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Effects of fungicide treatment, N-fertilisation and harvest date on arabinoxylan, endoxylanase activity and endoxylanase inhibitor levels in wheat kernels

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Abstract

Fungicide treatment had a significant impact on endoxylanase activity and XIP levels, but did not affect arabinoxylan (AX) and TAXI levels. The different response of TAXI and XIP type inhibitors to fungicide treatment is interesting. N-fertilisation did not affect AX levels, but significantly increased TAXI and XIP type inhibitor levels. Wheat-associated microbial endoxylanase activity levels were also affected by nitrogen supply, but levels of the endogenous enzyme did not change, except when sprouting occurred. The weather conditions before harvest had no influence on total AX (TOT-AX) and inhibitor levels, but had a large impact on both microbial and endogenous endoxylanase activity and water extractable AX (WE-AX) levels. Under most conditions, endoxylanase activity levels were related to those of α-amylases, liquefaction numbers (LN) and specific weights. WE-AX levels were often weakly but significantly correlated with endoxylanase activity levels, indicating that it is possible that part of the WE-AX in wheat originates from AX degradation by endoxylanases in the field. These results clearly indicate that agronomic circumstances significantly affect the levels of AX, endoxylanases and their inhibitors in wheat, and consequently could affect wheat quality.
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Keywords: Wheat; Arabinoxylan; Endoxylanase; TAXI; XIP; Fungicide; N-fertilisation; Harvest date

1. Introduction

Wheat characteristics not only depend on genetic and climatological circumstances as described in Dornez et al. (2007), but also on agronomic conditions during wheat production. Agronomic inputs such as fungicide treatment and N-fertilisation have a major significant effect on the developing wheat during kernel filling and consequently strongly determine wheat quantity and quality characteristics (Dupont and Altenbach, 2003).

Fungicides are applied to avoid disease related yield losses and augment grain yields by prolonging grain filling and increasing kernel weights (Bertelsen et al., 2001;

Abbreviations: a.i., active ingredient; AX, arabinoxylan; AZCL-AM, azurine-cross-linked-amylose; AZCL-AX, azurine-cross-linked-arabinoxylan; GHF, glycoside hydrolase family; GS, growing stage; HFN, hagberg falling number; LN, liquefaction number; NSP, non-starch polysaccharide; TAXI, *Triticum aestivum* xylanase inhibitor; TKW, thousand kernel weight; TOT-AX, total arabinoxylan; WE-AX, water extractable arabinoxylan; XIP, WU-AX, water unextractable arabinoxylan; XIP, xylanase inhibiting protein

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Dimmock and Gooding, 2002; Ruske et al., 2003). As endoxylanases associated with wheat kernels to a large degree originate from microorganisms populating the outer wheat kernel layers (Dornez et al., 2006a), it can reasonably be assumed that those microbial endoxylanase activities would be higher when fungicide treatments are omitted. Furthermore, fungicide treatment may also affect endoxylanase inhibitor levels in wheat, as these are often thought to be involved in plant defence (Bellincampi et al., 2004). Indeed, it is unlikely that endoxylanase inhibitors play a regulatory role in plants as they are not able to inhibit endogenous endoxylanases and only inactivate fungal and bacterial endoxylanases. Endoxylanases of pathogenic microorganisms like *Fusarium graminearum* (Beliën et al., 2005) and *Botrytis cinerea* (Brutus et al., 2005), for example, were shown to be inactivated by wheat endoxylanase inhibitors. In this context, Igawa et al. (2004) reported that the expression of the major XIP (xylanase inhibiting protein) gene, *Xip-I*, is strongly induced in leaves infected by the powdery mildew fungus *Erysiphe graminis* and by wounding. In the case of TAXI (*Triticum aestivum* xylanase inhibitor), the *Taxi-I* gene does not respond to such stress signals and was considered to be a basal preexisting defence gene against fungal pathogens during seed development and germination. However, other TAXI genes such as *Taxi-III* and *Taxi-IV*, are pathogen inducible (Igawa et al., 2005). These findings might imply that omitting fungicides might increase significantly the levels of endoxylanase inhibitors in wheat.

N-fertilisation is one of the most investigated factors in wheat production. Although total albumin and globulin protein levels tend to be insensitive to applied N-fertilisation, no studies have determined whether this is true for individual albumin and globulin components such as endoxylanases and endoxylanase inhibitors (Dupont and Altenbach, 2003). Furthermore, Coles et al. (1997) already found some evidence that N-fertilisation in combination with a plentiful water supply can cause an increase in the levels of AX in wheat. Bodson et al. (2001) showed that changing the timing of nitrogen application, with dose reduction during tillering and transferring this amount to flag leaf application, not only increased grain protein contents, but also decreased disease risks, suggesting that N-fertilisation might impact microbial endoxylanase activity levels in wheat.

Besides fungicide treatment and N-fertilisation, the weather conditions before harvest may also affect wheat composition. Harvesting of wheat ideally takes place when the wheat kernel is sufficiently dry. However, in some years, wheat has to be harvested under wet conditions. This can lead to strong microbial contamination and/or preharvest sprouting. Based on the large influence of weather conditions at harvest on α-amylase activity levels and hence on Hagberg falling numbers (HFN), it can reasonably be expected that endoxylanase activity levels also change with harvest date.

The purpose of the present study was to examine whether the above described agronomic conditions (N-fertilisation, fungicide treatment and harvest date) have an impact on the AX, endoxylanase activity and endoxylanase inhibitor levels in wheat. The obtained partial correlation coefficients may help to identify which components are similarly affected by changing agronomic conditions.

2. Materials and methods

2.1. Wheat samples

The wheats were grown at an experimental site (Lonzée, Gembloux, Belgium) in three successive growing periods (2001/02, 2002/03 and 2003/04). Each sample was grown in four different plots of 16 m^2 on the same field in a fully randomised block design. Wheat kernels from the different plots were mixed to reduce location effects. Unless specified otherwise, wheat samples were sown in October and two fungicide treatments were applied at the flag leaf and the ear emergence stage [growing stages (GS) 37 and 59, respectively] (Zadocks et al., 1974). In trials in which N-fertilisation was not the factor studied, N-fertilisation (175 kg N/ha in 2001/02, 185 kg N/ha in 2002/03 and 185 kg N/ha in 2003/04) was given in three split applications, the first during tillering (GS 20–25), the second at the beginning of stem elongation (GS 30) and the third at flag leaf emergence (GS 37–39), in liquid form for the first two partial applications and in solid from the third partial application. Weed and insect control were achieved in all trials by applying appropriate herbicides and insecticides. Lodging was limited by using chlormequat chloride as growth regulator. Treatments that were not investigated in the trials were applied at the same time and in equal doses to make comparison between the samples possible.

2.1.1. Trial 1

In a first trial, the effect of fungicide treatment was examined for two wheat varieties by comparing wheat samples grown with two fungicide treatments applied at the flag leaf stage and the ear emergence stage, respectively, and wheat samples grown without fungicide treatments. As apparent endoxylanase activities were previously reported to be possibly related to the ear disease sensitivity of the wheat variety (Dornez et al., 2006b), a wheat variety that is very sensitive to ear diseases, Meunier, and a less sensitive one, Mercury were used in this trial. The fungicides were invariably made up of a triazole–strobilurin combination. Triazole fungicides inhibit sterol biosynthesis, thereby impairing the membrane production of fungi and work curative. Strobilurin fungicides, in contrast, exert their fungicidal action by blocking electron transport in the mitochondrial respiratory chain in fungi and work in a preventative rather than a curative way (Bertelsen et al., 2001). The fungicides were applied at the manufacturers' recommended field rates. In 2002, 1.0 l/ha epoxyconazol

[Opus, BASF Belgium, 125 g active ingredient (a.i.)/l], 1.0 l/ha azoxystrobin (Amistar, Syngenta Belgium, 250 g a.i./l and 1.0 l/ha metconazole (Caramba, BASF Belgium, 60 g a.i./l) were used. In 2003, 1.5 l/ha pyraclostrobin and epoxyconazol (Opéra, BASF Belgium, 133 g a.i./l and 50 g a.i./l, respectively) were used. In 2004, 1 l/ha epoxyconazol and 1 l/ha azoxystrobin were used. The fungicide protection applied to the crops was not similar each year but treatments were adapted to the risks of disease development in that year to obtain a maximum disease control. This was consistent with the objective of the fungicide trial, i.e. to compare absence and presence of fungicide protection and not to compare the effect of different dosages.

2.1.2. Trial 2

In a second trial, the impact of N-fertilisation was investigated for the wheat varieties Corvus and Folio. Folio is susceptible to preharvest sprouting, while Corvus is not. Nitrogen was applied under solid form as ammonium nitrate (27% N) in three different dosages. The total N-fertilisation applied to the wheat varieties was varied from 0 to 150 and 300 kg N/ha. These levels were equally distributed over the three split applications i.e. a first during tillering (GS 20–25), a second at the beginning of stem elongation (GS 30) and a third at flag leaf emergence (GS 37–39). The latter assures the survival of the photosynthetically active flag leaf, which is associated with increased grain yield.

2.1.3. Trial 3

In a third trial, the influence of harvest date and associated weather conditions was investigated for the wheat varieties Corvus, Folio and Meunier. For these varieties, harvesting was on 25 July and 1, 8, 15 and 23 August in 2002, on 22 and 28 July and 3, 7 and 12 August in 2003 and on 5, 9, 18, 23 and 30 August in 2004. The different harvest dates were hence spread over a 1-month period spanning the optimal harvest period. Corvus is resistant to preharvest sprouting and ear diseases, while Folio and Meunier are sensitive to preharvest sprouting and ear diseases, respectively.

2.2. Chemicals and reagents

Chemicals, bovine serum albumin and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of at least analytical grade. *Bacillus subtilis* glycoside hydrolase family (GHF) 11 endoxylanase (Grindamyl H640, Swissprot Accession # P18429) was from Danisco (Brabrand, Denmark). *Penicillium pupurogenum* GHF 10 endoxylanase (Swissprot Accession # Q9P8J1) was kindly made available by Prof. Jaime Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile). Azurine-cross-linked-arabinoxylan (AZCL-AX) and amylose (AZCL-AM) tablets were purchased from Megazyme (Bray, Ireland).

2.3. Methods

Determination of grain yield, specific weight and thousand kernel weight (TKW) was as previously described (Dornez et al., 2007). HFN values were determined according to the AFNOR NF V03-703 method (AFNOR, September 1997) and transformed to LN values by the equation of Perten (1964). Protein levels ($N \times 5.7$) were determined with the Dumas combustion method (Leco FP 2000, Leco, Garges-les-Gonesse, France) (ICC No. 167). TOT-AX and WE-AX levels were determined with gas-liquid chromatography as previously described (Dornez et al., 2007). α-Amylase activity levels were determined with the Amylazyme method (Anonymous, 1998) and endoxylanase activity and endoxylanase inhibitor levels were estimated by the Xylazyme-AX method as outlined elsewhere (Dornez et al., 2007).

2.4. Statistical analyses

All statistical analyses were performed using the Statistical Analysis System software 8.1 (SAS Institute, Cary NC, USA). The aim of the analyses was to study the effect of agronomic conditions on AX, endoxylanase activity and endoxylanase inhibitor levels in wheat kernels. Those agronomic conditions were experimentally fixed and hereby treated as fixed effects in the Analysis of Variance (ANOVA) model. In order to generalise results for all harvest years and genotypes, those two settings were considered as being random factors in the ANOVA model. In other words the considered harvest years and genotypes are treated as a sample from all possible years and genotypes, respectively. The choice for random rather than fixed factors was based on the review by Smith et al. (2005) on use of mixed effects models and how they compare to fixed effects in agronomy. Interaction terms, all being random effects, are considered as part of the residual and, hence, cannot be tested. A likelihood ratio test was used to identify the significant sources of variance (Verbeke and Molenberghs, 2000). Maximum likelihood (ML) was used to estimate the variance components and related significances for the considered fixed (agronomic) effect. After a positive omnibus test, post hoc analyses were conducted in order to detect differences among experimental settings. A Tukey multiple comparison procedure was used with a 5% family significance level (Kutner et al., 2004). The results of this type of analysis are given under 'All years' in the tables of Section 3. Besides these combined models, mixed effects models for each year were performed separately. In those analyses, agronomic conditions were again treated as the fixed effect, whereas genotype was considered as being the random effect. Model selection and tests were identical to those described above. The linear correlations between the different parameters were calculated using Multivariate

Analysis of Variance (MANOVA). The correlation coefficients reported in the different trials were partial correlations after correcting for the possible effects of wheat variety and harvest year as discrete covariates. However, some trials were too small to calculate the test statistics (e.g. Wilks' Lambda) indicating whether there was indeed an overall effect of wheat variety and harvest year. In spite of this, we used the corrected coefficients in all cases. Total endoxylanase activity was not included in the correlation tables as it is not an independently measured trait.

3. Results and discussion

3.1. Trial 1: fungicide treatment

3.1.1. AX levels

Fungicide treatment did not significantly affect TOT-AX or WE-AX levels in wheat (Table 1). Grosjean et al. (1999) also did not find an influence of fungicides on the water extract viscosity of wheat, which is often used as an indirect measurement of soluble non-starch polysaccharide (NSP) content. In contrast, Wang et al. (2004) suggested that certain fungicide combinations promote the synthesis of soluble NSP. However, their measurements were only based on a viscosity decrease and not on direct WE-AX measurements. Hence, it cannot be excluded that other components were responsible for the observed viscosity decrease. Indeed, Saulnier et al. (1995) already stated that the content of WE-AX was only poorly correlated with the viscosity of the aqueous extracts.

3.1.2. Endoxylanase activity levels

Overall, the microbial endoxylanase activity level in wheat almost doubled when fungicide treatment was omitted (Table 1). The effect was stronger for Meunier than for Mercury. Meunier is indeed much more sensitive to ear diseases than Mercury (Dornez et al., 2006b). This large increase in microbial endoxylanase activity when fungicide treatment is omitted is not surprising as phytopathogenic fungi secrete a variety of hydrolytic enzymes, such as cellulases, cutinases, pectinases, proteases and xylanases, during the infection process. They do so to facilitate invasion of grain and use polysaccharides as a carbon source for their own growth (Knogge, 1998; Stahl and Bishop, 2000; Matthäus et al., 2004). As the cereal cell wall, which is an important barrier to pathogenic microorganisms, predominantly consists of heteroxylan, endoxylanases are often thought to be the most important enzymes for infection of cereals. Indeed, Benamrouche et al. (2002) demonstrated that the inner bran, composed of

Table 1
Impact of presence (+) or absence (−) of fungicide treatment on water extractable (WE-AX) and total arabinoxylan (TOT-AX) levels, endogenous, microbial and total endoxylanase activity levels and TAXI and XIP type endoxylanase inhibitor levels in wheat

Wheat variety	Fungicide	Arabinoxylan (%)		Endoxylanase (EU/g)			Endoxylanase inhibitor (ppm)	
		TOT-AX	WE-AX	Endogenous	Microbial	Total	TAXI	XIP
2002								
Mercury	−	6.57	0.60	0.30	1.54	1.84	104	387
	+	6.58	0.64	0.18	1.22	1.40	92	320
Meunier	−	6.74	0.92	0.48	2.42	2.90	78	366
	+	6.62	0.86	0.33	0.45	0.78	97	318
Average	−	6.66[a]	0.76[a]	0.39[b]	1.98[b]	2.37[b]	91[a]	377[b]
	+	6.60[a]	0.75[a]	0.26[a]	0.84[a]	1.09[a]	95[a]	319[a]
2003								
Mercury	−	6.70	0.54	0.09	0.65	0.75	91	326
	+	6.25	0.55	0.09	0.44	0.52	102	319
Meunier	−	6.97	0.71	0.19	1.21	1.40	138	313
	+	6.11	0.70	0.09	0.66	0.75	126	307
Average	−	6.84[b]	0.63[a]	0.14[a]	0.93[b]	1.08[b]	115[a]	320[a]
	+	6.18[a]	0.63[a]	0.09[a]	0.55[a]	0.64[a]	114[a]	313[a]
2004								
Mercury	−	6.59	0.50	0.13	0.79	0.91	107	362
	+	6.80	0.50	0.08	0.52	0.60	118	281
Meunier	−	6.80	0.74	0.11	1.78	1.89	126	365
	+	6.88	0.67	0.07	1.09	1.16	136	312
Average	−	6.70[a]	0.62[b]	0.12[b]	1.29[b]	1.40[b]	117[b]	364[b]
	+	6.84[a]	0.59[a]	0.08[a]	0.81[a]	0.88[a]	127[a]	297[a]
All years								
Average	−	6.73[a]	0.67[a]	0.22[b]	1.40[b]	1.60[b]	107[a]	353[b]
	+	6.54[a]	0.65[a]	0.14[a]	0.73[a]	0.87[a]	112[a]	309[a]

For each year, values with the same letter in the same column are not significantly different from each other.

the nucleus, seed coat, tube cells and cross cells, and especially the aleurone layer are susceptible to endoxylanase hydrolysis. The outer bran, composed of hypodermis and epidermis, in contrast, was found to be rather resistant to endoxylanase hydrolysis.

Endogenous endoxylanase activity levels were also significantly affected by fungicide treatment. However, the reason for this is less obvious. Apparently, a change in fungal disease pressure can cause a varying response of the plant to production of endogenous endoxylanases. Matthäus et al. (2004) hypothesised that inoculation of wheat kernels with *F. culmorum* could stimulate the plant to increase the activity of hydrolysing enzymes that normally occurs during germination.

Total endoxylanase activity levels, calculated by summation of endogenous and microbial activity levels, also increased significantly when fungicide treatment was omitted.

3.1.3. Endoxylanase inhibitor levels

Based on their inhibition specificity (Dornez et al., 2007) endoxylanase inhibitors are often assumed to play an important role in plant defence. TAXI and XIP are not able to inhibit endogenous GHF 10 endoxylanases, but inhibit bacterial and fungal GHF 11 and fungal GHF 10 and 11 endoxylanases, respectively (Gebruers et al., 2004; Juge et al., 2004). Based on the results of Igawa et al. (2004, 2005), it can be assumed that the plant produces these kinds of inhibitors in response to fungal attack. Omitting fungicide treatment, however, did not significantly affect TAXI levels in wheat (Table 1). XIP levels, however, were significantly affected and were approximately 15% higher in wheat samples grown without fungicide treatment (Table 1). When the different harvest years were analysed separately, the differences in XIP levels between treated and untreated wheat were only significant in 2002 and 2004. The disease pressure in 2003 was presumably too low to induce XIP gene expression. These results are in good agreement with those of Igawa et al. (2004, 2005) who found that the *Xip-I* gene was pathogen inducible, while the *Taxi-I* gene was not. Our results further suggest that TAXI genes *Taxi-III* and *Taxi-IV,* found to be pathogen inducible by Igawa et al. (2004), code for TAXI isoforms which do not occur in quantities large enough to cause a significant overall effect.

3.1.4. Partial correlation coefficients

Table 2 lists the partial correlation coefficients between the measured traits for the fungicide trial after elimination of possible year and variety effects. A remarkably strong positive correlation was found between yield and specific weight, which can be explained by the positive effect of fungicide treatment on both parameters (Dimmock and Gooding, 2002). Surprisingly, no correlation could be found between α-amylase activity levels and LN values. This was due to the fact that α-amylase activity levels did not change in our trial, while LN values tended to be higher

Table 2
Partial correlation coefficients for the fungicide treatment trial for wheat varieties Mercury and Meunier from harvest years 2002, 2003 and 2004 after elimination of possible year and variety effects

	Yield	TKW	Specific weight	Protein	LN	α-Amylase	TOT-AX	WE-AX	Endogenous endoxylanase	Microbial endoxylanase	TAXI	XIP
Yield	–	0.66	0.87***	−0.20	−0.54	−0.05	−0.54	−0.26	−0.70*	−0.79*	0.26	−0.55
TKW		–	0.58	−0.10	0.50	0.04	−0.44	0.08	−0.44	−0.45	0.09	−0.12
Specific weight			–	0.13	−0.67*	−0.07	−0.42	−0.42	−0.90**	−0.67	0.34	−0.39
Protein				–	−0.14	0.36	−0.01	−0.52	−0.28	0.43	0.60	0.39
LN					–	0.01	0.06	0.48	0.42	0.69*	−0.43	0.17
α-Amylase						–	−0.05	−0.66	−0.15	0.05	0.71*	−0.02
TOT-AX							–	0.06	0.29	0.31	0.08	−0.04
WE-AX								–	0.51	0.32	−0.86**	0.18
Endogenous endoxylanase									–	0.45	−0.46	0.45
Microbial endoxylanase										–	−0.14	0.65
TAXI											–	−0.18
XIP												–

***P-value <0.0001.
**P-value <0.01.
*P-value <0.05.

when fungicides were omitted. However, LN values seemed to be correlated with microbial endoxylanase activity levels. It is hence necessary to be aware of the fact that high LN and hence low HFN values are not necessarily caused by preharvest sprouting and accompanying elevated α-amylase activity levels, as already indicated by Lenartz et al. (2003), but could also depend on the levels of wheat-kernel-associated microbial endoxylanases or other influencing factors such as, for example, starch levels and starch properties.

Moderate negative correlations were found between yield and endoxylanase activity levels. Fungicide treatment in general increased wheat yield, whereas it decreased the wheat endoxylanase activity levels. Therefore, this correlation is in line with what could be expected. Specific kernel weight was negatively correlated with endoxylanase activity levels, more specifically with endogenous endoxylanase activity levels. TAXI levels were strongly negatively correlated with WE-AX and positively correlated with α-amylase activity levels. Endogenous endoxylanase activity levels were not significantly correlated with the microbial enzymes. As fungicide treatment had a much larger impact on microbial than on endogenous endoxylanase activity levels, this is not surprising.

3.2. Trial 2: N-fertilisation

3.2.1. AX levels

N-fertilisation did not have a significant impact on TOT-AX or WE-AX levels (Table 3). Coles et al. (1997), who previously studied the impact of N-fertilisation on AX levels in wheat, found higher TOT-AX levels per kernel when additional nitrogen was made available during grain filling and when water availability was not limiting. They

Table 3
Impact of nitrogen dosage (0–150–300 kg N/ha) on water extractable (WE-AX) and total arabinoxylan (TOT-AX) levels, endogenous, microbial and total endoxylanase activity levels and TAXI and XIP type endoxylanase inhibitor levels in wheat

Wheat variety	N-dosage (kg N/ha)	Arabinoxylan (%)		Endoxylanase (EU/g)			Endoxylanase inhibitor (ppm)	
		TOT-AX	WE-AX	Endogenous	Microbial	Total	TAXI	XIP
2002								
Corvus	0	6.27	0.63	0.12	0.74	0.86	87	211
	150	6.75	0.65	0.16	0.99	1.15	90	221
	300	6.50	0.68	0.21	1.47	1.68	108	283
Folio	0	6.97	0.72	0.24	0.84	1.07	97	302
	150	6.97	0.77	0.37	1.32	1.68	112	295
	300	6.78	0.82	1.18	3.43	4.61	116	311
Average	0	6.62^{a}	0.68^{a}	0.18^{a}	0.79^{a}	0.97^{a}	92^{a}	257^{a}
	150	6.86^{a}	0.71^{b}	0.27^{a}	1.16^{a}	1.42^{a}	101^{ab}	258^{a}
	300	6.64^{a}	0.75^{c}	0.70^{b}	2.45^{b}	3.15^{b}	112^{b}	297^{b}
2003								
Corvus	0	6.95	0.49	0.02	0.25	0.27	118	222
	150	7.27	0.50	0.02	0.24	0.26	136	285
	300	7.07	0.46	0.02	0.28	0.30	150	351
Folio	0	6.28	0.55	0.10	0.49	0.59	145	366
	150	6.26	0.58	0.09	0.65	0.74	143	363
	300	6.28	0.53	0.09	0.79	0.88	149	373
Average	0	6.62^{a}	0.52^{ab}	0.06^{a}	0.37^{a}	0.43^{a}	132^{a}	294^{a}
	150	6.77^{a}	0.54^{b}	0.06^{a}	0.45^{ab}	0.50^{ab}	140^{ab}	324^{ab}
	300	6.68^{a}	0.50^{a}	0.06^{a}	0.54^{b}	0.59^{b}	150^{b}	362^{b}
2004								
Corvus	0	7.12	0.42	0.04	0.25	0.29	91	244
	150	6.37	0.39	0.02	0.19	0.21	99	264
	300	6.57	0.39	0.02	0.24	0.26	113	314
Folio	0	7.42	0.59	0.05	0.30	0.34	122	365
	150	7.06	0.57	0.05	0.37	0.43	129	376
	300	6.99	0.51	0.06	0.53	0.59	134	397
Average	0	7.27^{a}	0.51^{a}	0.05^{a}	0.28^{a}	0.32^{a}	107^{a}	305^{a}
	150	6.72^{a}	0.48^{a}	0.04^{a}	0.28^{a}	0.32^{a}	114^{ab}	320^{a}
	300	6.78^{a}	0.45^{b}	0.04^{a}	0.39^{b}	0.43^{a}	124^{b}	356^{b}
All years								
Average	0	6.84^{a}	0.57^{a}	0.09^{a}	0.48^{a}	0.57^{a}	110^{a}	285^{a}
	150	6.78^{a}	0.58^{a}	0.12^{ab}	0.63^{ab}	0.75^{a}	118^{b}	301^{a}
	300	6.70^{a}	0.56^{a}	0.26^{b}	1.12^{b}	1.39^{b}	128^{c}	338^{b}

For each year, values with the same letter in the same column are not significantly different from each other.

thought the longer survival of the photosynthetically active leaf and ear tissue to be responsible for the higher assimilation of AX. However, even when our results were expressed per kernel, we could not find such an increase. This shows that further research is needed to clarify the effect of N-fertilisation on TOT-AX levels in wheat. The overall result for WE-AX levels seemed to be in agreement with the observations of Grosjean et al. (1999) who did not observe an effect of N-fertilisation on wheat extract viscosity. However, in our trial, the effect of N-fertilisation seemed to depend on harvest year. WE-AX levels increased with increasing N-fertilisation in 2002, while they decreased in 2004, but the differences were very small.

3.2.2. Endoxylanase activity levels

The impact of N-fertilisation on endogenous endoxylanase activity levels in wheat was significant in 2002, but not in 2003 or 2004 (Table 3). This was caused, probably, by the occurrence of sprouting in 2002, similar to what is typically observed for α-amylase activity levels (Detje, 1992). The impact of N-fertilisation on microbial endoxylanase activity levels in wheat was more pronounced for Folio than for Corvus (Table 3). At first sight, it might seem surprising that extra supply of nitrogen to the wheat plant only affects microbial and not endogenous endoxylanase activities. However, it is well established that the effect of N-fertilisation on protein levels of wheat is mainly caused by a large increase of the storage protein levels. In addition, there is already some evidence that increasing levels of ammonium nitrate nitrogen applied to wheat can increase the incidence of *Fusarium* head blight or *Fusarium*-infected grain (Edwards, 2004; Ma et al., 2004). Finally, Bodson et al. (2001) also reported that the management of nitrogen application can clearly influence the development of wheat diseases. Total endoxylanase activity levels, which are mainly determined by microbial endoxylanase activity levels and only to a lesser extent by endogenous ones, were affected in a similar way by N-fertilisation as microbial endoxylanase activity levels.

3.2.3. Endoxylanase inhibitor levels

N-fertilisation significantly increased both TAXI and XIP type inhibitor levels (Table 3). Apparently, when more nitrogen is available, the wheat plant is able to invest more in the production of these plant defence molecules. However, the increase of inhibitor levels with increasing N-fertilisation was only significant for the wheat variety Corvus and not for the wheat variety Folio. Overall, TAXI levels increased on average from 110 to 128 ppm, while XIP levels increased from 285 to 338 ppm.

3.2.4. Partial correlation coefficients

Table 4 lists the partial correlation coefficients between the measured traits for the N-fertilisation trial after elimination of possible year and variety effects. Wheat yield was strongly positively correlated with protein level but negatively with TKW. A very strong correlation was

Table 4
Partial correlation coefficients for the N-fertilisation trial for wheat varieties Corvus and Folio from harvest years 2002, 2003 and 2004 after elimination of possible year and variety effects

	Yield	TKW	Specific weight	Protein	LN	α-Amylase	TOT-AX	WE-AX	Endogenous endoxylanase	Microbial endoxylanase	TAXI	XIP
Yield	–	−0.68**	0.52*	0.85***	0.12	0.03	−0.18	−0.15	0.19	0.31	0.62*	0.53*
TKW		–	−0.05	−0.86***	−0.44	−0.33	−0.10	−0.19	−0.50	−0.62*	−0.79**	−0.73**
Specific weight			–	0.45	−0.47	−0.71**	−0.57*	−0.29	−0.40	−0.18	0.29	0.33
Protein				–	0.23	0.02	−0.24	−0.12	0.30	0.48	0.80**	0.76**
LN					–	0.81**	0.12	0.45	0.99***	0.91***	0.13	−0.07
α-Amylase						–	0.37	0.37	0.79**	0.61*	0.01	−0.19
TOT-AX							–	0.56*	0.07	−0.06	0.11	−0.03
WE-AX								–	0.43	0.43	0.22	−0.04
Endogenous endoxylanase									–	0.95***	0.18	−0.02
Microbial endoxylanase										–	0.32	0.13
TAXI											–	0.90***
XIP												–

*** P-value <0.0001.
** P-value <0.01.
* P-value <0.05.

found between the different types of endoxylanase activity levels proving that they are both affected by N-fertilisation. α-Amylase activity was well correlated with LN and specific weight. α-Amylase activity levels and in particular LN values also seemed to be correlated with endoxylanase activity levels, especially with the endogenous ones. TAXI and XIP levels were very strongly correlated with each other and both types of endoxylanase inhibitor levels were also positively correlated with protein levels, indicating that both types of endoxylanase inhibitor levels increased by nitrogen application. Both types of endoxylanase inhibitor levels and protein levels were negatively correlated with TKW.

3.3. Trial 3: harvest date

3.3.1. AX levels

The weather conditions at harvest had no impact on TOT-AX levels while later harvesting tended to increase the WE-AX levels in wheat (Table 5). However, the increase in WE-AX levels with increasing harvest date was only significant in 2002 and 2004, not in 2003. The difference in WE-AX levels between the first and the fifth harvest date was higher in 2002 (0.20%) than in 2004 (0.14%). The progressive increase in WE-AX levels is most likely caused by the high levels of endogenous endoxylanases present in the 2002 and 2004 wheat samples (see below), which hydrolyse the AX cell walls. Endogenous endoxylanases, caused by preharvest sprouting, degrade AX in aleurone and endosperm cell walls of wheat. This may explain why, in the 2003 samples, no significant differences in WE-AX levels were observed. To ensure that the solubilisation of AX by endoxylanases did not occur during wholemeal extraction, a control experiment was performed, in which wholemeal samples were boiled for 2 h

Table 5
Impact of harvest date on water extractable (WE-AX) and total arabinoxylan (TOT-AX) levels and on endogenous, microbial and total endoxylanase activity levels in wheat

Wheat variety	Harvest date	Arabinoxylan (%)		Endoxylanase (EU/g)		
		TOT-AX	WE-AX	Endogenous	Microbial	Total
2002						
Corvus	25/07	6.35	0.55	0.21	1.98	2.18
	01/08	6.54	0.51	0.14	0.78	0.92
	08/08	6.66	0.54	0.16	2.79	2.95
	15/08	6.59	0.62	0.37	1.05	1.41
	23/08	6.48	0.68	0.43	1.90	2.32
Folio	25/07	7.20	0.59	0.27	1.34	1.60
	01/08	6.76	0.60	0.49	1.33	1.81
	08/08	6.72	0.73	1.02	4.41	5.43
	15/08	6.90	0.74	0.83	1.70	2.54
	23/08	7.07	0.79	1.14	2.36	3.50
Meunier	25/07	6.17	0.60	0.14	0.80	0.94
	01/08	6.29	0.65	0.14	0.87	1.01
	08/08	6.34	0.80	0.34	2.16	2.50
	15/08	6.72	0.78	0.21	1.26	1.47
	23/08	6.41	0. 87	0.43	2.16	2.59
Average	25/07	6.57[a]	0.58[a]	0.21[a]	1.37[a]	1.57[a]
	01/08	6.53[a]	0.59[a]	0.26[ab]	0.99[a]	1.25[a]
	08/08	6.57[a]	0.69[b]	0.51[c]	3.12[b]	3.63[b]
	15/08	6.74[a]	0.71[b]	0.47[bc]	1.34[a]	1.81[a]
	23/08	6.65[a]	0.78[c]	0.67[c]	2.14[c]	2.80[b]
2003						
Corvus	22/07	6.30	0.45	0.02	0.14	0.16
	28/07	6.64	0.47	0.05	0.36	0.41
	03/08	6.62	0.46	0.04	0.27	0.31
	07/08	6.71	0.45	0.05	0.28	0.33
	12/08	6.48	0.44	0.03	0.25	0.29
Folio	22/07	6.73	0.60	0.05	0.22	0.27
	28/07	6.84	0.56	0.07	0.46	0.54
	03/08	7.22	0.59	0.10	0.63	0.73
	07/08	7.05	0.57	0.09	0.50	0.59
	12/08	6.78	0.59	0.11	0.66	0.76
Meunier	22/07	6.71	0.59	0.02	0.21	0.23
	28/07	6.88	0.64	0.03	0.40	0.43
	03/08	6.70	0.63	0.05	0.41	0.46
	07/08	6.64	0.64	0.05	0.36	0.42
	12/08	6.35	0.60	0.04	0.34	0.38
Average	22/07	6.58[a]	0.55[a]	0.03[a]	0.19[a]	0.22[a]
	28/07	6.79[a]	0.56[a]	0.05[ab]	0.41[b]	0.46[b]
	03/08	6.85[a]	0.56[a]	0.06[b]	0.44[b]	0.50[b]
	07/08	6.80[a]	0.55[a]	0.06[b]	0.38[b]	0.45[b]
	12/08	6.54[a]	0.54[a]	0.06[b]	0.42[b]	0.48[b]
2004						
Corvus	05/08	6.72	0.38	0.03	0.16	0.19
	09/08	6.50	0.39	0.02	0.17	0.19
	18/08	6.90	0.42	0.06	2.11	2.17
	23/08	6.89	0.50	0.14	1.96	2.10
	30/08	6.56	0.52	0.11	1.70	1.81
Folio	05/08	7.07	0.49	0.05	0.23	0.27
	09/08	6.92	0.48	0.04	0.27	0.31
	18/08	7.24	0.57	0.13	1.97	2.09
	23/08	6.94	0.73	0.31	1.62	1.93
	30/08	6.39	0.73	0.37	1.38	1.76
Meunier	05/08	7.09	0.50	0.03	0.20	0.23
	09/08	6.00	0.54	0.04	0.27	0.31
	18/08	6.48	0.69	0.09	3.13	3.21
	23/08	6.25	0.74	0.13	1.85	1.98
	30/08	6.97	0.77	0.33	1.86	2.19
Average	05/08	6.96[a]	0.46[a]	0.04[a]	0.20[a]	0.23[a]
	09/08	6.47[b]	0.47[a]	0.03[a]	0.24[a]	0.27[a]
	18/08	6.87[ab]	0.56[b]	0.09[a]	2.40[b]	2.49[b]
	23/08	6.69[ab]	0.66[c]	0.19[b]	1.81[c]	2.00[bc]
	30/08	6.64[ab]	0.67[c]	0.27[c]	1.65[c]	1.92[c]
All years						
Average	1	6.70[a]	0.53[a]	0.09[a]	0.59[a]	0.67[a]
	2	6.60[a]	0.54[a]	0.11[ab]	0.55[a]	0.66[a]
	3	6.76[a]	0.60[b]	0.22[bc]	1.99[b]	2.21[b]
	4	6.74[a]	0.64[c]	0.24[c]	1.18[c]	1.42[c]
	5	6.61[a]	0.67[c]	0.33[c]	1.40[c]	1.73[bc]

For each year, values with the same letter in the same column are not significantly different from each other.

with 80% ethanol to inactivate the enzymes present in wholemeal, prior to determination of WE-AX levels in extracts of these samples. Although endoxylanase activity levels were reduced drastically, WE-AX levels did not change (results not shown). The results confirmed that the AX degradation by endoxylanases did not occur during sample preparation, but in the field.

3.3.2. Endoxylanase activity levels

The weather conditions at harvest largely affected the wheat endoxylanase activity levels (Table 5). Microbial endoxylanase activity levels showed a similar profile as a function of harvest date for the three varieties in each harvest year. In 2002, activities were the highest at date 3 and 5. In 2004, a rather similar profile for endoxylanase activity was found, with all varieties showing much higher microbial activity levels from the third date onwards. In the 2003 samples, microbial endoxylanase activity levels were very low in all three varieties, again indicating that the weather conditions in that year were unfavourable for wheat pathogens. However, the microbial endoxylanase activity levels increased when harvesting was postponed to a later date. Remarkably, the profile of the microbial endoxylanase activity levels in the different harvest years seemed to correspond with the level of rainfall received some days before (Fig. 1), although the trend is less clear in the 2003 harvest year, in which rainfall amounts and endoxylanase activity levels were extremely low. Endogenous endoxylanase activity levels were also affected by the weather conditions at harvest, but, in contrast to microbial endoxylanase activity levels, these activity levels did not decline with harvest date and their profile more resembled that of the α-amylase activity levels (Fig. 1, Table 5). Total endoxylanase activity levels, which are predominantly determined by microbial endoxylanase activity levels, showed the same trend as the microbial enzymes (Table 5).

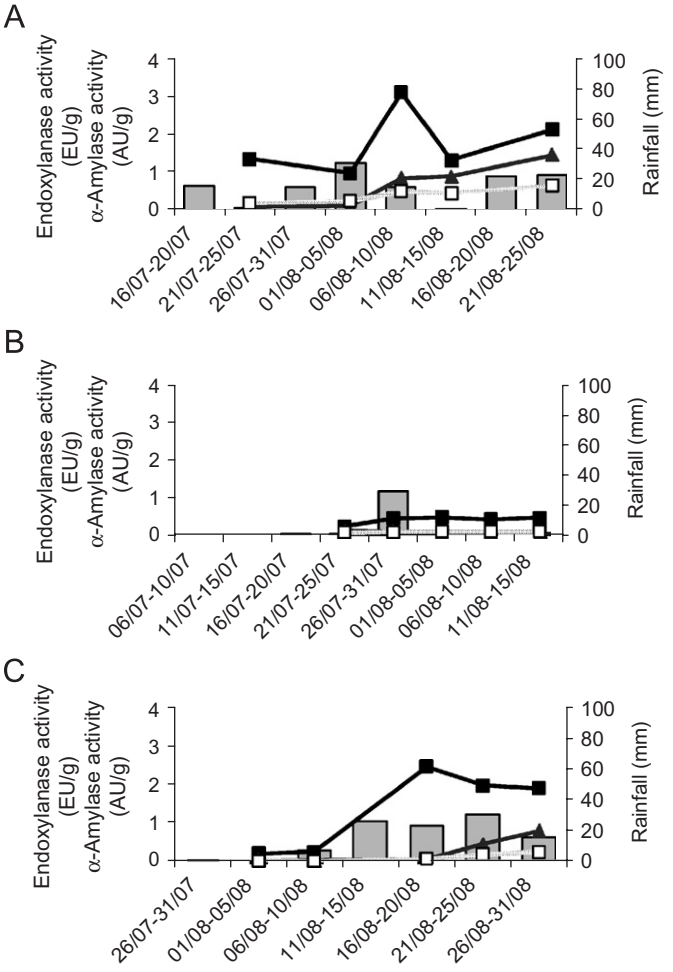

Fig. 1. Average microbial endoxylanase activity levels (EU/g, black squares), endogenous endoxylanase activity levels (EU/g, white squares) and α-amylase activity levels (AU/g, black triangles) for the three wheat varieties and total rainfall data (mm, bars) for July and August 2002 (A), 2003 (B) and 2004 (C).

3.3.3. Endoxylanase inhibitor levels

Weather conditions at harvest had no impact on the inhibitor levels of wheat as already reported by Dornez et al. (2006b) for the 2002 and 2003 harvest years. The same was found for the 2004 harvest year (results not shown). As inhibitor levels are more than 70% determined by genotype and much less by harvest years (Dornez et al., 2007), it seems logical that weather conditions around harvest do not largely affect the inhibitor levels in wheat wholemeal samples.

3.3.4. Partial correlation coefficients

Table 6 lists the partial correlation coefficients between the measured traits for the harvest date trial after elimination of possible year and variety effects. A strong correlation was again found between LN and α-amylase activity levels. Endogenous endoxylanase activity levels were well correlated with α-amylase activity levels and LN, presumably because they were all induced by preharvest sprouting. Microbial endoxylanase activity levels were less well correlated with α-amylase activity levels, LN and endogenous endoxylanase activity levels, possibly due to the fact that endogenous endoxylanases and α-amylase activity levels do not decline as a function of time, while microbial endoxylanase activity levels fluctuate strongly.

WE-AX levels were correlated with both types of endoxylanase activity levels and with α-amylase activity levels and LN. TAXI and XIP levels were not correlated with each other. As both do not change very much with harvest date, this is not surprising. Both types of inhibitor levels however were weakly correlated with specific weight.

3.4. Relevance of the present findings

From this study, it is clear that agronomic factors like fungicide treatment, N-fertilisation and harvest date can have an effect on the levels of WE-AX, endoxylanase activity and endoxylanase inhibitor of wheat. Omitting fungicide treatment or increasing N-fertilisation dosage did not affect AX levels, but did augment microbial

Table 6
Partial correlation coefficients for the harvest date trial for wheat varieties Corvus, Folio and Meunier from harvest years 2002, 2003 and 2004 after elimination of possible year and variety effects

	TKW	Specific weight	Protein	LN	α-Amylase	TOT-AX	WE-AX	Endogenous endoxylanase	Microbial endoxylanase	TAXI	XIP
TKW	–	−0.08	−0.25	0.17	0.18	0.22	0.37*	0.23	0.28	−0.20	−0.20
Specific weight		–	0.26	−0.28	−0.36*	−0.03	−0.34*	−0.29	−0.16	0.51**	0.44**
Protein			–	0.03	−0.11	−0.17	−0.28	0.04	0.04	0.18	0.19
LN				–	0.75***	0.06	0.36*	0.84***	0.34*	0.00	−0.23
α-Amylase					–	−0.01	0.68***	0.86***	0.39*	−0.21	−0.15
TOT-AX						–	−0.07	−0.02	0.01	−0.04	−0.07
WE-AX							–	0.60***	0.58***	−0.28	−0.22
Endogenous endoxylanase								–	0.52**	−0.11	−0.26
Microbial endoxylanase									–	−0.28	−0.22
TAXI										–	0.05
XIP											–

*** P-value < 0.0001.
** P-value < 0.01.
* P-value < 0.05.

endoxylanase activities by a factor of 2 to 2.5. However, this increase was rather small compared to the more than 15-fold difference in microbial endoxylanase activity levels found between wheat varieties. The effect of agronomic factors on endogenous endoxylanases was also limited. Inhibitor levels increased by approximately 15–20% when N-fertilisation was increased. Omitting fungicide treatment also increased XIP levels by approximately 15%, but did not alter TAXI levels. The different response of the two types of endoxylanase inhibitors is interesting from a plant physiological point of view.

Again, these differences in inhibitor levels are rather small compared to the differences found between wheat varieties and harvest years and are presumably not large enough to affect wheat functionality.

Postponing harvest gave higher WE-AX levels, possibly as a result of the accumulation of water unextractable AX (WU-AX) products produced by wheat-associated endoxylanases in the field. It was furthermore clear from the results that the weather conditions at harvest were to a large extent responsible for both endogenous and microbial endoxylanase activity levels in wheat. In 2004, postponing harvest for two weeks caused a 10-fold increase in endoxylanase activity levels. Endoxylanase inhibitor levels, in contrast, were independent of weather conditions at harvest time.

In conclusion we can say that the impact of agronomic factors, such as fungicide treatment and N-fertilisation, on the observed variation in the levels of AX, endoxylanase activities and endoxylanase inhibitors in wheat wholemeal was rather limited compared to the impact of genetic variability and harvest conditions (Dornez et al., 2007).

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