Pathogenicity, Symptom Development, and Mycotoxin Formation in Wheat by *Fusarium* Species Frequently Isolated from Sugar Beet

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ABSTRACT

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Crop rotations with putative non-host crops such as sugar beet are often recommended to reduce *Fusarium* head blight (FHB) in cereals. However, recent observations have shown pathogenic, endophytic, and saprotrophic colonization of sugar beet with various *Fusarium* spp. Therefore, strains of seven species frequently isolated from sugar beet were tested for pathogenicity on wheat. Species-specific symptoms on heads and kernels were evaluated and the grains were analyzed for 20 mycotoxins with liquid chromatography–tandem mass spectrometry.

Fusarium graminearum, *F. culmorum*, and *F. cerealis* from sugar beet caused typical FHB symptoms and mycotoxin contamination with deoxynivalenol and nivalenol, while a high incidence of black point was observed in heads inoculated with *F. tricinctum* or *F. equiseti*. Black point kernels revealed 3.4 to 14.5 times higher mycotoxin concentrations than symptomless grains, containing enniatin B1 at 38,000 µg/kg, moniliformin at 4,900 µg/kg, and 2-amino-14,16-dimethyloctadecan-3-ol at 5,500 µg/kg, as well as monoacetoxyscirpenol at 2,600 µg/kg and nivalenol at 3,800 µg/kg. Monitoring of these latter two species in the field is hampered by the lack of typical head symptoms after infection. In further experiments, the impact of sugar beet residues on FHB severity and the correlation between mycotoxin contamination of cereal lots and the amount of black point have to be evaluated.

Fusarium head blight (FHB) has become one of the most important fungal diseases of small grain cereals worldwide. Losses of ≈\$3 billion due to reduction in yield and quality have been estimated in wheat and barley solely in the United States during the 1990s (68). In addition, the contamination of cereal-based food and feed with various mycotoxins affecting human and animal health is of great concern. Some countries have decided maximum levels for the occurrence of mycotoxins in unprocessed and processed cereal products intended for human consumption. In the European Union, such maximum levels are in effect for deoxynivalenol (DON), zearalenone (ZEA), and fumonisins (2). Whereas fumonisins are considered to be potentially carcinogenic, DON, like other trichothecenes, is a strong inhibitor of protein biosynthesis in mammals and affects the gastrointestinal tract. In contrast, ZEA binds to the estrogen receptors and interferes with the endocrine system (5).

The main causal agent of FHB in Central Europe and the United States is *Fusarium graminearum* (teleomorph *Gibberella zeae*), a potent producer of DON and ZEA (8,51). The typical symptoms include partial or complete bleaching of the ear (“white heads”) and the occurrence of shriveled and discolored (“scabby”) kernels. Nevertheless, at least 17 *Fusarium* spp. have been isolated from diseased wheat heads (9,52) and, depending on the infecting species, the symptoms and mycotoxins produced can be much more diverse. For example, various *Fusarium* spp. have also been frequently isolated from black point kernels (16,45). Black point describes a brown to black kernel discoloration of small grain cereals which is observed in nearly all growing regions. The

causes of black point are controversially discussed in the relevant literature. On the one hand, fungi (i.e., *Alternaria* spp., *Cochliobolus sativus*, and *Fusarium* spp.) were often found to be associated with the typical symptoms whereas, on the other hand, abiotic stress and climatic conditions during grain filling are supposed to be the trigger (30,43,45).

Fusarium infection and mycotoxin contamination of wheat is generally linked with environmental conditions and agricultural practices. Weather conditions (i.e., temperature, humidity, and rainfall) prior to anthesis and from flowering through the soft dough stage are crucial for inoculum production and head infection (22,69). Crop rotation, tillage, growth of resistant varieties, and fungicide application play a major role in FHB management (23). Crop residues on the soil surface have been shown to be the main inoculum source (39,46,56). Small-grain cereals pose a medium risk, whereas maize is supposed to be the worst crop to precede wheat with regard to FHB severity and DON contamination (21,61). As a result, crop rotations with so-called non-host crops, such as sugar beet, potato, or vegetables, have been recommended to reduce inoculum density (1).

However, recent observations have challenged this beneficial impact of sugar beet because pathogenic, endophytic, and saprotrophic colonization of sugar beet with typical FHB pathogens such as *F. graminearum*, *F. culmorum*, *F. cerealis*, and *F. avenaceum* has been detected during growth and storage (15,7,31,32). In some years and regions of Germany and Switzerland, an increased incidence of *F. culmorum* (41) and *F. graminearum* (33) was detected in wheat grown after sugar beet. In a crop rotation trial in Germany, the DON content of wheat succeeding sugar beet or potato was twice as high as the DON content of wheat following wheat or barley (51). Greenhouse inoculations revealed that *F. graminearum* strains isolated from sugar beet and potato were also pathogenic on wheat and caused mycotoxin profiles and amounts comparable with strains isolated from wheat (11,13).

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Fusarium spp. colonizing sugar beet are more or less as numerous as the species occurring in wheat. In total, 13 different *Fusarium* spp. have been isolated from freshly harvested and stored sugar beet in Lower Saxony, Germany (15). However, except for *F. graminearum*, the pathogenicity of sugar-beet-derived *Fusarium* spp. on wheat has not yet been determined.

The purposes of this study were to (i) determine the pathogenicity on wheat of the seven most frequently isolated *Fusarium* spp. from sugar beet, (ii) identify species-specific symptom development and mycotoxin production in wheat, and (iii) examine a possible association between mycotoxin contents and the observed black point symptoms caused by *F. equiseti* and *F. tricinctum*.

MATERIALS AND METHODS

Fungal isolates and inoculum preparation. Single-spore isolates of the seven most frequently isolated *Fusarium* spp. from sugar beet, as described in a previous study (15), were used for the inoculation of wheat heads: *F. cerealis* strain number O45 (DSM23356), *F. culmorum* O29 (DSM23354), *F. equiseti* O19 (DSM23353), *F. graminearum* O14 (DSM23352), *F. oxysporum* O38 (DSM23355), *F. redolens* O1 (DSM23351), and *F. tricinctum* O63 (DSM23357). The strains were previously characterized for their pathogenicity to sugar beet and their mycotoxin profiles on autoclaved rice grains (15), and had been deposited at the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. As reference strain for the evaluation of pathogenicity and aggressiveness, *F. graminearum* strain Fg142 from wheat, which had caused severe FHB symptoms in previous experiments (R. Gödecke, unpublished data), was used. The strains were maintained as conidia suspensions in 15% (vol/vol) glycerol at -80°C . Prior to use, the strains were subcultured on potato dextrose agar (PDA) at 22°C in the dark. For the inoculum preparation, Erlenmeyer flasks containing water and 2% (wt/vol) wheat straw were autoclaved twice and inoculated with two plugs of an actively growing culture on PDA (7 to 10 days old). After 14 days, the spore suspensions were filtered through two layers of cheesecloth and the spore concentrations were adjusted with sterile tap water to 2.5×10^5 spores/ml with 0.2% Tween 20. No distinction was made between macro- and microconidia.

Pathogenicity assay. *Fusarium*-susceptible red hard spring wheat 'Kadrlj' (SW Seed, Svalöf, Sweden) was sown in 9-by-9-cm pots filled with standard potting mixture (sand, potting soil, and steamed compost in a ratio of 3:12:4) in the greenhouse. All secondary shoots were removed in order to obtain equally developed plants with one tiller each per pot. The plants were arranged in a completely randomized design and the experiment was conducted twice to confirm the reproducibility of the results. In all, 576 plants (64 per treatment) were used in the first trial set-up and 720 plants (80 per treatment) in the second trial set-up; however, plants displaying deformed or short ears were excluded. The plants were fertilized weekly with Hakaphos (15% N, 10% P_2O_5 , 15% K_2O , and 2% MgO) (Compo, Münster, Germany) from the beginning of stem elongation to the dough stage and kept at 20 and 14°C (day and night, respectively) with a photoperiod of 16 h and an average humidity of 50%.

At anthesis, when half of the anthers were extruded, wheat heads were inoculated individually by spraying the spore suspension with a hand atomizer to the point of runoff. Heads sprayed with sterile distilled water were used as negative controls. After inoculation, the heads were covered with transparent polyethylene bags to maintain 100% humidity for 48 h. The heads were visually scored for symptoms on husks and rachis on a daily basis. The symptoms caused by the different strains varied widely; however, in order to assess pathogenicity, each affected spikelet showing a single brown spot or complete bleaching was defined as diseased. Because the term FHB is normally applied to more

severe symptoms, a disease index (DI) was determined. The DI was calculated at 7, 14, and 21 days postinoculation (dpi) according to the following formula: $\text{DI}(\%) = (\text{disease severity}/100) \times (\text{disease incidence}/100) \times 100$. Disease severity refers to the percentage of symptom-bearing spikelets per head, while disease incidence represents the percentage of symptom-bearing heads per treatment.

Postharvest examination of kernels. At maturity, the inoculated plants from the second trial set-up were randomly subdivided into four replicates with 20 plants each for kernel examination. The wheat heads were harvested and threshed by hand and the grains of each replicate and treatment were scored for kernel symptoms. All shriveled, moldy, or white and red discolored kernels were considered as scabby, while black point was defined according to Hanson and Christensen (30) as a dark discoloration normally restricted to the embryo and adjacent areas, sometimes extending to the crease. Subsequently, the grains were used for reisolation and mycotoxin analysis. For the fungal reisolation, 25 kernels per treatment were surface sterilized in a sodium hypochlorite solution ($\approx 1.5\%$ available chlorine) for 2 min, rinsed three times in sterile distilled water, and blotted dry on sterile filter paper. The kernels were placed on PDA and incubated at 22°C under normal daylight conditions for 10 days. Species identification was based on morphological traits on Spezieller Nährstoffarmer Agar (SNA) cultured at 25°C in the dark (27,40,50). The fungal isolates were compared with the originally inoculated strains.

Mycotoxin analysis. Because of low seed set in some treatments, grains from two replicates each were pooled and ground with a centrifugal mill (ZM 200; Retsch, Haan, Germany) to a fine flour. The samples were stored at -20°C until analysis at the Center for Analytical Chemistry, Department for Agrobiotechnology (IFA, Tulln, Austria), following a liquid chromatography–tandem mass spectrometry-based method which has been described previously (59,60). The amounts of mycotoxins given in Table 1 are the means of the two independent results. All of the samples were tested for DON, DON-3-glucoside, 3- and 15-acetyldeoxynivalenol, nivalenol (NIV), fusarenon X (FUS X; syn. 4-acetylivalenol), diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), ZEA, aurofusarin (AURO), beauvericin (BEAU), enniatins (ENNI) A, A1, B, B1, and B2, as well as moniliformin (MON), equisetin (EQUI), 2-amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol), and avenacin Y (AVENA). The external calibration was performed with a liquid multitoxin standard. A blank sample was spiked at one concentration level in triplicate. When the recovery was out of range ($100 \pm 10\%$), the results were corrected. The limit of detection was calculated for each of the toxins.

Mycotoxin formation in black point kernels infected with *F. equiseti* and *F. tricinctum*. In a separate experiment, the *Fusarium*-susceptible wheat 'Tybalt' (Saaten Union, Leopoldshöhe, Germany) was spray inoculated on the heads with a mixture of three strains each of *F. equiseti* (O17, O19, and O90) and *F. tricinctum* (O32, O35, and O63), with a final concentration of 2.5×10^5 spores/ml in order to determine the association between the specific mycotoxin formation and the occurrence of black point. In all, 72 plants per treatment were inoculated and arranged in a completely randomized design. Plant growth conditions, inoculation procedure, and mycotoxin analysis were performed as described above, except for the average humidity in the greenhouse, which was 60%. After harvest, the grains of all heads were pooled and classified into healthy-looking and black point kernels. Mycotoxin analysis of these fractions was restricted to the species-specific mycotoxins detected in the inoculation experiment described above (i.e., NIV, MAS, DAS, and EQUI for *F. equiseti*-infected kernels as well as ENNI A, A1, B, B1, and B2, MON, 2-AOD-3-ol, and AVENA for *F. tricinctum*-infected kernels).

Data analysis. Statistical analysis was carried out with SAS (version 9.1; SAS Institute, Cary, NC). Because FHB incidence and severity may vary between trial set-ups, the two trials were analyzed together. Single plant scores ($n = 134$ to 144) were used for comparison of DI at each rating date. The data did not fulfill the conditions of normality; therefore, the relative effect (RE) for each plant was calculated and an analysis of variance-type statistic (ATS) was performed. REs do not possess a dimension and can have values between upper and lower limits (10,54), which were included in Table 2. The REs were the basis for the following nonparametric test for factorial designs as described by Brunner and Munzel (10) and Shah and Madden (54). For multiple comparisons between treatments, least square means for the ranks of marginal effects were computed (adjustment = Tukey-Kramer) and the SAS macro MULT (<https://www.uni-hohenheim.de/bioinformatik/beratung/toolsmacros/sasmacros/mult.sas>) was used to assign letters. The estimated relative treatment effects with appropriate confidence limits for the REs of the single heads were calculated with the SAS macros LD_CI (http://www.ams.med.uni-goettingen.de/de/sof/ld/LD_CI.SAS) and OWL (<http://www.ams.med.uni-goettingen.de/amsneu/download/longit/OWL.SAS>), respectively. Kernel symptoms were expressed as the percentage of scabby or black point kernels in each of the four independent replicates. Analysis was conducted with PROC GLM of SAS.

Differences among means, as determined by Tukey's pairwise test, were used for the differentiation of treatments. Box plots were designed with STATISTICA 9 (Stat Soft, Tulsa, OK).

RESULTS

Pathogenicity and symptom development of sugar beet isolates on wheat. No symptoms were observed in the water control whereas sugar beet isolates of *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. tricinctum*, and *F. equiseti* proved to be pathogenic on 'Kadrlj' spring wheat. For all pathogenic strains, a constant increase in the median DI was observed from 7 to 21 dpi (Table 2); however, the disease assessment varied widely. *F. graminearum* from sugar beet proved to be the most aggressive strain in the study, although no significant differences were found among the relative effects of the *F. graminearum*, *F. cerealis*, and *F. culmorum* strains tested (Table 2). The DIs induced by the *F. cerealis* strain (5 to 85% at 21 dpi) were generally on a lower level than the DIs induced by the *F. graminearum* strains from wheat (0 to 100% at 21 dpi) or sugar beet (0 to 96.4% at 21 dpi) and the *F. culmorum* strain (0 to 98.7% at 21 dpi); however, the symptom development was quite similar.

The most typical symptoms were completely bleached spikelets and the brown discoloration of rachis segments. Ripening was

TABLE 1. Mycotoxins detected in grains of wheat 'Kadrlj' after head inoculation with *Fusarium* spp. isolated from sugar beet compared with a *Fusarium graminearum* reference strain from wheat (means of two samples)

Mycotoxin ^y	Mycotoxins (µg/kg) per species (strain) ^z								LOD (µg/kg)
	<i>F. graminearum</i> (Fg142, wheat)	<i>F. graminearum</i> (O14)	<i>F. culmorum</i> (O29)	<i>F. cerealis</i> (O45)	<i>F. equiseti</i> (O19)	<i>F. tricinctum</i> (O63)	<i>F. oxysporum</i> (O38)	<i>F. redolens</i> (O1)	
DON	10,520	22,200	89	28	—	—	—	—	5
DON-3-G	1,571	2,241	17	15	—	—	—	—	0.5
3-ADON	70	115	—	—	—	—	—	—	2
15-ADON	116	161	—	—	—	—	—	—	30
NIV	—	11	8,239	5,670	634	—	—	—	5
FUS X	—	—	755	1,024	370	—	—	—	5
MAS	—	—	—	—	78	—	—	—	4
DAS	—	—	—	—	3	—	—	—	0.7
ZEA	2	1	—	5	—	—	—	—	0.5
AURO	487	49	1,403	450	—	748	—	—	40
BEAU	—	—	TR	TR	—	TR	9	40	0.05
ENNI A	—	—	—	—	—	302	49	30	0.1
ENNI A1	—	—	—	—	—	2,576	26	24	0.1
ENNI B	—	—	—	—	—	3,264	TR	3	0.01
ENNI B1	—	—	—	—	—	4,820	7	13	0.2
ENNI B2	—	—	—	—	—	80	—	—	0.1
MON	—	—	—	—	—	1,650	—	—	20
2-AOD-3-ol	—	—	—	—	—	317	—	—	8
AVENA	—	—	—	—	—	630	—	—	60
EQUI	—	—	—	—	3,891	—	—	—	16

^y DON: deoxynivalenol, 3-ADON: 3-acetyldeoxynivalenol, 15-ADON: 15-acetyldeoxynivalenol, DON-3-G: deoxynivalenol-3-glucoside, NIV: nivalenol, DAS: diacetoxyscirpenol, MAS: monoacetoxyscirpenol, ZEA: zearalenone, AURO: aurofusarin, BEAU: beauvericin, ENNI: enniatin, MON: moniliformin, 2-AOD-3-ol: 2-amino-14,16-dimethyloctadecan-3-ol, AVENA: avenacein Y, EQUI: equisetin.

^z Values below the limit of detection (LOD) are given as "—" and TR = traces <1 µg/kg.

TABLE 2. Disease indices (median [Med] and relative effect [pi] with upper [pU] and lower [pL] limits of confidence) after artificial inoculation of wheat 'Kadrlj' with seven *Fusarium* strains from sugar beet compared with a *Fusarium graminearum* reference strain from wheat^z

Treatment	n	7 dpi				14 dpi				21 dpi			
		Med (%)	pL	pi	pU	Med (%)	pL	pi	pU	Med (%)	pL	pi	pU
<i>F. graminearum</i> (Fg142, wheat)	136	22.4	0.59	0.62	0.65 CD	50.0	0.76	0.78	0.81 D	67.3	0.81	0.84	0.86 D
<i>F. graminearum</i> (O14)	134	15.0	0.57	0.60	0.64 C	57.6	0.73	0.77	0.81 D	74.9	0.80	0.83	0.87 D
<i>F. cerealis</i> (O45)	139	20.0	0.63	0.65	0.67 CD	40.0	0.74	0.76	0.78 D	45.0	0.78	0.80	0.82 D
<i>F. culmorum</i> (O29)	139	24.6	0.65	0.67	0.69 D	44.3	0.76	0.78	0.80 D	54.1	0.79	0.81	0.83 D
<i>F. tricinctum</i> (O63)	139	0.0	0.29	0.31	0.33 B	8.8	0.47	0.50	0.52 C	16.3	0.55	0.57	0.60 C
<i>F. equiseti</i> (O19)	139	0.0	0.33	0.36	0.39 B	2.7	0.40	0.43	0.46 B	6.2	0.45	0.48	0.51 B
<i>F. oxysporum</i> (O38)	139	0.0	0.21	0.22	0.23 A	0.0	0.22	0.23	0.24 A	0.0	0.24	0.25	0.27 A
<i>F. redolens</i> (O1)	140	0.0	0.20	0.21	0.22 A	0.0	0.21	0.22	0.23 A	0.0	0.24	0.25	0.27 A
Water	144	0.0	0.20	0.21	0.21 A	0.0	0.20	0.21	0.21 A	0.0	0.20	0.21	0.21 A

^z Relative effects within columns followed by the same letter are not significantly different (adjustment Tukey-Kramer, $\alpha = 0.05$); dpi = days postinoculation.

accelerated and typical white heads without any kernel formation were observed in the heads inoculated with the *F. graminearum* strains, independent of the initial host plant. The symptoms caused by *F. cerealis* had less devastating effects than *F. graminearum* because larger parts of the heads stayed green, apart from some discolored spots. The symptoms caused by the *F. equiseti* and *F. tricinctum* strains were much less conspicuous. In most cases, only slight discolorations on the palea or lemma were noted, which sometimes became visible only when the florets were twisted (Fig. 1). *F. tricinctum* typically induced necroses on the very edge of the glumes in some spikelets (Fig. 1) and led to a delayed disease development because the initial symptoms were seldom noted before 10 dpi. After this period, the disease developed fast and the mean DI at 14 dpi ($10.5 \pm 9.4\%$) was eight times higher than at 7 dpi ($1.3 \pm 2.1\%$). Generally, inoculation with the *F. equiseti* and *F. tricinctum* strains resulted in a significantly lower DI compared with the *F. graminearum*, *F. culmorum*, and *F. cerealis* strains tested. The two strains of *F. redolens* and *F. oxysporum* hardly produced any symptoms and their relative effects did not differ from the water control (Table 2); however, all the species inoculated were reisolated from surface-sterilized grains at 84 to 100% (data not shown). The lowest reisolation rates were obtained for *F. graminearum* while recovery from *F. redolens* and *F. oxysporum* reached 100%. No fungal growth was observed for the water control.

Kernel symptoms. Similarly, the disease symptoms on the kernels varied greatly, depending on the inoculated strains (Fig. 2). In addition to the expected scabby kernels typical for FHB,

sometimes a dark discoloration of the embryo and adjacent areas was observed and identified as black point. The *F. graminearum* strain from sugar beet caused significantly more scabby kernels (mean $52.9 \pm 4.1\%$) than the *F. graminearum* strain from wheat ($32.5 \pm 4.8\%$). In comparison, *F. culmorum*-inoculated heads displayed a significantly lower incidence of scabby kernels ($24.4 \pm 2.0\%$). Black point symptoms generally occurred on a lower level than scab and were only sporadically noted for *F. graminearum* and *F. culmorum*. The grains from the ears inoculated with strains of *F. equiseti* and *F. tricinctum* were almost healthy looking, except for the observation that they displayed the highest incidence of black point kernels, with averages of $5.8 \pm 0.5\%$ and $6.4 \pm 1.5\%$, respectively (Fig. 2). *F. cerealis* took an intermediate position as the amount of black point ($4.1 \pm 0.8\%$) as well as of scabby kernels ($18.5 \pm 4.2\%$) differed significantly from the water control (Fig. 2).

Mycotoxin contamination of grains. Grains inoculated with the *F. graminearum*, *F. culmorum*, and *F. cerealis* strains contained high amounts of trichothecenes, whereas only traces of ZEA were detected (Table 1). More than twice as much DON was detected in the grains from the heads inoculated with the *F. graminearum* strain from sugar beet compared with the reference strain from wheat. Simultaneously, DON-3-glucoside occurred at a ratio of 10.1% (sugar beet strain) and 14.9% (wheat strain) of the DON concentration. Low amounts of DON-3-glucoside were also detected in the kernels inoculated with the NIV-producing isolates of *F. culmorum* and *F. cerealis*. A simultaneous occurrence of DON and NIV was observed for these strains as well as

Fig. 1. Characteristic symptoms caused by *Fusarium tricinctum* and *F. equiseti* isolated from sugar beet on wheat 'Kadrlj' following spray inoculation. **A**, Necrotic edges of glumes were typical symptoms of *F. tricinctum*. **B to D**, Apart from that, slight discolorations on palea and lemma, sometimes visible only when twisting glumes or florets, were usually the only sign of infection with *F. equiseti* and *F. tricinctum*.

for the *F. graminearum* strain from sugar beet. However, the production of the “minor” trichothecene in either the DON or NIV chemotypes did not exceed 1.1%. NIV and its derivative FUS X were mainly found in the grains infected with *F. culmorum*, *F. cerealis*, and *F. equiseti* (Table 1). In addition to NIV and the type A trichothecenes MAS and DAS, high amounts of EQUI were also detected in the *F. equiseti*-infected grains. The grains infected with *F. tricinatum* revealed AURO, MON, 2-AOD-3-ol, and AVENA as well as considerable concentrations of enniatins (Table 1). Enniatins, together with BEAU, were also found in the grains infected with the *F. oxysporum* and *F. redolens* strains, although the amounts were comparatively low (Table 1).

Mycotoxin formation in black point kernels infected with *F. equiseti* and *F. tricinatum*. The symptom development for *F. equiseti* and *F. tricinatum* on ‘Tybalt’ was the same as described above for the individual strains inoculated on ‘Kadrlj’. However, with 16.6% (*F. equiseti*) and 38.6% (*F. tricinatum*), the incidence of black point kernels in ‘Tybalt’ was higher than in the previous experiment, where not only a different cultivar but also a single strain instead of the strain mixture for inoculation was used. The mycotoxin content of the healthy-looking and black point kernels differed by ratios of 3.4 to 14.5 (Tables 3 and 4). The highest increase was observed for 2-AOD-3-ol, with 5,500 µg/kg in the black point kernels. In contrast, AVENA was not detected in healthy-looking kernels (Table 4). However, with MAS at 560 µg/kg and NIV at 490 µg/kg (Table 3) as well as ENNI B1 at

6,700 µg/kg and MON at 470 µg/kg (Table 4) in addition to the presence of other toxins in small amounts, the symptomless and apparently healthy kernels contained considerable mycotoxin concentrations.

DISCUSSION

Apart from *F. graminearum* (13), sugar beet isolates of four additional *Fusarium* spp. proved to be pathogenic on wheat heads. The *F. graminearum* strain from sugar beet was the most aggressive one applied in this study, even more aggressive, although not significantly, than the control strain isolated from wheat. Due to the facts that only one strain per species was tested and *Fusarium* strains may vary greatly in their aggressiveness (47), no general conclusions on species level can be drawn.

TABLE 3. Comparison of mycotoxin contents in different kernel fractions of wheat ‘Tybalt’ inoculated with *Fusarium equiseti*

Kernel fraction	Mycotoxin content (µg/kg) ²			
	NIV	DAS	MAS	EQUI
Healthy-looking kernels	490	7	560	200
Black point kernels	3,800	39	2,600	710
Ratio	7.6	5.4	4.6	3.6

² NIV: nivalenol, DAS: diacetoxyscirpenol, MAS: monoacetoxyscirpenol EQUI: equisetin.

Fig. 2. Percentage of **A**, scabby kernels and **B**, black point kernels induced in wheat ‘Kadrlj’ following artificial inoculation with strains of seven *Fusarium* spp. isolated from sugar beet compared with a *Fusarium graminearum* strain from wheat. Means with the same letter are not significantly different ($\alpha = 0.05$, Tukey pairwise test).

Nevertheless, *F. graminearum* strains from sugar beet and potato also proved to be more aggressive than cereal isolates in a previous study (13). Phylogenetic analyses might help to understand whether these differences in aggressiveness have a genetic base. However, based on 10 variable number tandem repeat markers, Burlakoti et al. (12) found no evidence for large genetic variation in *G. zeae* populations isolated from barley, wheat, potato, and sugar beet in the Upper Midwest of the United States. *F. graminearum* is not known for pronounced host specificity (27,47); cereal-pathogenic *Fusarium* isolates have also been obtained from common weeds (35), representing alternative hosts, as well.

An average of three tons of sugar beet residues (whole beet tubers, removed crowns, and root fragments) per hectare remain in the field with conventionally applied harvesting techniques (29). Regarding the observation that the cereal-pathogenic species *F. culmorum*, *F. cerealis*, and *F. graminearum* prevailed in sugar beet subjected to long-term storage (15), the impact of sugar beet residues on FHB infection of the succeeding wheat culture needs to be evaluated. Hypothesizing that these species are also prevalent in sugar beet debris (which is nothing else than long-term stored and injured sugar beet tissue), these residues might provide a potential inoculum source.

Generally, no statistically significant differences were detected among the strains causing typical FHB symptoms. This is mostly due to the overlapping variances and the weak, nonparametric test method, because the mean (data not shown) and median DIs revealed quantitatively rather great differences (e.g., sugar beet isolates of *F. cerealis* and *F. graminearum* at 21 dpi). Based on the visual assessment, the aggressiveness of the strains tested decreased in the following order: *F. graminearum* > *F. culmorum* > *F. cerealis* > *F. tricinatum* > *F. equiseti*. *F. oxysporum* and *F. redolens* hardly produced any symptoms under the given environmental conditions. However, all seven *Fusarium* strains isolated from sugar beet were able to penetrate the wheat husks and infect the heads at least endophytically after spray inoculation because reisolation was possible in high percentages for all of them. This indicates a certain amount of aggressiveness also for the putatively apathogenic strains of *F. redolens* and *F. oxysporum*.

In a previous study, the mycotoxin profiles of 34 single-spore isolates from sugar beet belonging to 13 *Fusarium* species, including the here used strains, were determined in rice culture (15). No differences, apart from the normal intraspecific variation, were detected when compared with *Fusarium* spp. isolated from other hosts (19). This was confirmed by the present analysis in planta. The sugar beet strain of *F. graminearum* used in this study led to a DON contamination of wheat grains twice as high as was observed for the reference strain from wheat. DON is supposed to be an essential factor for aggressiveness (3,4) whereas, in general, NIV producers are thought to be less aggressive (25,48,49). However, the DI and percentage of scabby kernels caused by the NIV-producing strains of *F. culmorum* and *F. cerealis* did not differ significantly from the DON-producing *F. graminearum* strain from wheat. This is similar to the results of Carter et al. (14), who found no difference in the aggressiveness of DON- and NIV-producing isolates in wheat, although the NIV chemotypes were more aggressive in maize.

In addition to the high DON contents found, significant amounts of DON-3-glucoside were detected in the *F. graminearum*-in-

fecting grains. Glucose-conjugated or “masked” mycotoxins escape routine detection methods but are transformed to their toxic precursors after hydrolysis in the gastrointestinal tract and might contribute to human and animal mycotoxicosis (26). Interestingly, low levels of DON-3-glucoside also were detected in addition to minor concentrations of DON in the NIV-producing strains of *F. culmorum* and *F. cerealis*. Concomitant production of DON and NIV has been reported before (58). However, to the best of the authors’ knowledge, co-occurrence of NIV and masked DON has not been described before. Nevertheless, slight background contamination cannot be excluded in greenhouse experiments and these results will have to be confirmed by further investigations.

A constant increase in the DI from 7 to 21 dpi was observed for all five pathogenic strains tested. For *F. graminearum*, *F. culmorum*, and *F. cerealis*, this could be explained by the fungi’s systemic growth via the rachis and subsequent secondary infections of other spikelets. Discoloration of rachis segments was observed in the majority of the heads inoculated with these species.

The infection process of *F. culmorum* and *F. graminearum* has been comprehensively studied by light, confocal laser scanning, and transmission electron microscopy (34,37,38). In contrast, such studies are lacking for *F. equiseti* and *F. tricinatum*. These latter two species have been reported as causing only necrosis in inoculated spikelets after point inoculation (57,66). In the present investigation, the *F. tricinatum* strain had the longest latency. It might be possible that the strains’ difficulty in penetrating into the floret might have led to external hyphal growth along the glumes and delayed primary infections. This may also explain the typical symptom of necrotic glume edges, becoming visible ≈10 dpi, found with this species.

The symptom descriptions for the so-called less important species of the FHB complex are ambiguous in the literature. Kane et al. (36), after applying high inoculum densities of *F. tricinatum*, reported that the number of kernels per head, unlike the 1,000-kernel weight, was reduced, although no severe symptoms were observed. In contrast, Vargo et al. (65) described the symptoms of *F. tricinatum* as resembling FHB, except that the kernels were not affected. For the same reasons, *F. equiseti* and *F. poae* have also been classified as being weakly pathogenic compared with *F. graminearum*, *F. culmorum*, and *F. avenaceum* (24). Only a few heads inoculated with *F. equiseti* and *F. tricinatum* strains in this study revealed typical FHB symptoms (i.e., bleaching of single spikelets). Scabby kernels were not produced. The less obvious symptoms on the palea and lemma sometimes only became visible when the glumes or florets were twisted. Monitoring of such symptoms in the field would be most laborious and might be considered to be unnecessary because the yield is supposed to be not affected.

However, after spray inoculation in the greenhouse, the kernels revealed a high amount of toxigenic secondary fungal metabolites, including type A trichothecenes, NIV, FUS X, and MON. Because grains from inoculated heads were almost healthy-looking, there is a high probability that naturally infected kernels might pass the different steps of grain processing, leading to the occurrence of “hidden” mycotoxins in food and feed. The low level of black point was the only visible sign of a disorder in the grains from *F. equiseti*- or *F. tricinatum*-inoculated heads after harvest. Although various *Fusarium* spp. have been isolated from

TABLE 4. Comparison of mycotoxin contents in different kernel fractions of wheat ‘Tybalt’ inoculated with *Fusarium tricinatum*

Kernel fraction	Mycotoxin content (µg/kg) ^z							
	ENNI A	ENNI A1	ENNI B	ENNI B1	ENNI B2	MON	2-AOD-3-ol	AVENA
Healthy-looking kernels	810	4,000	2,700	6,700	160	470	380	<LOD
Black point kernels	6,200	20,000	9,100	38,000	1,700	4,900	5,500	130
Ratio	7.7	5	3.4	5.7	10.6	10.4	14.5	...

^z ENNI: enniatin, MON: moniliformin, 2-AOD-3-ol: 2-amino-14,16-dimethyloctadecan-3-ol, AVENA: avenacein Y, LOD: limit of detection.

black point kernels (17,45), until now, a correlation with symptom development has been verified only for *F. proliferatum* and *F. avenaceum* (16,28).

Because the baking quality of cereal lots with high levels of black point is unaffected (42,53), black point is often referred to as an aesthetic disorder only important for grain grading. However, black point kernels infected with *F. avenaceum* displayed higher MON concentrations than healthy-looking kernels (28). Desjardins et al. (20) detected significant amounts of black point and FUM in wheat kernels infected with *F. proliferatum*. This is consistent with the results of the present study because all the detected mycotoxins were increased in black point kernels. Kernels of 'Tybalt' with black point symptoms exhibited 3.4 to 14.5 times higher mycotoxin contents compared with symptomless kernels. The greatest increase was observed for 2-AOD-3-ol, a sphingosine analogue, which has only recently been described (64). The maximum concentration in naturally infected grains was reported to be quite low, with only 56 µg/kg in a Finnish wheat sample (63). It might be of interest to analyze the occurrence of this toxin in cereal lots exhibiting a greater incidence of black point because, in this study, 5,500 µg/kg were found in such kernels.

The assumption that black point is just a physiological stress response without fungal association (44,67) is contradictory to the detection of greater amounts of mycotoxins in black point kernels, indicating higher fungal activity. Moreover, in this study, black point was only associated with *F. equiseti*, *F. tricinctum*, and *F. cerealis*, whereas this symptom was only sporadically observed in the grains inoculated with strains of other *Fusarium* spp. and was lacking in the water control, all of which were kept under the same humidity conditions. It remains to be seen whether black point kernels infected with *Alternaria* spp. and *Cochliobolus sativus* also exhibit greater mycotoxin amounts. Therefore, as long as further potentially associated fungi or triggering factors are not known, a possible mycotoxin contamination of cereal lots with high levels of black point has to be assumed. Color screening, usually performed for grading or breeding purposes (6,18,55), and sorting of grain might help to decrease mycotoxin content.

The influence of crop rotation on the less important components of the FHB complex is not well understood because, usually, only typical FHB heads are sampled in field surveys. However, a significant difference was detected in the range of species from diseased and randomly sampled grains (62). Because of their rather minimal symptoms, the impact of species like *F. equiseti* and *F. tricinctum* on mycotoxin contamination might be underestimated. Surveys less restricted to white heads and DON might contribute to a more impartial view in determining the risk deriving from *Fusarium* infections in wheat and any associated mycotoxin contamination.

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