoxin production

F. graminearum and *F. culmorum* isolates of different aggressiveness were inoculated on wheat in a field experiment to ascertain their influence on mycotoxin content and yield in grain in the presence and absence of irrigation.

Aggressiveness expresses the quantity of a disease caused by pathogenic microorganisms (Vanderplanck 1984). Aggressiveness and mycotoxin production are usually considered as separate traits of pathogenicity. However, mycotoxins are thought to be involved in plant pathogenesis (Desmond et al. 2008; Langevin et al. 2004), and thus have a significant impact on the aggressiveness (Miedaner et al. 2008). Therefore, in this study, a rating system for the aggressiveness of fungal isolates of the two toxigenic *Fusarium* species, *F. culmorum* and *F. graminearum,* was established, combining both the length of necrotic lesion on inoculated leaf segments and the in vitro mycotoxin production in a cluster analysis. Altogether, the isolates showed considerable variation in their aggressiveness displayed through these traits. Such differences have already been documented in studies with *Fusarium* isolates in Europe (Toth et al. 2004) and Canada (Gilbert et al. 2001).

In vitro assessment of aggressiveness versus effects in the field

The level of aggressiveness among the *Fusarium* isolates determined in vitro was not reflected in the inoculation field experiments. However, the weather conditions in 2007 promoted fungal growth as well as the presence of mycotoxins in field crops. Thereby, the most aggressive *Fusarium* isolates (groups 3 and 4), determined in vitro, did not cause the most severe disease levels. They did not produce the highest fungal colonization rate, the highest DON contamination or the highest yield losses. There was no correlation between the rating of in vitro aggressiveness and the level of disease incited by the isolates in wheat plants in the field. In the dry year 2008, none of the differently aggressive *Fusarium* groups was able to colonize the wheat plants and to produce mycotoxins. It seems that the behavior of a toxigenic *Fusarium* isolate in a living plant is clearly different from that in an in vitro system. This is very likely due to different environmental conditions and interactions between the inoculated isolates and fungi of the natural microflora in the field. Under field conditions, autochthonous and inoculated filamentous fungi compete for nutrients and infection sites on wheat ears, and the results of this competition may be decreased fungal biomass in grain, but increased mycotoxin production as already reported (Xu et al. 2007).

Sucrose-cleaving enzyme activities

Extracellular invertase was the only sucrose-cleaving enzyme that was correlated with pathogen infection in 2007, which indicated an active response of the plant to the pathogen attack. Its increased activity provides energy for plant defence responses, but simultaneously ensures a pathogen-induced supply of carbohydrate to the fungus (Roitsch 2004; Roitsch et al. 2003). However, this was only true when the water supply was adequate, i.e. the fungal spores germinated owing to water availability. The activity of this enzyme has already increased 7 DAI, at a time when the fungal pathogens could hardly been established in their host plant (see Fig. 3a). This is confirmed by investigations of Berger et al. (2004) who found that the expression of the sink-specific extracellular invertase occurs immediately after pathogen attack. Vacuolar and cytoplasmic invertase isoenzymes have functions in various aspects of the plant life cycle, but they are not involved in response to pathogens (Roitsch and González 2004). The sucrose synthase is responsible for maturation processes in developing seeds (Koch 2004). Therefore, it was active at the time of sampling. However, the regulation of this enzyme is completely different from that of invertases (Koch 2004). And this is also reflected in a significant decrease in enzyme activity after the attack of a highly aggressive fungus under dry conditions (Fig. 4d).

The data suggest a relationship between invertase activity and in vitro isolate aggressiveness: the higher the extracellular invertase activity, the more aggressive (deter-

mined in vitro) was the infecting isolate (Fig. 4a). A study published by Peltonen (1998), who found similar reactions in the phenylalanine ammonia-lyase in barley and wheat, supports our findings. It is a new aspect in this context, however, that our data indicate that this relationship depends on the water supply.

The measured enzyme activities, like the other parameters determined in this study, gave no indication of a fungal infection in 2008. The generally higher degrees of activity compared to those measured in 2007 might be due to accelerated maturation processes in the developing seeds (for review, see Roitsch and González 2004) under warmer and dryer conditions.

The influence of the weather

There is general agreement that air humidity, soil moisture and weather conditions at and after anthesis have the most important influence on the severity of *Fusarium* infection, disease development and mycotoxin production (Del Ponte et al. 2007; Müller et al. 2011). In this study, we investigated the impact of fungal inoculations on crop quality parameters over 2 years that were fundamentally different in climate. In 2007, there was a dry period during the entire anthesis and moderate precipitation during the early milk stages (a total of 30.3 mm), but a humid period 4 weeks before harvest (a total of 111.6 mm) (Fig. 1). We found that additional irrigation between anthesis and the ripening of the grain (a total of 69.0 mm) significantly enhanced fungal colonization and mycotoxin production. Consequently, the differences in fungal growth at irrigated and non-irrigated plots might be due mainly to the distinctions in water availability during anthesis and the beginning of the ripening period. This is in line with the results published by Lacey et al. (1999) and Koch et al. (2006) who reported that the greatest rate of ear infection and a crucial DON contamination were related to high irrigation or precipitation during anthesis. However, despite these significant differences between the irrigated and the non-irrigated plots, the wheat plants of the non-irrigated plots also showed relatively high levels of DON (Table 2). This might be due to the frequent rainfall in July and confirms the study by Mesterházy (2002) who demonstrated in a field trial that high levels of precipitation in the period of grain ripening also increase kernel infection, DON contamination and yield losses in wheat.

An extremely dry summer throughout the entire period between anthesis and ripening with a total of only 63 mm precipitation in June and July (Fig. 1) prevented wheat floret colonization by the inoculated *Fusarium* isolates and fungal growth in the spikelets in 2008. This was clearly indicated by the very low TRI-DNA contents in the wheat heads. Surprisingly, even an irrigation of 123 mm in total in June 2008 (Fig. 1) could not promote the intended *Fusarium* infection in the wheat ears.

Local prediction and outlook

The federal state of Brandenburg is characterized by a moderate, continental-dry climate and is one of the driest regions in Germany (Wessolek and Asseng 2006). Climate change calculations predict that the amount of precipitation in Brandenburg will increase in winter and decline in summer (Wiggering et al. 2008). This predicted climatic change and the results of this study lead to the conclusion that *Fusarium* infection risk and the resulting mycotoxin contamination will decrease. However, the climate change prediction as calculated by Wiggering et al. (2008) does not take into account extreme precipitation events, which are expected to increase in frequency (IPCC 2007). Such extreme events might enhance the risk of *Fusarium* infection and mycotoxin production. Therefore, it is very difficult to calculate the future mycotoxin risk in grain for this region. Perhaps the most significant secondary result of this study was the fact that a typical mycotoxin year may be followed by a year totally free of mycotoxin contamination in grains.

The present study demonstrates once more that fungal growth and mycotoxin production of *Fusarium* in wheat grains are strongly dependent on weather conditions in anthesis and ripening. When water is available for germination of the fungal spores, the attack of the pathogen fungi stimulates the extracellular invertase activity as a very early defence response of the plant. The aggressiveness of *Fusarium* isolates infesting the crop is very likely crucial in plant pathogenesis and mycotoxin production. However, aggressiveness is obviously more complex than in vitro assessed by two traits in this study. And furthermore, the conditions of field experiments are too complex as well to deduce profound conclusions from two completely different growing seasons.

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