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Head infection and accumulation of *Fusarium* toxins in kernels of 12 barley genotypes inoculated with *Fusarium graminearum* isolates of two chemotypes

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Abstract

Twelve barley genotypes were inoculated with two *F. graminearum* isolates of different chemotype I_1 #148 (producing nivalenol/deoxynivalenol) and I_2 #108 (deoxynivalenol/acetyldeoxynivalenol). For both I_1 and I_2 isolates, respectively, reductions (%) in number of kernels head^{-1} 10.6 and 14.3; yield 39.6 and 35.7; weight of 1000 kernels 36.9 and 23.2 were observed in inoculated plants from control values. Chemical analysis revealed the presence (average concentration mg kg^{-1}) of deoxynivalenol (1.3) and nivalenol (3.2) in kernels of all genotypes inoculated with the I_1 isolate, and zearalenone (0.2) in three samples. After inoculation with the I_2 isolate, deoxynivalenol (37.8) and zearalenone (0.4) were found in kernels of all genotypes, while 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol, respectively, were determined in five and four samples only. No significant correlation between examined characteristics was found for either the I_1 or I_2 isolate. The results obtained contribute information on the accumulation of toxins in cereal grain inoculated with *F. graminearum* isolates of different chemotypes.

Introduction

Fusarium graminearum Schwabe, *F. culmorum* (W.G.Sm.) Sacc. and *F. avenaceum* (Fr.) Sacc. are internationally recognized as the most common fungi causing head blight of barley (Parry et al., 1995). Pathogenicity of *F. graminearum* on barley or wheat is slightly lower when compared with *F. culmorum*, the most severe pathogen of the genus *Fusarium* (Perkowski et al., 1995 a; Wong et al., 1995). *Fusarium graminearum* strains are able to form several toxic secondary metabolites in kernels of infested cereals, the most important being deoxynivalenol (DON) with its acetylated derivatives (3-AcDON and 15-AcDON), nivalenol (NIV) and zearalenone (ZEA) (Miller et al., 1985; Mirocha et al., 1994; Perkowski et al., 1990, 1995 a). The profile of metabolites formed is pathogen chemotype dependent (Miller et al., 1991; Sydenham et al., 1991). Two chemotypes of *F. graminearum* are known: type I able to form DON with its acetylated derivatives and type II with NIV as the most important product of the trichothecene biosynthesis pathway. Since NIV is significantly more toxic than DON, the prevalance of each fungus chemotype is important in the toxicity of infested kernels (Yoshizawa and Marooka, 1977). The aim of this work is study head infection and toxin accumulation in different barley genotypes under field conditions after artificial inoculation with *F. graminearum* isolates of chemotypes I and II.

Materials and methods

Source of barley kernels

Eight cultivars (Apex, Aramir, Ars, Bielik, Diva, Havila, Koru, Roland) and four lines (MOB-487, NAD-685, NAD-785, POB-984) of spring barley were inoculated under field conditions at the experimental station RZD Czesławice near Lublin, Poland. Barley

cultivars used in the inoculation experiment are very common in the region of Lublin (South-East part of Poland) and originally were introduced in Poland (cvs Bielik and Ars), in The Netherlands (Apex, Aramir, Diva and Havila), in Sweden (Roland) and in the UK (Koru). All inoculated lines were of Polish origin and were tested in The Cereal Breeding Station, Czesławice, Poland. Two isolates of *F. graminearum*, N° 148 = I_1 and 108 = I_2, were used for inoculation. The isolates were obtained from the culture collection at the Department of Plant Pathology, Agricultural University of Lublin, Poland. Pathogenicity of the above isolates was tested by the germination potential of Apex cultivar kernels, following inoculation with *F. graminearum*. Isolates #148 and #108, respectively, reduced seed germination ability by up to 36 and 23% of germinating seedlings. The *Fusarium* toxin potential of the strains was determined by *in vitro* cultures on barley kernels (cv. Apex, 45% moisture, 20 °C, 4 weeks). *F. graminearum* isolate N° 148 = I_1 was of chemotype II and was able to form NIV (with small amounts of DON, ZEA), while N° 108 = I_2 isolate was of chemotype I with biosynthetic abilities of DON, 3-AcDON, 15-AcDON and ZEA.

Inoculum was prepared according to a modification of the method of Mesterhazy (1978). Growing medium (1 l) was prepared from water, starch (2 g), water extract of 0.5 kg barley leaves, and was autoclaved (1 h, 121 °C and 1 atm) before use. *F. graminearum* isolates were grown on the above medium according to the method of Kiecana (1988). The medium was inoculated with mycelium of two week-old cultures of isolates #148 and #108 and was incubated for two weeks at 18–20 °C with natural (ambient) light. After incubation, stirred (10 min) inoculum was filtered through cheese-cloth, and the supernatant of the conidial suspension (5×10^6 spores ml^{-1}) used for inoculation.

Each barley cultivar was sown on April 19, 1991 and grown separately on 10 m^2 experimental plots bordered with rows and paths. Eighty heads of barley (20 heads $replicate^{-1}$) were inoculated with *F. graminearum* four days after anthesis (stage 10.5 on the Feekes scale) from 50 plants (July 1–5, 1991). Inoculum, prepared as described above, (1 ml $head^{-1}$) was applied with a laboratory sprayer. The same cultivars sprayed with distilled water were used as a control group. After inoculation or water spraying, the heads were protected with plastic bags for 24 h to avoid water evaporation and spread of inoculum. Weather conditions during the experiment did not significantly differ from the multiannual average. Mature heads were collected on August 19, 1991 and threshed manually. Yield (Y), number of kernels $heads^{-1}$ (NK) and 1000 kernel weight (TKW) were measured for four replicates separately, and were statistically correlated with results of *Fusarium* toxin content in the kernels.

Chemical analysis

Fusarium toxins (DON, 3-AcDON, 15-AcDON, NIV and ZEA) were extracted from finely ground samples as described by Tanaka et al., (1985). Trimethylsilyl (TMS) esters of the metabolites were prepared as described by Scott et al., (1986) and analyzed by gas chromatography with ^{63}Ni electron capture detection (GC-ECD) after splitless injection on a Shimadzu C-R4Ax Chromatopac Gas Chromatograph fitted with a 25 m × 0.25 mm Permabond SE-54-DF-0.25 column (Machery Nagel). The carrier gas was helium (80 kPa) and the make-up gas nitrogen (45 ml min^{-1}). The injection temperature was 250 °C, while the temperature programme for the column was 60 °C for 1 min, then raised to 120 °C (10 °C min^{-1}) and to 280 °C (4 °C min^{-1}), and held at this temperature for 20 min. Detector temperature was 330 °C. Retention times (min) of the derivatized metabolites were as follows: DON – 29.60; 3-AcDON – 35.75; 15-AcDON – 37.05 and NIV – 40.90. *Fusarium* mycotoxins were determined by capillary gas chromatography combined with a selective mass detector (ion trap) working in CI – mode with methane as a reagent gas. Trifluoroacylated derivatives were prepared by reaction with trifluoroacetic acid anhydride as described by Schwadorf and Müller (1991). Detection limits for the metabolites were 1–5 μg kg^{-1} (<1–5 μg = ND), depending on toxin analyzed and content of impurities in the extracts. Recoveries (from barley spiked with 200 and 500 ng of toxic metabolites) were 78–89%. Part of the Florisil column eluate (Tanaka et al., 1985) was subjected to ZEA analysis by fluorescence detection after high-performance liquid chromatography (HPLC) on a Lichrosorb Si 60 column (5 μm, 25 cm × 4 mm, Merck) at 25 °C with mobile phase (water – saturated dichloromethane / 1-propanol 98.5: 1.5 v/v), flow rate 2 ml min^{-1}. A fluorescence detector (Model 1046 A, Hewlet Packard, Germany) at an excitation 236 nm and emission 450 nm wave-length was used. 'External standard' method with 'valley-to-valley' integration was selected. Retention time of ZEA was 4.6 min while the detection limit was 0.5 μg kg^{-1}.

Table 1. The average reduction (%) from control values of yield (Y), number of kernels head^{-1} (NK), 1000 kernel weight (TKW) and concentration (mg kg^{-1}) of *Fusarium* toxins (DON, NIV, 3-AcDON, 15-AcDON, ZEA) in 12 barley genotypes (8 cultivars and 4 lines) inoculated with two *F. graminearum* isolates of different chemotype (I_1 = *F.g.* N°148, I_2 = *F.g.* N°108)

Cultivars or lines	Isolate															
	I_1								I_2							
	Reduction (%)			Concentration (mg kg^{-1}) of *Fusarium* mycotoxins					Reduction (%)			Concentration (mg kg^{-1}) of *Fusarium* mycotoxins				
	Y	NK	TKW	DON	NIV	3-AcDON	15-AcDON	ZEA	Y	NK	TKW	DON	NIV	3-AcDON	15-AcDON	ZEA
Apex	48.0	5.6	41.8	0.42	0.84	ND	ND	ND	16.8	4.9	7.4	10.57	ND	ND	ND	0.49
Aramir	37.3	8.5	38.5	0.59	0.94	ND	ND	ND	21.7	4.9	14.9	6.78	ND	ND	ND	0.38
Ars	36.4	2.2	44.7	0.81	0.82	ND	ND	ND	49.4	10.5	45.6	66.44	ND	ND	ND	0.83
Bielik	35.2	15.0	25.6	0.15	0.41	ND	ND	ND	35.9	15.5	20.0	68.50	ND	0.68	ND	0.70
Diva	36.3	10.5	33.7	0.11	0.80	ND	ND	ND	36.6	19.3	17.2	11.09	ND	ND	ND	0.38
Havila	32.0	10.4	26.1	0.80	0.84	ND	ND	ND	36.8	16.1	12.8	29.11	ND	ND	ND	0.51
Koru	13.0	7.8	22.1	0.71	3.91	ND	ND	ND	33.0	9.1	10.5	0.41	ND	ND	ND	0.06
Roland	46.4	6.2	44.5	4.17	7.07	ND	ND	ND	52.3	28.3	37.5	8.24	ND	0.54	1.10	0.12
MOB-487	54.8	18.6	48.5	2.80	2.92	ND	ND	0.25	33.5	12.1	25.6	105.24	ND	0.77	5.54	0.36
NAD-685	50.0	21.5	44.8	1.53	5.67	ND	ND	0.13	45.6	15.5	31.2	89.73	ND	0.68	1.96	0.65
NAD-785	34.7	9.8	28.7	1.00	5.50	ND	ND	ND	56.1	8.1	45.7	52.46	ND	0.45	2.15	0.68
POB-984	52.3	9.4	43.7	2.57	8.09	ND	ND	0.13	6.7	24.0	10.2	4.72	ND	ND	ND	0.15
Average	39.6	10.6	36.9	1.30	3.15	ND	ND	0.17	35.7	14.3	23.2	37.77	ND	0.62	2.44	0.44

Results

Agronomic performance

All barley genotypes inoculated with either I_1 or I_2 *F. graminearum* isolate exhibited bleached heads, the typical symptom of fusariosis. Kernels collected from infected heads were small, shrivelled and soft, while kernels from the control group did not exhibit symptoms of the disease. Yield (Y), number of kernels head^{-1} (NK) and weight of 1000 kernels (TKW) were used as criteria of genotype resistance to fusariosis. All results indicated that the two isolates of *F. graminearum* used for inoculation were different (Table 1). Reductions (%) of Y (39.6) and TKW (36.9) for I_1 isolate were higher when compared with I_2, respectively 35.7 and 23.2%, while the number of kernels head^{-1} was reduced similarly for I_1 (10.6%) and I_2 (14.3%) isolates. Y and TKW only were significantly correlated for both strains (Table 2).

Chemical analysis of the mycotoxins

The following toxic secondary metabolites DON, 3-AcDON, 15-AcDON, NIV and ZEA were detected in kernels of barley genotypes inoculated with I_1 and I_2 strains (Table 1). NIV, DON and ZEA at average concentrations (mg kg^{-1}) of 3.2, 1.3 and 0.2, respectively, were found in samples of barley kernels inoculated with the I_1 isolate. Amounts of toxins were correlated, with a coefficient of 0.74 at $P < 0.01$. After inoculation with the I_2 isolate, the following toxic metabolites (at average concentrations mg kg^{-1}) were determinated: DON (37.8); 3-AcDON (0.6); 15-AcDON (2.4) and ZEA (0.4). NIV was not detected in these samples (I_2 isolate). Amounts of DON formed by I_1 and I_2 isolates were not significantly correlated.

Discussion

Results achieved in the described experiments indicate that average yield reductions from control values (%), (Table 1), after inoculation with isolate I_1 and I_2 respectively, were as follows: Y 40 and 36; NK 11 and 14; TKW 37 and 23; this indicates broadly similar pathogenicity of the two isolates. Lower values of TKW were observed in the case of isolate I_2; however, correlation was significant for both isolates for TKW/Y (I_1 = 0.83, I_2 = 0.81, $P < 0.01$). Published information on barley fusariosis after artificial inoculation is limited, and indicates (Mirocha et al., 1994, Perkowski et al., 1995 a, 1996) that infected kernels exhibit similar but stronger symptoms of the disease when compared with samples naturally infested with the pathogen. Similar observations were published for

Table 2. Correlation coefficients between yield (Y), weight of 1000 kernels (TKW), number of kernels head^{-1} (NK), deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) for 12 barley cultivars and lines inoculated with two *F. graminearum* isolates of different chemotype (I_1 = *F.g.* N° 148, I_2 = *F.g.* N° 108)

I_1						I_2				
	Y	NK	TKW	DON	Correlation		Y	NK	TKW	DON
NK	0.32	–	–	–	coefficient	NK	0.34	–	–	–
TKW	0.83**	–0.04	–	–	$I_1 \neq I_2$	TKW	0.81**	–0.15	–	–
DON	–0.69*	0.27	–0.80**	–	($P < 0.05$)	DON	–0.32	0.47	–0.52	–
NIV	–0.49	0.40	–0.62*	0.74**		ZEA	–0.32	0.59	–0.55	0.62*

* significant at $P < 0.05$
** significant at $P < 0.01$

wheat and rye (Snijders and Perkowski 1990, Wong et al., 1995; Perkowski et al., 1995 b). Field experiments with artificially inoculated cereals is a good model for elucidation of the pathogen's effect on yield, the profile of formed toxins and for understanding the mechanism of fusariosis and biosynthesis of toxic *Fusarium* metabolites. No significant correlations (significance level $P < 0.05$) were observed either for NK/Y, TKW or between these values for I_1 and I_2 isolates. We have previously presented similar observations for a strongly pathogenic *F. culmorum* isolate (Perkowski et al., 1996). Yield reductions obtained in the current work after inoculation of barley with *F. graminearum* indicate that pathogenicity of these isolates is lower compared to *F. culmorum* (Perkowski et al., 1996); this confirms earlier observations (Mirocha et al., 1994; Wong et al., 1995, Perkowski et al., 1995a)

Chemical analysis revealed the presence of *Fusarium* toxins in samples inoculated with both I_1 and I_2 isolates. As expected, the profile of toxic metabolites was dependent on the chemotype of the isolate. *F. graminearum* has been differentiated into two populations designated as groups 1 and 2 (Francis and Burgess, 1977). Isolates of group 1 are usually associated with diseases of the crowns of plants and do not form perithecia in culture. Members of group 2 are associated with diseases of the aerial plant parts and readily form perithecia of *Gibberella zeae* (Schw.) Petch in culture (Francis and Burgess 1977). Another classification based on the profile of toxic metabolites including estrogenic zearalenone (ZEA) and trichothecenes (DON, 3-AcDON, 15-AcDON, NIV) was suggested by Ichinoe et al., (1983). The NIV chemotype of *F. graminearum* is able to form NIV and fusarenone-X (FUS-X) while the DON chemotype produces DON and 3-AcDON. Miller et al., (1991) suggested the following more complex groups of chemotypes: IA (DON, 3-AcDON), IB (DON, 15-AcDON) and II (NIV, diacetyl NIV). Sugiura et al., (1990) observed that *F. graminearum* isolates of chemotype I are able to form DON with both isomers of its acetylated derivative (3-AcDON and 15-AcDON), while chemotype II NIV and low amounts of DON. We obtained similar results in our earlier experiments with naturally infected wheat samples (Perkowski et al., 1990).

The I_1 isolate (belonging to the NIV chemotype) in samples of all cultivars and lines, formed NIV at an average concentration (mg kg^{-1}) of 3.2 (from 0.4 Bielik to 8.1 POB-984), and DON at an average concentration of 1.3 (from 0.1 Bielik to 4.2 Roland). In three samples, small amounts of ZEA were detected. The correlation coefficient for amount of NIV/DON was 0.74 (at $P < 0.01$). A similar coefficient of 0.68 (at $P < 0.05$) was found in a barley inoculation experiment with another *F. graminearum* (N° 122) isolate (Perkowski et at., 1995a). The I_2 isolate belonging to the DON chemotype formed DON at relatively high concentrations in all genotypes of barley, but since the toxicity of the metabolite is 14-fold lower than NIV (Yoshizawa and Morooka, 1977), the average toxicity of samples inoculated with I_1 and I_2 (taking into consideration levels of contamination) is probably the same. In contrary to artificial inoculation experiments, it is very hard to forecast the presence of toxins in samples naturally infested with fungi of the genus *Fusarium*. Results of screening analyses of *Fusarium* toxins in cereals of South/Central Europe indicate simultaneous occurrence of DON and NIV. In Poland, among 48 analyzed wheat and barley samples (Ueno et al., 1985), 29% exhibited the presence of DON while 83% had NIV. In another study, Goliński et al., (1996) found DON and NIV in 50 and 30% of samples, respectively, and in 16% of the samples simultaneous presence of the metabolites was found. In other countries the relation (%) of DON/NIV positive samples was as follows: Germany (Müller et al., 1993) 85/28 in 373 wheat and

barley samples, Norway and Holland (Sundheim et al., 1988; Tanaka et al., 1990) 67/100 and 90/79 in 102 and 29 samples, respectively, and from samples collected internationally (Tanaka et al., 1988) 45/48 in 292 cereal samples. The fact that *F. graminearum* and *F. culmorum* are very common pathogens of cereals explains the high percentage of DON positive samples. Recent publications indicate that in addition to *F. culmorum* and *F. graminearum* (Perkowski et al., 1992; Mirocha et al., 1994), *F. crookwellense* (Goliński et al., 1988) and *F. poae* (Pettersson 1991) are able to form NIV. This could explain the high percentage of the metabolite-positive samples obtained. In the experiment described above we observed that in all cultivars and lines, the profile of toxins formed was very similar for the same chemotype of the fungus used for inoculation. We did not find significant correlations (at $P < 0.05$) between the same variable in experiments with I_1 and I_2 isolates. However, results indicate that the cvs Aramir, Koru, Havila, Diva and Apex are resistant to fusariosis, whereas lines MOB-487 and NAD-685 are very susceptible to the disease.

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