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TOOLS FOR *FUSARIUM* MYCOTOXIN REDUCTION IN FOOD AND FEED CHAINS
RESEARCH PAPERS

Factors of wheat grain resistance to Fusarium head blight

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Summary. Fusarium head blight (FHB), caused by *Fusarium graminearum*, is an important wheat disease that affects grain yield and conformation, and contaminates grains with mycotoxins, including the trichothecene deoxynivalenol (DON). The impacts of *Fusarium* infections on grain filling, grain deformation and rheological properties were assessed under different environmental conditions. Genotypes with elevated grain anthocyanin content were used. Resistance of seven wheat varieties and breeding lines was assessed with artificial infections in the field. Grains from infected and control plots were assessed for proportion of *Fusarium* damaged kernels, grain filling (thousand kernel weight) and DON accumulation. Biochemical and rheological properties of harvested grain were also assessed. Grain resistance to *Fusarium* has several components, including resistance against DON accumulation, deformation and stability of grain filling. These mechanisms are interdependent but act independently. Resistance against DON contamination was highly influenced by environmental conditions, but environment had little effect on the other resistance components. Anthocyanins and protein concentrations were unchanged in infected grains, suggesting that FHB does not affect grain biosynthesis processes but impacts the transport of assimilates caused by changes in grain composition. We suggest that this is the reason for the alterations of rheological properties. The greater the grain resistance, the less was the impact on dough properties. This study suggests that the resilience of rheological properties under FHB infection pressure is an additional component of grain resistance to the disease.

Key words: resistance types, grain filling, anthocyanin, rheological properties, resilience.

Introduction

Fusarium head blight (FHB), caused by different species of *Fusarium*, is one of the most important diseases of wheat. Besides considerable yield losses, infections lead to contamination of grain with mycotoxins and to grain morphological changes, produce unsuitable for consumption and trade (McMullen *et al.*, 2012). In temperate climates, *F. graminearum* Schwabe is the prevalent FHB-causing species, and this fungus produces, among others, the mycotoxin deoxynivalenol (DON) (Parry *et al.*, 1995). At host flowering and with high relative humidity, ascospores or conidia of the fungus penetrate at the base of the florets to infect the floral tissues. Subsequently, the fungus spreads throughout the spikes via xylem and phloem causing characteristic symptoms on the spikes and colonising the developing grains (Kang and Buchenauer, 2000).

Management strategies for FHB aim to avoid primary infection from debris of previous crops (in particular maize) (Vogelgsang *et al.*, 2011), and the use of resistant varieties (Mascher *et al.*, 2005). Resistance of wheat against FHB includes a large number of resistance mechanisms (Ravensdale *et al.*, 2014). Schroeder and Christensen (1963) described "type 1 resistance" as resistance to primary infection, and "type 2 resistance" as resistance against spread the pathogen

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throughout the host inflorescences (spikes). This concept was subsequently extended with types of kernel resistance, yield stability (Mesterhazy, 1995) and resistance against mycotoxin accumulation (Miller *et al.*, 1985).

The presence of shrivelled, misshaped and so-called scabby grains reflects *Fusarium* infection of grain. The proportion of healthy looking grains is a measure of grain resistance, termed "type 3 resistance" (Jones and Mirocha, 1999). The underlying symptoms can be differentiated, firstly in grains that have been damaged by the infection, so-called *Fusarium* damaged kernels (FDK), and secondly grains that are poorly filled (Mesterhazy, 1995; Foroud and Eudes 2009). Poor grain filling is quantified by measuring the difference in thousand kernel weight (TKW) of infected and non-infected grains. Reduced TKW is part of type 3 resistance, but is also a component of "type 4 resistance" defined as the conservation of yield despite pathogen presence (Foroud and Eudes 2009; Almeida *et al.*, 2016). Type 5 resistance is considered the capacity of host plants to impede the production of mycotoxins by the pathogen, and to detoxify the mycotoxin (Miller *et al.*, 1985; Boutigny *et al.*, 2008). *Fusarium* infection can also alter the biochemical constituents of grains resulting in deterioration of rheological and baking quality traits (Dexter *et al.*, 1996; Häller Gärtner *et al.*, 2008). Resistance mechanisms against changes in grain biochemistry have not yet been described.

All of these resistance types are partly interdependent, but are probably based on distinct mechanisms and are likely to be independently inherited. (Mesterházy *et al.*, 1999; Bai *et al.*, 2000). Furthermore, strong genotype-environment interactions have been observed in FHB outcomes and trichothecene accumulation (Miedaner *et al.*, 2001). For these reasons, the evaluation of grain resistances towards FHB remains difficult in a modern breeding context. Usually, only symptoms on the wheat ears, and sometimes mycotoxin accumulation (particularly DON), are taken into account by plant breeders (Foroud and Eudes, 2009). However, these few resistance parameters do not span all facets of resistance, and may be insufficient when looking for specific combinations of traits in breeding lines or when phenotyping for resistance in mapping populations.

Between host flowering and maturity, developing grains are supplied with all required nutrients such as sugars, minerals, and amino-acids (Feillet, 2000). Subsequently, these nutrients are transformed into starch, storage protein, functional proteins and other constituents. The effects of *Fusarium* pathogen on these biochemical mechanisms have not been studied.

This study aimed to investigate the impacts of *Fusarium* infection on grain filling and modifications in rheology of flour from infected grains. We have tested seven wheat varieties and breeding lines with different resistance levels against FHB in field tests with artificial inoculations at three experimental sites in Switzerland. Four genotypes with coloured grains, due to elevated anthocyanin content (Abdel-Aal *et al.*, 2007), were included in the study. Anthocyanins are synthesised by grain tissue during maturation, starting at the milk-dough stage (Knievel *et al.*, 2009; Žofajová *et al.*, 2012). Biosynthesis of these compounds depends on the availability of photosynthesis assimilates (Bustos *et al.*, 2012), and is conditioned by abiotic stresses (Gordeeva *et al.*, 2013). In the present study, coloured grains were used to measure the impacts of a biotic stress on grain filling and biosynthesis of anthocyanins and proteins.

Materials and methods

Plant material

Seven winter wheat genotypes, including five registered cultivars and two breeding lines, have been studied in this experiment (Table 1). The cultivars 'Arina', 'Combin', 'Hanswin' and 'Vanilnoir' show different FHB resistance levels in the field (Häner *et al.*, 2014; Mascher, unpublished). ACW 083, ACW 271, 'Indigo' and 'Vanilnoir' have coloured grains due to the presence of anthocyanins in the grain pericarps.

The cultivar 'Purendo 38' has dark blue grains with anthocyanins in the grain pericarps and endosperms. This additional genotype was employed for comparison of anthocyanin synthesis production in two field tests (at Changins and Vouvry). Due to high susceptibility to yellow rust, this variety could not be included in the resistance evaluation.

Fungal isolates and production of inoculum

Fusarium graminearum strains used in the current study were the single spore isolates FG 1145[1] (isolat-

[1] Strains deposited in the Agroscope data base http://myco scope.bcis.ch/

Table 1. Description of winter wheat genotypes investigated in this study. + = resistant, - = susceptible, and Ø = non-significant resistant comportment.

Genotype	Resistance level	Baking quality class	Coloration of the grains	Breeder	Pedigree
ACW 083	unknown	unknown	dark	Breeding line Agroscope	TABOR/NS1201/6/ZENITH/BRST-KV4666-56//17R4/5/VUKA/4/PROBUS MS×2/ FLINOR/3/BEZOSTAYA 1//CAN3842/ HEINE 7/7/KONINI
ACW 271	unknown	unknown	dark	Breeding line Agroscope	TABOR/NS1201/6/ZENITH/BRST-KV4666-56//17R4/5/VUKA/4/PROBUS MS×2/ FLINOR/3/BEZOSTAYA 1//CAN3842/ HEINE 7/7/KONINI
'Arina'	+	I	clear	Agroscope/DSP (CH)	MOISSON//CANADA3842-3663/Heines-VII
'Combin'	-	I	clear	Agroscope/DSP (CH)	VIRTUE/3/ZENITH/NS611// LICHTI/4×PROBUS/4/W'ST480-73/3/ ZENITH/NS611//LICHTI/4×PROBUS
'Hanswin'	Ø	I	clear	Agroscope/DSP (CH)	OBELISK/TAMARO//PEGASSOS
'Indigo'	unknown	unknown	dark	KWS Ltd (UK)	
'Vanilnoir'	+	Top	dark	Agroscope/DSP (CH)	RUNAL/W'ST479-77//KONINI
'Purendo 38'	unknown	unknown	blue	Crop Development Center, Saskatoon, (CAN)	

ed in 2006, Canton Vaud, Switzerland), FG 13[1] (1998, Canton Zug) and FG 0410[2] (2005, Canton Schaffhausen). The three strains were isolated from symptomatic wheat ears, and were chosen to represent the average level of virulence of various *F. graminearum* strains isolated in Switzerland (Martin *et al.*, unpublished). Conidia of the strains were stored in a 1:1 mix of water with glycerol at -80°C. Routinely, the strains were cultured on Potato Dextrose Agar (PDA, BD Difco) for 1 week in the dark at 4°C. For mass production of conidia of each isolate, two discs (5mm diam.) from a well-grown colony were transferred to 200 mL of liquid V8-medium (1:5, V8 juice (Campbell Soup Company): distilled water, +2 g sodium carbonate L^{-1}) in a 1 L capacity Erlenmeyer flask. Cultures were incubated for 7 d at 24°C, on a shaker at 200 rpm in the dark. The culture was then filtered through sterile cheesecloth to remove all mycelium, and conidia were collected by centrifugation at 4,500 rpm for 10 min. The resulting pellet was re-suspended in sterile, demineralised water. These preparations were either used immediately or stored at -80°C as described below.

[2] Strain deposited at the CBS http://www.cbs.knaw.nl/fusarium/

Field experiments and artificial inoculations

Field experiments were conducted in 2014 at three locations in Switzerland: Changins (VD), Vouvry (VS), and Reckenholz (ZH) (Table 2). All winter wheat genotypes were sown in 1 m^2 microplots on 5 lines with a Seedmatic seeding machine (HegeMaschinen) in autumn 2013. Artificial inoculations took place when 50% of the plants inside each microplot were at flowering stage (BBCH 65). Inoculum was prepared just before the inoculations by mixing equal proportions of liquid cultures of each of the three *F. graminearum* strains and add-

Table 2. Locations and environmental conditions for three field experiment locations (data from 01/06/2014 to 31/07/2014). (source: http://www.meteoswiss.admin.ch)

Environmental characteristics	Changins	Vouvry	Reckenholz
Coordinates (North/Est)	46°24′36″/6°14′06″	46°20′16″/6°53′28″	47°16′30″/8°26′45″
Altitude (m)	455	387	494
Daily mean temperature (°C)	18.4	18.0	18.2
Total degree-day (°C)	1119.8	1100.9	1112.0
Daily mean rainfall (mm)	4.0	5.6	4.1
Total rainfall (mm)	245.6	344.0	247.5
Daily mean humidity (%)	70.3	77.1	72.3

ing 0.0125% of TWEEN®20 (Sigma-Aldrich Chemie GmbH). Concentration of conidia and volume of suspension prepared were adjusted to 1.5×10^7 conidia per microplot. Suspensions were applied with a hand sprayer (Spray-matic 1.25P, Birchmeier) at dawn. According to the climatic conditions, the plots were irrigated to maintain humidity on the ears for at least 24 h. A high pressure/low volume overhead spray irrigation system was available at Changins. In Vouvry and Reckenholz, water (600 L ha^{-1}) was sprayed manually with a back-pack sprayer. A second inoculation took place 2 d later.

Disease assessments on spikes

Disease severity and incidence were recorded on 30 randomly marked spikes in each plot. Disease severity was recorded by counting the number of infected spikelets, and disease incidence was scored by counting the number of infected spikes. The severity was expressed as percentage of infected spikelets relative to the total number of spikelets. The first assessments were carried out 15 d after the last inoculation, and then at 3 d intervals. At least three assessments were carried out for each plot.

Harvest and milling

All wheat plots were harvested at full maturity (BBCH 89) using the locally available facilities. In Reckenholz, a combine harvester (HEGE 140, Mähdreschwerke GmbH) was used. The airflow on the harvester was reduced to recover a maximum of kernels. In Changins and Vouvry, spikes were harvested by hand. After threshing with a laboratory thresher (Saatmeister, Kurt Pelz), grains were dried to 14% moisture and cleaned using a vertical airflow (Baumann Saatzuchtbedarf) to remove dust and other debris. All grains were stored at 4°C and processed in Changins. Two hundred gram sub-samples were extracted after 3 times homogenisation with a riffle divider (Schieritz & Hauenstein AG). Samples were milled separately with a sample mill (1093 Cyclotec Sample Mill, FOSS) to obtain wholemeal flour. The flours were stored at -20°C until used for further analyses.

Analyses of grains

Morphological analysis of grains

The thousand kernel weight (TKW) of each grain sample was measured with a MARVIN optical grain counter (Digital Seed Analyser, GTA Sensorik GmbH) and a balance (Mettler PM2000). The ratio of *Fusarium* damaged kernels (FDK) in each sample was determined by counting the number of shrivelled and misshapen grains for one hundred randomly chosen grains.

DON content

Content of deoxynivalenol was determined in wholemeal flour using a DON ELISA kit (Ridascreen® FAST DON, R-Biopharm AG), according to the manufacturer's instructions. Samples with high levels of contamination were diluted 10 times in double distilled water before analysis.

Anthocyanin concentrations

The total anthocyanin concentrations in grains were measured in wholemeal flour as described elsewhere (Eticha *et al.*, 2011) with modifications. Extractions were each carried out on 2.5 g of wholemeal flour with 20.0 mL of methanol/hydrochloric acid solution (85:15, v/v) in a 250 mL capacity flask. The mixture was homogenized for 20 min with a magnetic stripper and stored at 4°C for 20 min. The mixtures were transferred in 50 mL plastic tubes, centrifuged at 4,000 rpm for 5 min, and then stored at 4°C for 20 min. The supernatants were filtered into 50 ml volumetric flasks (S-Pak Filters, 0.45µm diam. 47 mm, on a Swinnex Filter Support 47 mm, Millopore SA). The extraction processes were repeated once on the pellet. Before measuring, 25 mL of each supernatant was mixed and stored on ice cubes in the dark. Measuring was done within 2 h. The absorbance of the extract was measured at 538 nm with a VIS/UV spectrometer (UViLine 9400, SCHOTT Instruments). The anthocyanin concentration was calculated based on a calibration curve obtained with cyanidin chloride as a standard (Sigma-Aldrich Chemie GmbH) according to Abdel-Aal and Hucl (1999). All anthocyanin concentrations were expressed in milligrams of equivalent of cyanidin chloride kg^{-1} (Abdel-Aal and Hucl 1999).

Protein content

The protein contents (%) of infected and non-infected grains were analysed by near-infrared reflectance spectroscopy (NIRS) using a NIRFlex N-500 (Büchi Labortechnik AG). The protein calibration of the NIRFlex was regularly adjusted with 50-100 wheat samples from different varieties and origins. Basis analyses were made with the Kjeldahl method, according to ICC standard method No. 105/2. The coefficient of confidentially of the calibration is $R^2 = 0.93$ (Cécile Brabant, pers. comm.). The protein content of infected and non-infected samples fitted into the range of the NIRS calibration.

Rheological properties

Dough stability duration (min), dough softening (Farinograph Units; FUs) and water absorption capacity (%) during kneading were measured using the microdough LAB farinograph (model 2800, Perten Instruments). The measurements were based on the microdough LAB 120 rpm method 02.01 (Perten Instruments Method Description for microdoughLAB, Perten Instruments). For each sample, wholemeal flour (4 g) was placed into the "120 rpm kneading device". Distilled water was automatically added until the dough reached the consistency of 650 FUs. The volume of added water was recorded. The dough stability duration is the time the dough consistency remains at 650 FUs, while dough softening corresponds to the decrease in FUs after 10 min of kneading.

Experimental set up and statistical analyses

Crop planting, inoculation methodology and disease assessments were the same at the three field sites. The trials each consisted of three replicates with, and three without, artificial inoculation, planted in a split-plot design.

Data of disease incidence and severity were integrated with the number of observation days (number of days between infection and the last scoring) and divided by the number of the observation day, and were thus expressed as relative area under the disease pressure curve (AUDPCrel). Variations of TKW and differences in rheological properties of grains due to the infections were analyzed for each infected sample by calculating the difference (%) with the average of the three non-infected samples of the same wheat genotype from the same location. All calculations were conducted on Microsoft® Excel 2013.

Statistical analyses were carried out using statistical R software (R Core Team, 2015). Analyses of variances (ANOVA) of resistance indicators were used to compare the effects of genotype and environment on inoculated plants. Data for disease incidence, and DON content were square root transformed to obtain normal distributions. The effect of an infection on grain properties was investigated using analyses of variance for the three factors presence of inoculation, genotype, or environment. Data of dough stability and anthocyanin content were square root transformed to obtain normal distributions. Comparison of TKW losses and relative differences in rheological properties of dough were carried out with two factor ANOVAs on the factors genotype and environment. When significant, multiple comparisons analyses were based on Tukeys HSD (package "agricolae", de Mendiburu, 2015). Correlations between the parameters were investigated using Pearson correlations on normalized data.

Results

FHB severity and incidence

The assessments of disease severity and disease incidence were carried out in inoculated and in non-inoculated (control) plots. Symptoms were significantly more severe in Changins than in Reckenholz or Vouvry ($P<0.05$) (Figure 1). Genotypes gave different resistance levels to infection, but no genotype × environment interactions were detected. Overall, the cultivars 'Combin' and 'Indigo' showed greater disease scores than 'Vanilnoir' and the breeding lines ACW 083 and ACW 271. 'Arina' and 'Hanswin' showed intermediate incidence and severity. No FHB symptoms were found in the non-inoculated plots.

Proportion of Fusarium *damaged kernels (FDK)*

Generally, all samples from inoculated plots, including all varieties in all environments, contained damaged grains in various proportions (Figure 2). The proportion of FDK was significantly less in samples from Vouvry than from the other sites ($P < 0.05$). In non-inoculated plots, 1–2% of the grains showed morphological damage attributed to *Fusarium*. 'Combin' presented the greatest proportion of *Fusarium* damaged kernels in all environments, while the 'Vanilnoir' was the least affected.

Thousand kernel weight (TKW)

Grain filling was measured as TKW, and differences in TKW between grains from inoculated and non-inoculated plots revealed the impacts of the infection on grain filling. In Changins and Reckenholz, infection had significant impacts on grain filling ($P<0.05$) (Table 3). For example, losses in TKW reached 25% for 'Combin' in Reckenholz and Changins. In contrast, TKW was hardly affected at the Vouvry site. Over all three environments, the genotypes with coloured grains (ACW 083, ACW 271, and 'Vanilnoir') showed the smallest losses in TKW due to inoculation.

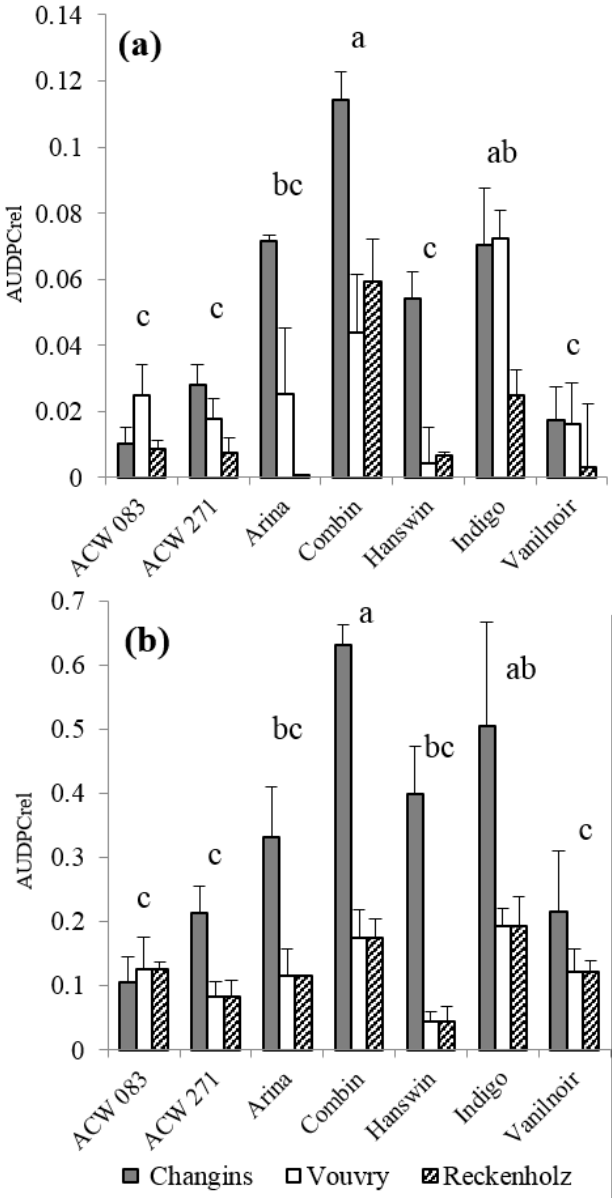

Figure 1. Means of rel. AUDPC for (a) FHB severity and (b) FHB incidence on 7 wheat genotypes in Changins, Vouvry and Reckenholz. Bars accompanied by with the same letter are not statistically different ($P<0.05$).

Accumulation of DON

The accumulation of the mycotoxin DON is considered key for the evaluation of the severity of kernel infection by *F. graminearum*. No DON was detected in non-inoculated samples. The content in DON was significantly ($P<0.05$) conditioned by the environment (20% contribution to variability) (Figure 2). In Reckenholz, Changins and Vouvry, the average content in DON in grains was 31.9 mg kg^{-1} for Reckenholz, 20.7 mg.kg^{-1} for Changins and 12.3 mg kg^{-1} for Vouvry. Over the three environments, the greatest DON contents were measured in grains from 'Combin' and 'Indigo', while grains of 'Arina',

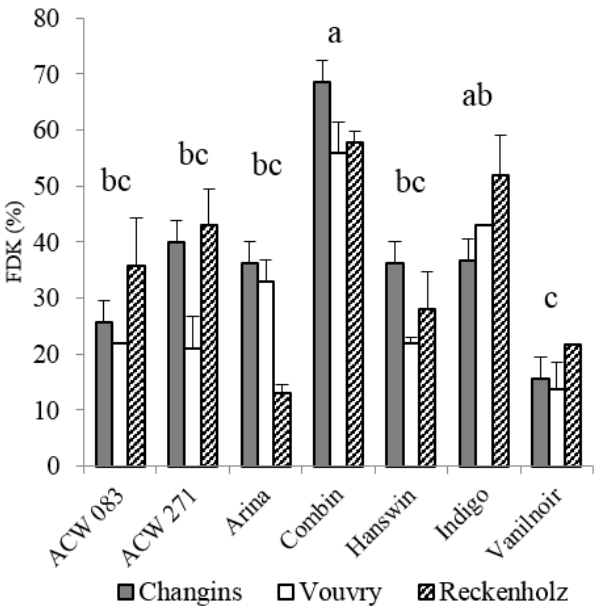

Figure 2. Mean *Fusarium* damaged kernel (FDK) ratios of three replicates, for seven wheat genotypes grown at three different field sites. Bars accompanied by the same letter are not statistically different (P<0.05).

Table 3. Means of Thousand Kernel Weights (TKW), losses (%) for all genotypes across at the three field sites (environments). Data followed by the same letter are not significantly different (P<0.05).

Genotype	TKW losses (%)			Mean	
	Changins	Vouvry	Reckenholz		
ACW 083	9.0	8.3	7.0	8.1	c
ACW 271	8.9	6.3	11.3	8.8	c
Arina	23.7	7.1	8.1	13.0	bc
Combin	24.7	16.8	25.8	22.4	a
Hanswin	15.0	-2.9	8.8	11.9	bc
Indigo	19.6	6.6	17.8	14.7	ab
Vanilnoir	8.4	1.7	5.7	5.3	c
Mean	15.6 (a)	7.8 (b)	12.1 (g)	12.0	

Table 4. Classification of the different types of resistance for the wheat genotypes used in this study. + = resistant, - = susceptible, and Ø = non-significant resistant comportment.

Genotype	Resistance of the spikes	Resistance category		
		DON accumulation	*Fusarium* incidence of grains	Reduction of the grain filling
ACW083	+	Ø	Ø	+
ACW271	+	Ø	Ø	+
'Arina'	Ø	+	Ø	Ø
'Combin'	-	Ø	-	-
'Hanswin'	Ø	+	Ø	Ø
'Indigo'	Ø	-	Ø	Ø
'Vanilnoir'	+	+	+	+

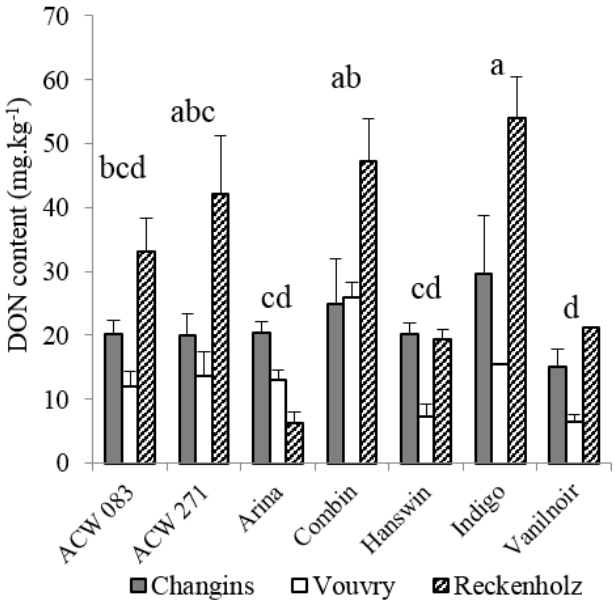

Figure 3. Mean deoxynivalenol (DON) concentrations in grains from seven wheat genotypes grown at three field sites (environments). Bars accompanied by the the same letters are not statistically different (P<0.05).

'Hanswin' and 'Vanilnoir' contained significantly less (P<0.05) DON.

These analyses allowed the genotypes to be classified according to their different types of resistance (Table 4). 'Vanilnoir' was the most resistant for all types of resistance while 'Combin' was generally the most susceptible. Overall, these types of resistance were affected by environmental con-

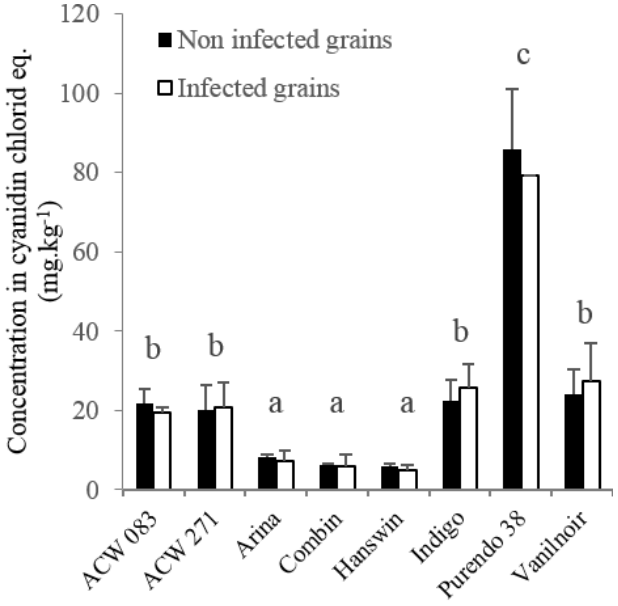

Figure 4. Mean anthocyanin concentrations (as cyanidin chlorid equivalent) in infected and in non-infected wheat grains from seven different wheat varieties. The values were calculated across three different field sites (environments). Bars accompanied by the same letter are not statistically different (P<0.05).

ditions but to different extents: impacts of *Fusarium* infection were significantly less at Vouvry regarding FDK ratio and TKW losses while DON accumulation were greater at Reckenholz for all the tested wheat genotypes.

Impacts of *Fusarium* infections on grain biochemical and dough rheological properties

Anthocyanin concentrations were measured in wholemeal flour samples from all genotypes, including the cultivar 'Purendo 38', all locations and from inoculated and non-inoculated plots. Anthocyanins were detected in all genotypes. In the non-coloured varieties 'Arina', 'Combin' and 'Hanswin', anthocyanin concentrations were approx. 10 mg kg^{-1}. The contents were between 20 and 35 mg kg^{-1}in the genotypes with coloured grains, and were an average of 85 mg kg^{-1} for the blue genotype 'Purendo 38' (Figure 4). Anthocyanin concentrations in grains were affected by environmental conditions, explaining 9% of anthocyanin concentration variability (Table 5). The concentrations were significantly less in grains from Vouvry irrespective of the genotypes. The *Fusarium* inoculations did not change the anthocyanin concentrations in the grain samples (Table 5).

The total protein contents ranged between 11% and 16% Statistical analyses revealed significant

Table 5. Composition of the variances (%) between the different factors (genotype, inoculation, site/environment and interactions) affecting the parameters: anthocyanin concentration and protein content, water absorption, dough stability and dough softening during kneading. Significance levels: *** at P<0.001, **: at P<0.01, * at P<0.05, ns: Not significantly different (P>0.05).

Factor	Anthocyanin concentration (%)	Total protein content (%)	Water absorption (%)	Dough stability duration (%)	Dough softening during kneading (%)
Genotype	77.1 ***	42.5 ***	53.6 ***	18.8 ***	8.2 ***
Inoculation	ns	6.5 ***	5.9 ***	48.4 ***	68.8 ***
Environment	9.4 *	20.8 ***	0.9 *	3.8 ***	2.8 ***
Interactions					
Genotype×Inoculation	ns	4.1 **	3.7 ***	11.1 ***	4.6 ***
Genotype×Environment	3.2 ***	6.6 **	6.1 ***	6.5 ***	4.1 ***
Inoculation×Environment	0.8 **	ns	13.1 ***	2.9 ***	5.0 ***
Genotype×Inoculation×Environment	2.9 ***	ns	ns	4.1 ***	3.9 ***

Table 6. Relative differences (%) in dough stability duration and dough softening during kneading resulting from *Fusarium* inoculation. Differences in dough stability duration are the means of relative reductions due to inoculation. Differences in dough softening during kneading are the means of relative increases due to inoculation. Means followed by the same letter are not significantly different ($P<0.05$)

Genotype	Differences in dough stability duration (%)					Differences in softening during kneading (%)				
	Changins	Vouvry	Reckenholz	Mean		Changins	Vouvry	Reckenholz	Mean	
ACW083	20.2	32.3	45.1	32.5	c	65.8	59.9	111.6	79.1	d
ACW271	29.1	19.5	38.5	29.0	c	34.6	110.3	173.4	97.7	cd
'Arina'	44.1	46.2	42.5	44.3	b	129.3	163.3	81.0	124.5	c
'Combin'	37.3	42.1	72.3	49.4	b	161.2	59.6	462.2	97.7	b
'Hanswin'	59.3	33.6	76.7	59.4	a	245.9	90.5	626.3	349.7	a
'Indigo'	54.7	58.8	74.6	63.8	a	137	216	313	224	b
'Vanilnoir'	35.2	5.0	33.8	24.6	c	105	27	95	75	d
Mean	40.0 (a)	33.9 (a)	54.8 (b)	43.3		135 (a)	79 (b)	260 (g)	150	

differences between genotype, experimental site and inoculation. The variance of protein content depended mainly on the experimental site (21%). Protein contents were on average less in samples from Reckenholz than in those from Changins or Vouvry, while the impact of the *Fusarium* inoculation accounted for only 7% of the total variance (Table 5). Significant interactions were detected between the genotypes and inoculation, accounting for 7% of the total variance, with the genotypes affected differentially.

Water absorption (%), dough stability duration (min) and dough softening (FU) were measured on flour from infected and non-infected grains. Water absorption showed significant differences between the genotypes (Table 5). The *Fusarium* inoculations (6% of total variance) and differences in environmental conditions (1%) had weak impacts on water absorption (Table 5).

The *Fusarium* inoculations gave strong effects on dough stability duration and dough softening during kneading, irrespective of the genotypes and the sites. Analyses of variances revealed that inoculation explained 48% of the stability duration variances and 68.8% and softening variances (Table 5). The effects of the different genotypes on the two parameters (respectively, 18.8 and 8.2% of the total variance) were weaker than the effect of the inoculations. . The impacts of environmental conditions on stability duration and softening variances (respectively 4 and 2.8% of the total variances) were also weak. The significant interactions between genotype and inoculation indicated that rheological properties of grains were not affected to the same extent in all the genotypes. The relative differences in dough stability duration and dough softening caused by the disease were calculated to reflect the differences in FHB impacts between genotype (Table 6). Overall, *Fusarium* inoculations decreased the duration of dough stability and increased dough softening, and the genotypes were affected differentially. For dough stability duration, 'Hanswin' and 'Indigo' were more affected compared to the other genotypes, with mean reductions across all environments of 59% in 'Hanswin' and 64% in 'Indigo' (Table 6). Differences in dough stability duration were less in ACW 083, ACW 271 and 'Vanilnoir'. The greatest impact of *Fusarium* inoculations on dough softening was measured for 'Hanswin' with more than a three-fold increase for samples from inoculated plots compared with uninoculated plots (Table 6). Impacts of inoculation on dough softening were less for ACW 083 and 'Vanilnoir with decreases, respectively of 79 and 75%. Environmental conditions also affected the impacts of inoculation, as overall differences in dough stability duration and in dough softening were greatest in samples from Reckenholz (Table 6).

Table 7. Pearson correlation coefficients between observed indicators of resistance and relative differences in grain rheological properties caused by *Fusarium* inoculations. *** significant at P=0.001, ns = not significant (P>0.05).

Indicators of resistance	FHB severity on spike	FHB incidence on spike	FDK (%)	DON content (mg.kg^{-1})	TKW losses (%)	Differences in dough stability duration (%)
FHB incidence on spike	0.92***					
FDK (%)	0.61***	0.51***				
DON content (mg.kg^{-1})	0.47***	0.34***	0.59***			
TKW losses (%)	0.72***	0.64***	0.61***	0.57***		
Differences in dough stability duration (%)	ns	ns	0.46***	0.55***	0.41***	
Differences in softening during kneading (%)	ns	ns	0.47***	0.54***	0.36***	0.88***

Abbreviations: FDK = *Fusarium* damaged kernel ratio; DON = deoxynivalenol; TKW = thousand kernel weight.

Links between the components of resistance and rheological properties

The relationships between the different FHB resistance components have been examined with Pearson's correlation analysis (Table 7). Disease severity and incidence on the spikes were only weakly correlated with the symptoms on grains. The different components of grain resistance (FDK ratio, TKW loss and DON accumulation) were linked, yet the correlations were moderately strong (0.57 to 0.61; P<0.001) but not complete. Differences caused by infections in dough stability duration and softening indicate significant and positive correlations with FDK ratio, DON content and TKW losses. This suggests that varieties that accumulate more DON and show reduced grain filling were also more impacted by changes in their rheological properties than less affected varieties. No correlations were detected between differences in rheological properties and the symptoms scored on the spikes in the field.

Discussion

In this study, seven wheat genotypes were assessed for spike and grain resistance to FHB caused by *F. graminearum*, in multi-locality field experiments with artificial inoculations. Theses differing environmental conditions have challenged the resistance reactions and allowed examination of resistance and genotype × environment interactions under different infection conditions.

The genotypes showed different levels of disease severity and incidence. While the overall intensity of the infections was different at each of the three sites, the ranking of the varieties was the same across all sites and no genotype × environment (G × E) interactions were detected. Intensity of infection was the least at Vouvry, intermediate at Reckenholz and greatest at Changins. This was likely to be due to the climatic conditions, as Vouvry was characterized by low temperatures and high relative humidity, and at Changins the infections were favoured by the permanent irrigation facility.

The mycotoxin DON was detected in all inoculated samples across all sites. Even at Vouvry, with very low disease severity and incidence, elevated concentrations of DON were detected in the grain samples. Therefore, DON accumulation in grains was not directly linked to type 1 or type 2 resistance of the host spikes, confirming findings in other environments and with other wheat genotypes (Liu *et al*., 1997; Mesterhàzy *et al*., 1999; Mesterhàzy, 2002). The accumulation of DON was also affected by the wheat genotype and by statistically significant G × E interactions. These results emphasise the important role of grain resistance for wheat breeding, in order to enhance food safety. Besides the accumulation of DON, resistance to FHB effects is characterised by ability to withstand grain deformations, detected as FDK, and to maintain grain volumes, measured as TKW losses

or test weight (hectolitre weight). Our observations indicate that the *Fusarium* infections cause deformations in varying percentages and reduce the TKW of all genotypes across all environments depending on the severity strength of infection and the degree of varietal resistance. As for symptoms on spikes, the ranking of resistance of the varieties, in terms of FDK and TKW, was the same in all environments, thus excluding G × E interactions.

These results indicate that grain resistance to FHB is made of several resistance components. The correlation coefficients between these factors were medium, ranging from 0.41 and 0.61, and indicating only moderate links. This was illustrated with the cultivar 'Hanswin', which accumulated only low concentrations of DON despite developing average amounts of FHB on the spikes and considerable numbers of *Fusarium* damaged kernels. 'Hanswin' mechanisms that impede DON accumulation, or that favour DON degradation (Miller *et al.*, 1985; Boutigny *et al.*, 2008; Boutigny *et al.*, 2010). Overall, these observations suggest that all kernel resistance types are interacting and may be interdependent, and that they can act individually, confirming other reports (Mesterházy *et al.*, 1999; Mesterházy 2002; Langevin *et al.*, 2004; Snijders 2004).

This study also focused on the impacts of FHB on grain protein content and the dough rheology. Since *Fusarium* infections impede translocation of assimilates to grain, we have used different genotypes with coloured grains to provide knowledge of grain filling, and the impacts of FHB on biochemical processes in the grain. Anthocyanins and proteins are synthesised in the grains (Knievel *et al.*, 2009; Feillet 2000; Bustos *et al.*, 2012; Žofajová *et al.*, 2012), and changes in anthocyanin biosynthesis in grains is part of abiotic stress responses (Gordeeva *et al.*, 2013). In the present study, for all genotypes and across all environments, the concentration of anthocyanins has not changed due to infection. This also held true for the cultivar 'Purendo 38', which contains anthocyanins in the grain endosperms and the pericarps. Although the concentration of anthocyanins in the grains depended on the environment (Chalker-Scott, 1999), we detected only very weak G × E interactions (3.2% difference) and no genotype × infection interactions. The total protein content of the grains was hardly affected by the infection, and was overshadowed by the dominant genotype and environment factors. Similar studies on the impacts of infection on grain protein content have showed either slight increases (Boyacioğlu and Hettiarachchy, 1995) or slight decreases (Häller Gärtner *et al.*, 2008). However, these previous studies were conducted in single environments. The present study demonstrated the interactions between genotype, environment and infection, and revealed negligible impact of the *Fusarium* infection on the grain protein content. Similarly, Wang *et al.* (2005) concluded that the total protein content was not impacted by *Fusarium* infection, but they showed that seriously infected grains had lower glutenin contents. However, high disease pressure does not influence sulphur speciation of glutenins that are responsible for the functionality of the gluten network and the resulting baking quality (Andrews and Skerritt, 1996; Birzele *et al.*, 2003; Prange *et al.*, 2005). DON is mainly found in the grain pericarps and diffuses gradually into the core of the grains (Häller Gärtner *et al.*, 2005) Therefore, flour is generally less contaminated with DON than the bran (Cheli *et al.*, 2013), with the exception being for heavily infected grains (Bechtel *et al.*, 1985). It is therefore likely that the pathogen hardly penetrates through the outer grain layers. The reduced glutenin content described by Wang *et al.* (2005) may be due to the degradation activity of *Fusarium* proteases, once in contact with the gluten and activated after addition of water during dough preparation (Nightingale *et al.*, 1999; Wang *et al.*, 2005). In the present study, the measures with the automatic kneading device did not exceed 10 min. per sample. Hence, it is unlikely that the reduced dough performance in flour from infected grains was due to the fungal enzymatic activity.

The assessment of dough stability duration and softening by kneading revealed degradation of dough properties by *Fusarium* infections in all the wheat genotypes assessed, with important differences between the genotypes. This is in accordance with previous observations of the rheological and baking quality performance in spring wheat varieties (Häller Gärtner *et al.*, 2008). Wang *et al.* (2005) associated alteration of bread-making quality with damage on starch granules and storage proteins caused by *Fusarium* pathogens. Overall, the greater the grain resistance to FHB effects, the lower was the impact on dough properties. The concentration of DON was the best indicator for the reductions in dough properties.

Our results have shown that *Fusarium* infections do not affect the biosynthesis of proteins and antho-

cyanins nor physiological processes in wheat grains. However, the spread of the pathogen in the spikes associated with the reduction of grain filling demonstrated that the infection modulates the transport of assimilates and consequently changes grain composition. These observations confirm that the evaluation of resistance of grain only with measurements of DON content, as often made in plant breeding programs, is not sufficient to fully characterise grain resistance.

Grain resistance is not a simple trait but is composed of several components, including resistance to DON accumulation, morphological changes and transport of photosynthesis products. This list must be completed with aspects of resilience of rheological properties. Future studies should focus on the impacts of infection on starch and other baking-quality determinants such as hemicelluloses and non-gluten proteins. Important aspects also include plant factors that modulate fungal pathogenesis factors, such as protease inhibitors (Pekkarinen *et al.*, 2000), DON accumulation (Boutigny *et al.*, 2010) and their roles in grain resistance. In this study, some coloured wheat genotypes displayed increased grain resistance to FHB effects. This may be due to the antioxidant activity of anthocyanins, known to enhance resistance in plants (Zhou *et al.*, 2007; Pani *et al.*, 2014). Additional studies are required to elucidate the potential roles of anthocyanins in resistance to FHB.

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