Occurrence of Deoxynivalenol (DON), 3-Acetyl-DON, Zearalenone, and Ergosterol in Winter Rye Inoculated with *Fusarium culmorum*

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ABSTRACT

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To investigate the occurrence of *Fusarium culmorum* mycotoxins and ergosterol (ERG) in winter rye, 29 single-cross hybrids were artificially inoculated at anthesis in 1992. Single-cross hybrids revealed a significant genotypic differentiation for head blight rating and relative grain-weight per spike. Deoxynivalenol (DON) content ranged from 0.72 to 28.0 mg kg^{-1} (average 10.8 mg kg^{-1}); 3-acetyl DON (3-Ac DON) ranged from 0.1 to 3.4 mg kg^{-1} (average 1.2 mg kg^{-1}); the two were closely correlated ($r = 0.79$, $P = 0.01$). Correlation coefficients between head blight rating or relative grain-weight per spike and DON or 3-Ac DON content were nonsignificant ($r = 0.34$–0.36). For a subset of 10 single-cross hybrids, the proportion of *Fusarium*-damaged kernels was closely correlated to relative grain-weight per spike ($r = -0.93$, $P = 0.01$), but showed nonsignificant correlations to the DON and 3-Ac DON contents ($r = 0.45$–0.60). For the other 19 single-cross hybrids, zearalenone (ZEA) and ERG contents ranged from 0.02 to 0.15 mg kg^{-1} (average 0.07 mg kg^{-1}) and 42.9 to 135.4 mg kg^{-1} (average 84.5 mg kg^{-1}), respectively. ERG content was significantly correlated with relative grain-weight per spike ($r = -0.52$, $P = 0.05$), and with DON, 3-Ac DON, and ZEA contents ($r = 0.57$–0.79). ERG-DON ratios varied between 3.6 and 14.4. Winter rye artificially inoculated with *F. culmorum* accumulated DON, 3-Ac DON in high amounts with considerable differentiation among 29 single-cross hybrids.

Trichothecene mycotoxins deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-Ac DON), and an estrogenic mycotoxin zearalenone (ZEA) are produced by *Fusarium culmorum* (W.G.Sm) Sacc. This species is considered most important in regions of moderate climate in Western and Middle Europe (Chelkowski 1989, Snijders 1990, Miedaner et al 1993). There is little published information about the occurrence of these mycotoxins in rye (Tanaka et al 1988, Gareis et al 1989, Scott 1990, Yoshizawa 1991).

The aim of this study was twofold: 1) analyze the accumulation of DON, 3-Ac DON, ergosterol (ERG), and ZEA in grain of 29 winter rye (*Secale cereale* L.) single-cross hybrids after *Fusarium culmorum* infection; and 2) compute correlations between these fungal metabolites and the resistance traits head blight rating and relative grain-weight per spike.

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MATERIALS AND METHODS

Genetic Materials

Twenty-nine single-cross hybrids established with homozygous inbred lines from the Petkus gene pool as seed parents, and inbred lines from the Carsten gene pool as pollinators, were used for this study. All crosses were established in 1991 using cytoplasmic male sterility (CMS) on adjacent plots isolated by polyethylene walls.

Field Trials

The single-cross hybrids were grown in 1992 at Stuttgart-Hohenheim (400 m above sea level, 8.5°C mean annual temperature, 685 mm mean annual precipitation). They were planted in three-row (0.625 m^2) drilled microplots in a noninoculated and an inoculated treatment block adjacent to each other. Within each block, the genotypes were grown as a 6×7 lattice design with three replicates.

To avoid infection with other pathogens, the plots were sprayed once for *Pseudocercosporella herpotrichoides* (Sportak Alpha, 1.5 l ha^{-1}, jointing stage), *Erysiphe graminis,* and *Puccinia recondita* (Simbo, 1.0 l ha^{-1}, shortly before heading). Application of a growth regulator (Terpal C, 1.5 l ha^{-1}, jointing stage) prevented lodging.

Inoculum Production and Inoculation

To avoid any specific interaction between *F. culmorum* isolates and host genotypes, a mixture of four *F. culmorum* isolates of

different geographic origin (FC9, FC10, FC18, FC19) were used for inoculation. Isolates were established in single spore culture and maintained as permanent cultures in test tubes containing sterilized soil. Conidia in small amounts were produced by plating mycelium from the permanent cultures on synthetic nutrient-poor agar (SNA) for each isolate (Nirenberg 1981). Mass production of conidia was done on wheat grain medium. Wheat grain was boiled with water for 15 min, excess liquid was removed, and 400 ml of grain was placed in 1,000-ml glass flasks. Flasks were sealed with aluminium foil and autoclaved at 121°C for 20 min. One flask of cooled wheat grain medium was inoculated with a spore suspension produced on one petri-dish (65 mm in diameter) during two weeks at 18°C in the dark on SNA. The inoculated wheat grain was incubated at 18°C in the dark for three weeks. Afterward, the colonized wheat grain was removed from the flasks, spread in a thin layer over the surface of plastic boxes, covered with a punctured polyethylene film, and placed under constant black light (Philipps 40W) for seven to ten days until sporulation. The spore-loaded wheat grain was air-dried for two to three days and stored in a refrigerator at 2°C until inoculation. To produce spore suspensions for inoculation, wheat grain material was put in tap water for 0.5 hr. Suspensions of the different isolates were adjusted to a concentration of 0.3×10^6 spores per ml and mixed in equal volumes. Each genotype was artificially inoculated at its respective flowering time with the mixture applied with a portable sprayer at ~80 ml m^{-2} in the evening (7:30–9:00 p.m.). The field was sprinkled by a mist irrigation device for 2 min at 30-min intervals in the morning after each inoculation (6:00–11.30 a.m.). The inoculation was repeated two days later to minimize the influence of weather conditions.

Disease Assessment

Head blight rating and relative grain-weight per spike were used for resistance assessment. Head blight ratings on a 1–9 scale (1 = no symptoms visible; 9 = 100% of spikelets and ears per plot infected) (Mielke 1988) were taken at three dates in the period from 17 to 26 days after inoculation. Arithmetic means across dates were used for further analysis. For determination of grain weight per spike, 20 primary spikes per row were harvested by hand, and all spikes per plot were threshed carefully as one sample.

The proportion of *Fusarium*-damaged kernels was also determined for a subset of 10 single-cross hybrids (Chelkowski 1989). *Fusarium*-damaged kernels are smaller and thinner than healthy kernels, are shrivelled, and are often covered with white or pink to carmin red mycelium. In contrast, visually healthy kernels are of the same size as kernels from the corresponding noninoculated treatment and show no visible discoloration. Kernels were assessed individually by a skilled person.

Chemical Analysis

Samples of infected rye kernels were analyzed for the trichothecenes DON, 3-Ac DON, 15-Ac DON, nivalenol (NIV), fusarenone-X (FUS-X) by thin-layer chromatography, high performance thin-layer chromatography (HPTLC), two-dimensional and quantitative TLC. ZEA and α and β-zearalenol were analyzed by liquid chromatography. For extraction and cleanup of samples, a method similar to that described by Tanaka et al (1985) was used.

Toxic fungal metabolites were detected by thin-layer chromatography. Before spotting of extracts and standards, the chromatography plates TLC (5553 Merck) and HPTLC (5633 Merck) were activated for 1 hr at 110°C and then dipped in a solution of aluminium chloride-water-methanol (10:15:90, w/v). The plates were dried for 12 hr at room temperature followed by 1 hr at 40°C. Plates were washed by development with chloroform-acetone-2-propanol (70:15:15) and air-dried for 2 hr. The content of DON was determined using chloroform-acetone-2-propanol (7.0:1.5:1.5, v/v) as a developing system. 3-Ac DON was detected by two-dimensional HPTLC with benzene-acetone (60:35, v/v) and toluene-ethyl acetate-formic acid (5:4:1, v/v) as the solvent system. Blue fluorescent spots of toxins were quantified using (50 μg/ml) standards dissolved in an ethyl acetate-methanol (9:1, v/v) solution. Each determination was replicated five times. The detection limit with this method was 0.02 mg/kg for DON, 3-Ac DON, 15-Ac DON, NIV, and FUS-X. Recovery tests for DON, 3-Ac DON, 15-Ac DON, NIV, FUS-X (n = 5) were (respectively) 85.0 ± 5.6; 76.0 ± 4.2; 75.0 ± 5.7; 88.0 ± 3.5; 92.0 ± 4.5. Further confirmation of the identity of toxic metabolites was performed by chromatography-mass spectrometry of trimethylsilyl derivatives.

A portion of the Florisil column eluate was subjected to analyses of ZEA, and α and β zearalenol by high-performance liquid chromatography with fluorescence detection. Liquid chromatography equipment made of the following parts was used: Constametric III metering pump; Spectro monitor TMD variable wavelength detector; CI-10 integrator; model SEK plotter (LDC/Milton Roy, Riviera Beach, FL), floppy 2031LP (Commodore Business Machines, Frankfurt, Germany), diode array detector–2140 Rapid Spectral Detector: LKB Wavescan EG software (LKB Producter AB, Bromma, Sweden); Spectral Search software (Nelson Analytical, Cupertino, CA); IBM PC AT; fluorescence detector–HP1046A programable fluorescence detector (Hewlett Packard, Germany); sample injector model 7125 syringe-loading sample injector with 20, 50, and 100 μl sample loops (Rheodyne, Cotati, CA).

The chromatographic separation was performed using a silica gel column (LiChrosorb Si 60). The mobile phase was water-saturated dichloromethane and 1-propanol (98.5:1.5, v/v). The column temperature and solvent flow-rate were set at 25°C and 2.0 cm^3/min, respectively. The fluorescence detector was set at an emission wavelength of 450 nm, and the excitation wavelength was 236 nm. On LiChrosorb Si 60 column, retention times are: zearalenone, ~4.6 min; α-zearalenol, ~13.0 min; and β-zearalenol, ~14.5 min. The detection limits were 0.5 ppb for ZEA, 1 ppb for α-zearalenol, and 10 ppb for β-zearalenol.

ERG

Sample extraction is similar to that described by Schwadorf and Müller (1989). ERG was estimated by high-performance liquid chromatography. The extraction residue was dissolved by brief sonification, in 1–5 ml of solvent, depending on the concentration of ERG expected. Flow rate of mobile phase (n-hexane and isoamyl alcohol, 95:5, v/v) was adjusted to 2.0 ml/min for LiChrosorb Si 60 column. The UV detector was set at 282 nm. Sample solution (10–100 μl) was injected within a linear response range (10–2,500 ng). On LiChrosorb Si 60 column, retention time of ERG was ~3.9 min. For identification and peak purity, control diode array technology was used. Sample solution was injected to enable (via IBM PC) continuous spectral acquisition over the complete UV spectrum during elution. Recovery of ERG was 99.4%. Values are reported on a dry weight basis.

Statistical Analyses

The field trial was performed with three replicates. Because head blight rating and relative grain-weight per spike showed significant (P = 0.01) correlations among the three replicates, the second replicate was selected for determination of the proportion of *Fusarium*-damaged kernels, ERG, and toxins. Grain-weight per spike from the inoculated treatment was calculated in relation to the noninoculated treatment to compensate for the differences in yield performance of the single-cross hybrids. This trait is called relative grain-weight per spike throughout. Entries followed a normal distribution for all traits assessed. For computing the coefficients of phenotypic correlation between head blight rating or relative grain-weight per spike and the proportion of *Fusarium*-damaged kernels, ERG, or toxins, only values of the second replicate of the experiment were used. The analysis assumed the effects of the genotypes as random.

RESULTS AND DISCUSSION

The results of the first subset of 10 single-cross hybrids indicated wide variation in *Fusarium*-damaged kernels (FDK), head blight rating, and relative grain-weight per spike (Table I). The analysis

TABLE I
Fusarium-Damaged Kernels (%), Head Blight Rating (1-9), Relative Grain-Weight per Spike (%), Deoxynivalenol (DON), and 3-Acetyldeoxynivalenol (3-Ac DON) Content in Two Fractions of Kernels (*Fusarium*-Damaged vs. Healthy Looking) of 10 Rye Genotypes Inoculated with *F. culmorum*

Genotype	*Fusarium*-Damaged Kernels %	Head Blight Rating	Relative Grain-Weight per Spike	DON in Kernels (mg/kg)			3-AcDON in kernels[a] (mg/kg)	
				Fusarium-Damaged	Healthy Looking	Average for Sample	*Fusarium*-Damaged	Average for Sample
1	25	2.8	71.96	4.4	0.1	1.18	0.9	0.23
2	49	2.7	52.78	5.0	0.1	2.50	0.9	0.44
3	50	4.5	48.59	10.1	0.1	5.10	2.0	1.00
4	60	4.2	48.58	6.3	0.1	3.82	0.8	0.48
5	62	3.8	52.03	1.1	0.1	0.72	0.2	0.12
6	62	5.0	45.73	2.7	0.2	1.75	0.5	0.31
7	73	7.0	41.28	3.0	0.3	2.27	0.3	0.22
8	74	5.7	38.11	12.0	0.3	8.96	1.0	0.74
9	75	5.3	44.97	22.5	0.8	17.08	1.4	1.05
10	78	4.2	40.42	28.0	0.1	21.86	2.2	1.72
Mean	61	4.5	48.44	9.5	0.2	6.52	1.0	0.63

[a]Not detected in healthy looking kernels.

TABLE II
Correlation Coefficients[a] Between *Fusarium*-Damaged Kernels, Head Blight Rating, Relative Grain-Weight per Spike, Deoxynivalenol (DON), 3-Acetyldeoxynivalenol (3-Ac DON), Ergosterol (ERG), and Zearalenone (ZEA) Analyzed in Two Subsets with 10 and 19 Genotypes, Respectively, After Inoculation with *Fusarium culmorum*

Correlated Traits	Subset I ($N = 10$)	Subset II ($N = 19$)
Fusarium-damaged kernels		
Head blight rating	0.707*[b]	...
Relative grain-weight per spike	−0.934**	...
DON	0.598	...
3-Ac DON	0.449	...
Head blight rating		
Relative grain-weight per spike	−0.731*	−0.587**
Relative grain-weight per spike		
DON	−0.485	−0.346
3-Ac DON	−0.452	−0.399
ERG	...	−0.515*
DON		
3-Ac DON	0.922**	0.686**
ERG		
DON	...	0.566*
3-Ac DON	...	0.790**
ZEA	...	0.587**
ZEA		
3-Ac DON	...	0.613**

[a]All correlation coefficients omitted are not significantly different from zero at $P \geq 0.1$; $r < 0.40$.
[b]* = Significant at $P = 0.05$; ** = significant at $P = 0.01$.

of FDK in rye is more difficult than it is in wheat. Infected rye kernels are less discolored and less shrivelled due to the narrower shape and smaller size of the kernels. Head blight rating and relative grain-weight per spike showed ranges similiar to those reported previously for winter rye (Miedaner et al 1993). The DON concentrations also revealed good differentiation among genotypes (0.7–21.9 mg kg^{-1}); 3-Ac DON was found in the FDK fraction of the samples only (Table I). This fraction also yielded considerably higher DON concentrations than average. However, healthy looking kernels contained DON concentrations above the detection limit. This was reported earlier for wheat, where up to 3.6 mg kg^{-1} of DON were found in this fraction (Perkowski et al 1990b). Other authors have reported that concentrations of DON, 3-Ac DON, NIV, and ZEA found in winter rye were rather low compared to infections of wheat and triticale with the same *Fusarium* species (Miller et al 1985; Shotwell et al 1985; Perkowski et al 1988, 1990b; Scott 1990). For example, Miller et al (1985) found DON contents in kernels ranging from 0.23 to 1.67 mg kg^{-1} for six resistant wheat varieties and from 0.024 to 0.58 mg kg^{-1} for six resistant rye varieties. However, they used a limited number of genetically heterogeneous rye populations. Our mycotoxin data represents a larger sampling of single-cross hybrids (genetically uniform) of winter rye. This different genetic structure of the entries may explain the higher mycotoxin concentrations reported here.

Coefficients of phenotypic correlation among the resistance traits FDK, head blight rating, and relative grain-weight per spike were significant and close (Table II). The high association between FDK and relative grain-weight per spike illustrates that the very laborious yield analysis can be replaced by a simple visual rating, similar to that proposed for wheat (Perkowski et al 1990a, Snijders and Perkowski 1990, Perkowski 1993, Perkowski and Chelkowski 1993). For screening large populations, FDK has the further advantage that only an artificially infected treatment is needed, a noninoculated control treatment is not necessary. Resistance traits and DON, 3-Ac DON concentrations correlated to a limited extent only. Although not significant, FDK and relative grain-weight per spike yielded, on average, closer correlations than did head blight rating. In wheat, closer correlations between FDK and DON content were reported (Shotwell et al 1985, Wang and Miller 1988, Perkowski and Chelkowski 1993, Mesterhazy and Bartok 1993). The low correlations between head blight rating or relative grain-weight per spike and DON content found in this study may partly be attributable to the *Fusarium* isolates, host genotypes, and environmental factors. Snijders and Perkowski (1990), for example, found correlations between head blight rating and DON content ranging from 0.55 to 0.96, depending on the *F. culmorum* isolate used. Similarily, Mesterhazy and Bartok (1993) reported moderate correlations between head blight rating or yield reduction and DON content for *F. graminearum* inoculation of 34 wheat genotypes (r = 0.50–0.62) only. Association between DON and 3-Ac DON was close (Table II).

Ten genotypes of the first subset were selected from a larger set of 29 genotypes due to their differentiation for resistance traits. Because the correlations between resistance traits and DON and 3-Ac DON contents were not very large, the mycotoxin concentrations of the further 19 genotypes of the same field experiment were analyzed. In addition, ERG and ZEA contents were determined. As expected from sampling procedure, the mean head blight rating and mean relative grain-weight per spike of both subsets showed nearly identical values (Tables I and III). The mean DON and 3-Ac DON contents were considerably higher in the second subset. ERG content ranged between 42.9 and 135 mg kg^{-1}; ZEA content was small for all samples (Table III). ERG is a principal component of fungal cell walls and was suggested for quantification of fungal mycelium content in infected samples (Seitz et al 1979). ERG content in visually healthy kernels varies from 0.95 ± 0.58 mg kg^{-1} in maize to 3.51 ± 2.16 mg kg^{-1} in oats (Müller and Schwadorf 1990). Thus, our data indicate a high amount of fungal colonization in all genotypes. This is in accordance with the 50% reduction of grain-weight per spike in

TABLE III
Head Blight Rating, Relative Grain-Weight per Spike, Ergosterol, and Mycotoxin Contents in Kernels for 19 Genotypes of Rye Inoculated with *Fusarium culmorum*

Genotype	Head Blight Rating[a]	Relative Grain-Weight per Spike (%)	Ergosterol (mg/kg)	Zearalenone (mg/kg)	Deoxynivalenol (mg/kg)	3-Acetyldeoxynivalenol (mg/kg)	Ergosterol/ Deoxynivalenol Ratio
11	3.3	58.06	70.76	0.066	5.51	1.32	12.84
12	3.8	53.60	72.05	0.033	6.52	0.56	11.05
13	3.8	67.77	53.57	0.046	8.85	0.60	6.03
14	4.0	44.73	95.54	0.045	13.80	1.17	6.92
15	4.0	47.35	52.84	0.015	14.78	1.16	3.57
16	4.0	52.22	97.11	0.112	20.24	1.96	4.80
17	4.3	52.46	77.61	0.050	17.33	1.61	4.48
18	4.5	45.21	88.87	0.044	14.98	1.57	5.92
19	4.5	45.65	135.37	0.071	28.00	3.43	4.83
20	4.7	54.88	82.18	0.062	8.15	1.18	10.08
21	4.7	37.01	93.92	0.083	12.12	1.83	7.75
22	4.7	41.20	103.86	0.057	13.55	0.76	7.66
23	4.7	44.27	129.29	0.147	18.48	2.38	7.01
24	4.8	60.62	70.92	0.103	6.78	1.34	10.46
25	5.0	55.02	42.88	0.045	6.24	0.53	6.87
26	5.0	42.67	102.88	0.077	7.14	1.97	14.41
27	5.0	43.99	79.60	0.091	22.90	1.96	3.48
28	5.2	41.77	61.74	0.025	12.63	0.66	4.89
29	5.8	34.72	94.39	0.115	9.83	2.03	9.60
Mean	4.5	48.59	84.49	0.068	13.04	1.47	7.51

[a]Scale of 1–9: 1 = healthy looking, 9 = 100% of spikelets and ears per plot infected.

the inoculated plots. Despite the high disease severity, ERG content showed considerable differences between genotypes. Similiar ranges were found for wheat genotypes (Miller et al 1985, Wang and Miller 1988, Snijders and Krechting 1992). ERG-DON ratio showed considerable variation among rye genotypes (Table III), which was similiar to earlier reports for wheat, where Miller et al (1985) found ERG-DON ratios varying from 2.4 to 13.3 for *F. graminearum* infections. This high variation illustrates that DON accumulation in cereal grain is not merely an effect of the amount of fungal mycelium within host tissue, but is greatly affected by the genotype. For example, genotypes 22 and 26 showed similiar ERG contents (Table III), but genotype 22 accumulated twice as much DON as genotype 26. Genotype 26 was less suitable for DON production or possessed mechanisms for DON degradation. On the other hand, genotype 15 showed one of the lowest ERG contents but had average DON content.

Similiar to the first subset, the second subset of rye genotypes also exhibited only weak, nonsignificant correlations among head blight rating or relative grain-weight per spike and ZEA, DON, and 3-Ac DON contents (Table II). Correlation between relative grain-weight per spike and ERG content was significant, illustrating that, in this experiment, yield reduction obviously better reflects fungal colonization of the kernels than head blight rating that showed no significant correlations to ERG or mycotoxin contents. Correlations among the fungal metabolites ERG, ZEA, DON, and 3-Ac DON were significant ($r = 0.57$–0.79) (Table II) with the exception of the correlation between DON and ZEA ($r = 0.230$).

In conclusion, 29 winter rye single-cross hybrids artificially inoculated with *F. culmorum* contained DON, 3-Ac DON, and a small amount of ZEA in the grain. Despite the high disease severity achieved in this experiment, the DON concentration tended to be lower than those reported for wheat artificially inoculated. 15-Ac DON and NIV were not detected. For all resistance traits and mycotoxin contents examined, wide continuous phenotypic variation was found among the tested single-cross hybrids. However, the weak correlations between FDK, head blight rating, or relative grain-weight per spike and toxins content require both to be considered during resistance selection.

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