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Effects of genotype, harvest year and genotype-by-harvest year interactions on arabinoxylan, endoxylanase activity and endoxylanase inhibitor levels in wheat kernels

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Abstract

The effects of genotype, harvest year and their interaction on the levels of arabinoxylans (AX), endoxylanases and endoxylanase inhibitors in wheat were studied using 14 varieties grown in three successive growing periods with diverse climatological conditions. Relations with more commonly evaluated wheat characteristics such as yield, thousand kernel weight, specific weight, protein level, Hagberg falling number (HFN) and α-amylase activity level were examined. Water extractable arabinoxylan (WE-AX) levels in wheat varied much more than total arabinoxylan (TOT-AX) levels. This variability was mainly genetically determined, but harvest year also had an important effect. Total endoxylanase activity levels varied more than a factor of 20 between the different wheat samples. Endogenous endoxylanases typically accounted for only 10–15% of this activity, while wheat-associated microbial endoxylanases accounted for the remaining 85–90%. However, when preharvest sprouting occurred, the contribution of endogenous endoxylanases could sometimes amount to over 40% of this total activity. Endogenous endoxylanase activity levels were mainly determined by the interaction of genotype and harvest year, while wheat-associated microbial endoxylanase activity levels were predominantly determined by genotype alone. Endogenous and microbial endoxylanase activity levels were strongly correlated, suggesting that wheat varieties which are susceptible to preharvest sprouting are often also susceptible to microbial contamination. The TAXI and XIP-type endoxylanase inhibitor levels varied by a factor of 8 and 1.8, respectively. They were mainly determined by genotype and were rather similar in the different growing periods.

Keywords: Wheat; Arabinoxylan; Endoxylanase; TAXI; XIP

1. Introduction

It is generally known that wheat yield and functional properties of the derived wheat flour can vary considerably as a result of genetic differences between wheat varieties. However, expression of genetically determined characteristics is often largely affected by environmental conditions. Climatological factors such as temperature, rainfall, and solar radiation exert large effects on the developing wheat

Abbreviations: AU, amylase unit; AX, arabinoxylan; AZCL-AM, azurine-cross-linked-amylose; AZCL-AX, azurine-cross-linked-arabinoxylan; CV, coefficient of variation; E_{590}, extinction at 590 nm; EU, endoxylanase unit; GHF, glycoside hydrolase family; HFN, Hagberg falling number; LN, liquefaction number; TAXI, *Triticum aestivum* xylanase inhibitor; TKW, thousand kernel weight; TLXI, thaumatin-like xylanase inhibitor; TOT-AX, total arabinoxylan; WE-AX, water extractable arabinoxylan; WU-AX, water unextractable arabinoxylan; XIP, xylanase inhibiting protein

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kernels during kernel filling and strongly determine wheat quantity and quality. On one hand, variability in wheat quantity is a major concern for farmers and, therefore, quite a lot of mathematical models relating wheat yield to environmental factors have been developed (Jamieson et al., 1998; Landau et al., 2000). Variability in the end-use quality of wheat on the other hand is one of the biggest challenges for the wheat milling and processing industries. Therefore, considerable effort had gone into investigations of the impact of growing conditions on the rate and duration of kernel filling and on the size and composition of the mature grain. A large volume of research, reviewed by Dupont and Altenbach (2003), has dealt with variations in starch and proteins. However, wheat quality is not only determined by these major constituents. Minor constituents like lipids, enzymes and non-starch polysaccharides such as AX can be of great importance too (Goesaert et al., 2005). AX are the most abundant cell wall non-starch polysaccharides in wheat and are built up of a xylan backbone substituted with arabinose residues and, especially for bran AX, with (4-*O*-methyl-)glucuronic acid residues (Courtin and Delcour, 2002; Schooneveld-Bergmans et al., 1999). They affect wheat functionality in bread making (Courtin and Delcour, 2002), gluten–starch separation (Frederix et al., 2004b), refrigerated doughs (Courtin et al., 2005), and animal feeds (Bedford and Schulze, 1998). In several wheat processing applications, endoxylanases are routinely added to improve processing or product quality. These enzymes hydrolyse the xylan backbone of AX internally and hence strongly impact their molecular weight, water extractability and functional properties (Courtin and Delcour, 2002). Endoxylanases associated with wheat kernels are mainly from microbial origin and to a smaller extent from plant origin (Dornez et al., 2006a). In addition, endoxylanases associated with wheat kernels occur in levels which are high enough to exert significant effects on processing (Dornez et al., 2006a, b). However, to date, only limited data on the variability of endoxylanase activity levels is available (Schmitz et al., 1974; Cleemput et al., 1995; Dornez et al., 2006b). Additionally, as the largest part of the wheat-associated endoxylanases is from microbial origin and is thus inactivated by endoxylanase inhibitors during the aqueous extraction of wheat preceding the activity measurements, the reported values presumably do not reflect the real variability in wheat-associated endoxylanase activity (Dornez et al., 2006a). Indeed, besides AX and endoxylanases, wheat also contains high levels of endoxylanase inhibitors. Such proteins can inactivate microbial endoxylanases and hence largely impact the endoxylanase functionality during wheat processing (Trogh et al., 2004; Frederix et al., 2004a). So far, three types of endoxylanase inhibitors are known: TAXI (*Triticum aestivum* xylanase inhibitor) (Debyser and Delcour, 1998), XIP (xylanase inhibiting protein) (McLauchlan et al., 1999) and TLXI (thaumatin-like xylanase inhibitor) (Fierens et al., 2007).

Despite the importance of AX, endoxylanases and endoxylanase inhibitors in biotechnological applications, very little is known about the variability of these components in wheat and about the contribution of genetic and climatological factors on this variability. However, this knowledge is of importance as wheat components that are highly controlled by genetic factors can be manipulated by the plant breeder, whereas those that are strongly determined by weather conditions cannot. Knowledge of the presence and magnitude of genotype-by-harvest year interactions is also important to the breeders in making decisions regarding the evaluation of newly developed wheat varieties (Mladenov et al., 2001).

Therefore, in this work, we analyzed the existing variability in the levels of the different types of AX, endoxylanases and endoxylanase inhibitors and determined the relative contributions of genotype, harvest year and genotype-by-harvest year interactions to this variability. In addition, correlations between AX, endoxylanase activity and endoxylanase inhibitor levels and wheat characteristics such as yields, thousand kernel weights, specific weights, protein levels, HFN and α-amylase activity levels were analyzed.

2. Materials and methods

2.1. Wheat samples

The wheat samples were grown at an experimental site (Lonzée, Gembloux, Belgium) in three successive growing periods (2001/02, 2002/03 and 2003/04).

The wheat varieties Agami, Apache, Buccaneer, Corvus, Cubus, Deben, Dream, Folio, Koch, Mercury, Meunier, Ordéal, Rialto and Robigus were sown on October 12 in 2001, October 11 in 2002 and/or October 17 in 2003 and harvested on August 15 in 2002, August 3 in 2003 and/or August 23 in 2004. Only six varieties i.e. Agami, Corvus, Dream, Folio, Mercury and Meunier were available in all three growing periods. Each variety 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. Nitrogen 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 and two fungicide treatments were applied. Good weed and insect control was achieved by applying appropriate herbicides and insecticides. Lodging was limited by using chlormequat chloride as growth regulator.

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 purpurogenum* 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. Determination of grain yields, specific weights and thousand kernel weights

Crops were harvested by a special plot combine supplied with a bagging system in the funnel allowing individualisation of the production of each plot. The content of each bag was cleaned with a laboratory thresher (Wintersteiger, Ried, Austria) followed by additional cleaning with a laboratory grain cleaner (Rationel Kornservice, Esbjerg, Denmark). The samples were subsequently stored in hermetically closed buckets. If the moisture of the grain was above 15%, it was first brought back under this norm by drying in a ventilated drying oven (MMM Medcenter Venticell, Munich, Germany). To prepare the samples for analysis, an equal weight of wheat kernels was taken from the four different plots in a randomised way and these kernels were mixed thoroughly.

Grain yield (kg/ha) corresponds to the total mass of grain harvested in each plot corrected to a 15% moisture base. Specific weights were determined with Grain Analysis Computer II (Dickey John, Auburn, USA). The coefficient of variation for the determination of specific weight was typically 0.5%.

Thousand kernel weights (TKW) were determined by counting 20 g of kernels with the Numigral Seed Counter (Tripette et Renaud, Paris, France). The coefficient of variation for the determination of TKW was typically 5%.

2.4. Grinding of wheat kernels

To obtain wholemeal, wheat kernels were ground in a Cyclotec 1093 sample mill (Dornez et al., 2006b). For determination of Hagberg Falling Numbers (HFNs), wheat kernels were ground using a Falling Number 3100 (Perten, Huddinge, Sweden) with a grid of 0.8 mm.

2.5. Determination of protein levels

Protein levels ($N \times 5.7$) of wholemeal samples were determined with the Dumas combustion method (Leco FP 2000, Leco, Garges-les-Gonesse, France) (ICC No 167) and expressed on dry matter basis. The coefficient of variation for the determination of protein level was typically 0.5%.

2.6. Determination of Hagberg falling numbers

HFN were determined according to the AFNOR NF V 03-703 method (AFNOR, 1997). The coefficient of variation for the determination of HFN was typically 5%. As HFN values are known to be reciprocally related with α-amylase activity levels, they were transformed to liquefaction number (LN) values by the equation of Perten (1964) i.e. $LN = 6000/(HFN-50)$. These LN values give a linear estimate of the α-amylase activity and are hence more suitable for correlation analyses.

2.7. Determination of α-amylase activity levels

α-Amylase activity levels in suspensions of wholemeal were determined with the Amylazyme method as described by the manufacturer (Anonymous, 1998) and expressed as α-amylase units (AUs) per gram making use of a reference curve to transform the extinction at 590 nm (E_{590}) in AU like in ICC method No 303. One AU is the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of *p*-nitrophenol from blocked p-nitrophenylmaltoheptaoside in 1 min at 40 °C and pH 5.2. The coefficient of variation for the determination of α-amylase activity levels was typically 3%.

2.8. Determination of arabinoxylan levels

Arabinoxylan levels of wheat wholemeal samples and aqueous extracts thereof were determined by gas–liquid chromatography of alditol acetates as described by Dornez et al. (2006c). AX levels were calculated as 0.88 times the sum of xylose and arabinose levels, after correction of the arabinose level for the presence of arabinogalactan-peptide (Loosveld et al., 1997). The coefficient of variation for the determination of both TOT-AX and WE-AX levels was typically 2%.

2.9. Determination of endoxylanase activity levels

Microbial, endogenous and total endoxylanase activity levels were determined using the washing method described by Dornez et al. (2006a). Intact wheat kernels (100 g) were shaken in threefold (Laboshake, VWR International) with 200 ml of sodium acetate buffer (25 mM, pH 5.0, 0.02% sodium azide) for 17 h. After washing, the liquids were recovered by sieving over a 2.0 mm sieve and used for further analysis. After the washing step, two rinsing steps were executed. In each step, kernels were shaken for 10 min in deionised water (800 ml). The rinsed wheat kernels were frozen with liquid nitrogen and freeze-dried. Endoxylanase activity levels in washing liquids and in extracts of ground washed kernels, prepared as described previously (Dornez et al., 2006a), were determined with the Xylazyme AX method (Megazyme) as described by Dornez et al. (2006b) and expressed in endoxylanase units (EU) per gram. As this analytical method is based on release of solubilised dyed substrates, one EU was defined as the amount of enzyme, present in the sample, needed to yield a ΔE_{590} of 1.0 after one hour incubation, under the

conditions of the assay. Endoxylanase activity levels determined in the washing liquids correspond to the wheat-associated microbial endoxylanase activities, while endoxylanase activity levels measured in extracts of ground washed wheat kernels correspond to endogenous endoxylanase activities. Summation of these activities gives the total endoxylanase activity associated with the wheat kernels. The coefficients of variation for the determination of microbial, endogenous and total endoxylanase activity levels were typically 13%, 23% and 15%, respectively.

2.10. Determination of endoxylanase inhibitor levels

Endoxylanase inhibitor activity levels were determined with the Xylazyme AX method (Megazyme) as described by Dornez et al. (2006b). TAXI and XIP inhibitor activity levels were measured with *Bacillus subtilis* and *Penicillium purpurogenum* endoxylanases, respectively. The coefficient of variation for the determination of both TAXI- and XIP-type inhibitor contents was typically 6%.

2.11. Statistical analyses

Two types of analyses were conducted to investigate the effects of harvest year and genotype. In a first analysis, the emphasis was on detecting significant differences among the tested cultivars and/or harvest years. As such, harvest and cultivar were treated as fixed factors in a two way analysis of variance (Kutner et al., 2004). 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. The aim of the second analysis was to quantify the contributions of harvest year, genotype and/or their interaction to the total variance in arabinoxylan, endoxylanase activity and endoxylanase inhibitor levels. Therefore, harvest, genotype and their interaction were considered as random factors in a random effects model. A likelihood ratio test was used to identify the significant sources of variance (Verbeke and Molenberghs, 2000). Maximum likelihood (ML) was used for model selection, whereas restricted maximum likelihood (REML) was used to estimate the variance components.

To study the linear correlations between all the different parameters, a Multivariate Analysis of Variance (MANOVA) was used. Hereby, harvest year was considered as a discrete covariate. The reported correlations are the partial correlations after correcting for the possible effect of harvest year. Total endoxylanase activity was not included in the correlations, as this parameter is not an experimentally measured value but a calculated one.

All statistical analyses were performed using the Statistical Analysis System software 8.1 (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Characteristics of growing conditions

The weather conditions during kernel filling and harvesting were completely different in the three harvest years. The 2002 harvest year was characterised by an extremely wet summer. The exceptionally high amounts of rain in August following a prolonged warm period in the weeks around harvest were responsible for the occurrence of preharvest sprouting (Ringlund, 1993) and increased disease pressure and loss of wheat quality. The 2003 harvest year, in contrast, had an atypically warm and dry summer. The elevated temperatures hastened kernel maturation. The yields were generally good and no preharvest sprouting was observed. In the 2004 harvest year, the beginning of the summer was cold and wet resulting in prolonged maturation and weak development of ear diseases. Harvest was strongly affected by rainfall giving rise to high humidity in the grains. Comparison between wheat samples from these three completely different harvest years hence allows good estimation of the impact of climatological conditions on the AX, endoxylanase activity and endoxylanase inhibitor levels in wheat.

3.2. Arabinoxylan levels

Table 1 shows the TOT-AX and WE-AX levels of the wheat varieties harvested in 2002, 2003 and 2004. TOT-AX levels varied from 5.81% to 7.56% and were on average 6.62% ± 0.37%, leading to a coefficient of variation (CV) of only 5.6%. There was hence only limited variation in TOT-AX levels. These values are very similar to those found in the literature. Saulnier et al. (1995) reported

Table 1
TOT-AX and WE-AX levels (%) in different wheat varieties harvested in three different years

Wheat variety	TOT-AX (%)			WE-AX (%)		
	2002	2003	2004	2002	2003	2004
Agami	6.96^{ab}	7.19^{a}	7.09^{a}	0.84^{bc}	0.68^{c}	0.65^{b}
Apache	6.40^{b}	6.28^{bc}	—	0.60^{e}	0.54^{ef}	—
Buccaneer	6.71^{ab}	6.12^{c}	—	0.90^{ab}	0.80^{a}	—
Corvus	6.76^{ab}	6.45^{bc}	6.54^{abc}	0.63^{de}	0.51^{f}	0.49^{d}
Cubus	—	—	6.69^{ab}	—	—	0.75^{a}
Deben	—	—	6.56^{abc}	—	—	0.63^{b}
Dream	6.85^{ab}	6.18^{c}	5.81^{c}	0.59^{e}	0.54^{e}	0.42^{e}
Folio	6.58^{ab}	6.96^{ab}	6.57^{abc}	0.74^{cd}	0.71^{b}	0.63^{bc}
Koch	—	—	6.80^{ab}	—	—	0.77^{a}
Mercury	6.58^{ab}	6.25^{bc}	6.80^{ab}	0.64^{de}	0.55^{e}	0.50^{d}
Meunier	6.62^{ab}	6.11^{c}	6.88^{ab}	0.86^{bc}	0.70^{bc}	0.67^{b}
Ordéal	6.82^{ab}	6.71^{abc}	—	0.64^{de}	0.63^{d}	—
Rialto	7.56^{a}	—	—	0.99^{a}	—	—
Robigus	—	—	6.09^{bc}	—	—	0.59^{c}

Values with the same letter in one column are not significantly different from each other.

TOT-AX levels ranging from 5.53% to 7.79% for 22 wheat varieties grown at different locations in France in one harvest year. The six varieties common to the three harvest years were used in the random effects model to calculate the contribution of genotype, harvest year and genotype-by-harvest year interaction to the observed variability. Table 2 shows that genotype explained 18% of the variance in TOT-AX level while harvest year had no influence at all. The interaction between genotype and harvest year accounted for 35% of the variance. A large portion of variation (46%) however could not be explained by our random effects model. No correlations for TOT-AX levels could be found between the different harvest years, which indicates a rather limited genotype effect (Table 3). This is in agreement with the findings of Andersson et al. (1993) who also could not find a relation between the non-starch polysaccharide level of wheat grain and the wheat variety. They found that the differences in non-starch polysaccharide composition in one variety grown at different locations in different years were just as large as the differences found between samples of different varieties. For the 6 varieties common to all harvest years, the average TOT-AX levels in 2002, 2003 and 2004 were 6.72%, 6.52% and 6.62%, respectively (Table 2). In contrast to Saastamoinen et al. (1989) and Coles et al. (1997) who found an increase in rye TOT-AX levels and a decrease in wheat TOT-AX levels, respectively, with increasing water supply, the differences in TOT-AX levels in our study between the harvest years were very small and not significant (Table 2).

WE-AX levels in wheat varied from 0.42% to 0.99% (Table 1). On average, the WE-AX level of wheat was 0.66% ± 0.13%. This again corresponds well with the WE-AX levels for 22 and 19 wheat varieties reported by Saulnier et al. (1995) and Martinant et al. (1999) which ranged from 0.36% to 0.83% and from 0.26% to 0.91%, respectively. The variability between wheat varieties is much higher for WE-AX (CV = 20%) than for TOT-AX levels (CV = 5.6%). In contrast to TOT-AX levels, the WE-AX levels in the different harvest years were strongly correlated, which indicates a large genetic contribution to the variability (Table 3). Indeed, from Table 2, it is clear that genotype accounted for 57% of the variance in WE-AX, while harvest year explained 37%. The interaction between harvest year and wheat variety was negligible, indicating that the magnitude of genotype response did not change in the different harvest years. This offers strong potential for breeding wheat varieties with low WE-AX levels suitable for applications such as animal feeding

Table 2
Ranges and average values per year of the levels of AX (%), endoxylanase activities (EU/g) and endoxylanase inhibitors (ppm) in wheat kernels from the 6 wheat varieties common in all three harvest years and estimations of the relative contributions (%) of harvest year, genotype and their covariation to the total variance (100%)

	Range			Means of harvest years			Explained variance (%)			
	2002	2003	2004	2002	2003	2004	Genotype (G)	Year (Y)	Covariation (G*Y)	Residual
Arabinoxylan										
TOT-AX	6.58–6.96	6.11–7.19	5.81–7.09	6.72[a]	6.52[a]	6.62[a]	18	0	35	46
WE-AX	0.59–0.86	0.51–0.71	0.42–0.67	0.72[a]	0.62[b]	0.56[b]	57	37	5	1
Endoxylanase										
Endogenous	0.18–2.53	0.05–0.27	0.06–0.47	0.70[a]	0.12[b]	0.14[b]	21	19	57	3
Microbial	0.45–3.72	0.24–1.54	0.52–2.78	1.49[a]	0.69[b]	1.17[ab]	63	15	19	3
Total	0.78–6.25	0.29–1.80	0.60–3.25	2.19[a]	0.81[b]	1.31[ab]	52	17	30	1
Inhibitor										
TAXI	28–107	17–126	58–138	81[b]	91[ab]	110[a]	77	16	3	4
XIP	263–331	253–353	193–355	299[a]	303[a]	281[a]	69	3	17	10

Values with the same letter in one row are not significantly different from each other.

Table 3
Coefficients of determination (R^2) between harvest years for AX, endoxylanase and endoxylanase inhibitor levels of the 6 wheat varieties common in the three harvest years

	Harvest years 2002 and 2003	Harvest years 2003 and 2004	Harvest years 2002 and 2004
TOT-AX	0.13	0.01	0.17
WE-AX	0.79	0.95	0.88
Endogenous endoxylanase	0.91	0.99	0.89
Microbial endoxylanase	0.77	0.81	0.91
Total endoxylanase	0.86	0.92	0.91
TAXI	0.96	0.96	0.98
XIP	0.80	0.69	0.70

(Bedford and Schulze, 1998). Saulnier et al. (1995) also suggested that genetic effects are important determinants of WE-AX levels in wheat grain based on similar relative variations found for WE-AX levels in varieties harvested in different years. Martinant et al. (1999) found a high heritability coefficient for WE-AX levels and suggested that this may indicate that only a few major genes are involved in the genetic variation of WE-AX levels. The rainy 2002 harvest year had significantly higher levels of WE-AX than the 2003 and 2004 harvests which were not significantly different from each other. These results are not in complete agreement with those of Grosjean et al. (1999) who found that the effects of precipitation were generally not significant. WE-AX are often thought to be building stones or degradation products of water unextractable AX (WU-AX) (Neukom, 1976; Courtin and Delcour, 2002). Therefore, it would be interesting to see if WE-AX levels relate to the endoxylanase activity levels present in the wheat kernels.

3.3. Endoxylanase activity levels

From previously published data (Dornez et al., 2006b) it is clear that apparent endoxylanase activity levels can vary enormously and that both genotype and climatological conditions are important determinants of this variability. In rye samples, Salmenkallio-Marttila and Hovinen (2005) also reported large differences in apparent endoxylanase activity levels. They observed much higher enzyme activity levels in a cold and rainy season than in a dry harvest season. However, extensive study of the factors that determine endoxylanase variability is lacking and, in addition, to the best of our knowledge, a distinction between variations in endoxylanase activity levels from endogenous and microbial origin has never been made. Keeping in mind that microbial endoxylanases are to large extent inhibited by wheat endogenous endoxylanase inhibitors (Dornez et al., 2006a), it is evident that apparent activity levels do not give sufficient information for wheat processing applications. Therefore, in this study, both types of endoxylanase activity levels were analyzed separately and total endoxylanase activity levels were calculated by summation of endogenous and microbial activity levels.

Table 4 lists the endogenous, microbial and total endoxylanase activity levels of the wheat varieties harvested in 2002, 2003 and 2004. Endogenous and microbial endoxylanase activity levels varied from 0.05 to 2.53 EU/g and from 0.24 to 3.76 EU/g, respectively. Total endoxylanase activity levels (calculated as the sum of endogenous and microbial endoxylanase activity levels) ranged from 0.29 to 6.25 EU/g. The total endoxylanase level of wheat was on average 1.81 EU/g. Endogenous endoxylanases accounted in 2003 and 2004 for approximately 15% and 10%, respectively, of the total endoxylanase activity. In 2002, sprouting led to a higher contribution of the endogenous endoxylanases. On average, endogenous endoxylanases contributed 30% of the total activity. For sprouting sensitive varieties, this contribution could amount to over 40%. The standard deviations of the endoxylanase activity levels of the different wheat samples over all harvest years were extremely high, sometimes even higher than the average values leading to CV of more than 100%. For endogenous, microbial and total endoxylanase activity levels, these standard deviations were 0.57, 1.01 and 1.50 EU/g, respectively, which corresponds to CV of 143%, 72% and 83%. Endogenous, microbial and hence also total endoxylanase activity levels were highly correlated between the different harvest years (Table 3), which indicates a significant effect of genotype.

Endogenous endoxylanase activity can be expected to be higher when sprouting of wheat kernels occurs, as wheat is known to produce this enzyme upon germination (Corder and Henry, 1989; Elliott et al., 2003). In 2002, a long period of dry and warm weather followed by heavy precipitation

Table 4
Endogenous, microbial and total endoxylanase activities (EU/g) in different wheat varieties harvested in three different years

Wheat variety	Endogenous endoxylanase (EU/g)			Microbial endoxylanase (EU/g)			Total endoxylanase (EU/g)		
	2002	2003	2004	2002	2003	2004	2002	2003	2004
Agami	2.53^{a}	0.27^{a}	0.47^{ab}	3.72^{a}	1.54^{a}	2.78^{a}	6.25^{a}	1.80^{a}	3.25^{a}
Apache	0.28^{ef}	0.07^{c}	—	0.81^{cd}	0.42^{ef}	—	1.09^{e}	0.49^{ef}	—
Buccaneer	0.91^{cd}	0.20^{ab}	—	1.84^{b}	1.12^{b}	—	2.75^{c}	1.31^{b}	—
Corvus	0.29^{ef}	0.07^{bc}	0.06^{d}	1.08^{bcd}	0.47^{de}	0.82^{c}	1.36^{de}	0.55^{de}	0.88^{c}
Cubus	—	—	0.09^{d}	—	—	1.19^{c}	—	—	1.28^{c}
Deben	—	—	0.56^{a}	—	—	2.38^{ab}	—	—	2.94^{a}
Dream	0.18^{f}	0.05^{c}	0.06^{d}	1.03^{bcd}	0.24^{f}	0.76^{c}	1.21^{e}	0.29^{f}	0.81^{c}
Folio	0.68^{de}	0.16^{abc}	0.12^{cd}	1.46^{bc}	0.80^{c}	1.04^{c}	2.14^{cd}	0.96^{c}	1.17^{c}
Koch	—	—	0.32^{bc}	—	—	2.81^{a}	—	—	3.13^{a}
Mercury	0.18^{f}	0.09^{bc}	0.08^{d}	1.22^{bcd}	0.44^{e}	0.52^{c}	1.40^{de}	0.52^{e}	0.60^{c}
Meunier	0.33^{ef}	0.09^{bc}	0.07^{d}	0.45^{d}	0.66^{cd}	1.09^{c}	0.78^{e}	0.75^{cd}	1.16^{c}
Ordéal	1.30^{bc}	0.24^{a}	—	3.76^{a}	1.14^{b}	—	5.06^{b}	1.38^{b}	—
Rialto	1.77^{b}	—	—	3.28^{a}	—	—	5.05^{b}	—	—
Robigus	—	—	0.22^{cd}	—	—	1.94^{b}	—	—	2.16^{b}

Values with the same letter in one column are not significantly different from each other.

before harvest caused germination of the kernels in the field and hence high endogenous endoxylanase activities. The weather conditions in 2003 and 2004 were more favourable and little or no sprouting of wheat was observed. A second important factor for sprouting is the sensitivity of the wheat variety. Indeed, some wheat varieties are very resistant while others are very sensitive to sprouting (De Pauw et al., 1993). Resistance to sprouting has often been associated with the intensity of primary dormancy of the wheat variety. Keeping this in mind, it was not surprising that the variance in endogenous endoxylanase activity levels was mainly determined by the covariation between genotype and harvest year (57%). This genotype-by-harvest year interaction can be explained by the fact that not all the wheat varieties showed the same response in the different harvest years. Indeed, both a sensitive wheat variety and unfavourable weather conditions are required to cause sprouting and hence high endogenous endoxylanase activity levels. Genotype and harvest year alone accounted each for approximately 20% of the total variance in endogenous endoxylanase activity levels.

Microbial endoxylanases on wheat kernels are produced by micro-organisms populating the outer wheat kernel layers. The microbial endoxylanase activity levels in wheat were mainly determined by genotype (63%) and to a lesser extent by harvest year (15%) and the covariation between genotype and harvest year (19%) (Table 2). This is not surprising as large differences in susceptibility of wheat varieties to wheat diseases exist (Hardwick et al., 2001; Edwards, 2004). It is hence obvious that the choice of the wheat variety is important if excessive amounts of microbial endoxylanases in wheat need to be avoided. The previously reported relation between the sensitivity to ear diseases and the wheat kernel associated microbial endoxylanase activity levels (Dornez et al., 2006b) was not that evident in the current series of wheat varieties: 4 out of 14 varieties (Deben, Folio, Koch and Robigus) had rather high microbial endoxylanase activity levels, despite their relatively good resistance to ear diseases. Meunier, in contrast, had low endoxylanase activity levels, although this variety is sensitive to ear diseases. However, the latter can possibly be explained by the protective action of the applied fungicide treatments (Dornez et al., 2007).

As total endoxylanase activity levels are calculated by summation of microbial and endogenous endoxylanase activity levels, with the former contributing more than the latter, it is obvious that total endoxylanase activity levels are mainly determined by genotype (52%), followed by the covariation between genotype and harvest year (30%) and harvest year (17%).

3.4. Endoxylanase inhibitor levels

TAXI and XIP levels from harvest years 2002 and 2003 (Dornez et al., 2006b) and of harvest year 2004 (results not shown) ranged from 17 to 138 ppm and from 193 to 355 ppm, respectively. On average wheat contained 97 ± 31 ppm TAXI and 294 ± 39 ppm XIP. The variability of TAXI levels (CV = 32%) is hence higher than the variability found for XIP levels (CV = 13%). XIP levels were about three times higher than TAXI levels, in agreement with earlier published data (Bonnin et al., 2005; Dornez et al., 2006b). TAXI- and XIP-type inhibitor levels were both highly correlated between the different harvest years (Table 3), which indicates a significant effect of genotype. Indeed, from Table 2 it is clear that the largest part of the variability in TAXI (77%) and XIP (69%) inhibitor levels could be attributed to genotype. Harvest year accounted for 16% of the variability in TAXI and 3% of the variability in XIP levels, while the covariation explained 4% of the variability in TAXI and 17% of the variability in XIP levels. The limited variability explained by harvest year is rather surprising. Based on their inhibition specificity, endoxylanase inhibitors are often thought to be involved in plant defence against endoxylanase producing micro-organisms (Bellincampi et al., 2004). In this context, Igawa et al. (2004, 2005) recently found evidence for the induction of both TAXI (*Taxi III* and *Taxi IV*) and XIP-type (*Xip I*) endoxylanase inhibitor genes by plant diseases and wounding in roots and leaves. As the climatological circumstances in 2002 were much more favourable for microbial growth than those in 2003 and 2004, higher inhibitor levels were expected in the former samples due to induction of endoxylanase inhibitor genes by fungal pathogens. However, TAXI and XIP inhibitor levels were both rather similar in the different harvest years. TAXI levels in 2002 were even significantly lower than in 2004, but the difference was small. However, our results are not necessarily in conflict with the results of Igawa et al. (2004, 2005) as the role of endoxylanase inhibitors in plant defence may differ with the plant tissue being infected, the stage of plant development, the type of pathogen or the type of enzyme secreted by the pathogen (Bellincampi et al., 2004).

3.5. Partial correlations between AX, endoxylanase activity and endoxylanase inhibitor levels and other grain characteristics

Our results demonstrate that genetic and environmental conditions both largely determine the WE-AX, endoxylanase activity and endoxylanase inhibitor levels in wheat. However, these parameters are generally not available as selection criteria for wheat end-users in need of an appropriate wheat variety for a specific application. Therefore, it is interesting to know whether the levels of AX, endoxylanase activities and endoxylanase inhibitors are related to more easily available wheat characteristics like yield, TKW, specific weight, protein level, HFN (or LN) and α-amylase activity level. Partial correlation coefficients were calculated between AX, endoxylanases and endoxylanase inhibitors on the one hand and these more commonly evaluated wheat parameters on the other

Table 5
Partial correlation coefficients for all wheat varieties from harvest years 2002, 2003 and 2004 after elimination of year effects

	Yield	TKW	Specific weight	Protein	LN	α-Amylase	TOT-AX	WE-AX	Endogenous endoxylanase	Microbial endoxylanase	TAXI	XIP
Yield	—	0.33	−0.36	−0.74***	0.39	0.38	−0.01	0.02	0.23	0.25	−0.07	−0.28
TKW		—	−0.43*	0.04	0.30	0.38	0.16	0.43*	0.29	0.35	0.41*	0.02
Specific weight			—	0.25	−0.35	−0.35	−0.28	−0.41*	−0.53**	−0.58**	−0.16	0.43*
Protein				—	−0.03	−0.01	0.18	0.18	−0.07	0.04	0.26	0.45*
LN					—	0.82***	0.13	0.15	0.68***	0.52***	0.15	−0.02
α-Amylase						—	0.21	0.08	0.50**	0.44*	0.26	0.03
TOT-AX							—	0.46*	0.40*	0.47*	0.31	−0.02
WE-AX								—	0.46*	0.47*	0.22	−0.07
Endogenous endoxylanase									—	0.83***	0.16	−0.15
Microbial endoxylanase										—	0.12	−0.07
TAXI											—	0.30
XIP												—

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

hand (Table 5). The effect of harvest year was hereby eliminated to avoid obtaining deceiving correlations.

Endogenous endoxylanases were strongly correlated with LN and α-amylase activity levels, suggesting they were produced by the wheat plant during preharvest sprouting. As it is generally known that preharvest sprouting and microbial contamination of wheat go hand in hand if weather conditions are unfavourable, it was not surprising to find strong correlations between microbial endoxylanase activity levels and sprouting related parameters (LN, α-amylase and endogenous endoxylanase activity levels).

Although these correlations could be expected, they were, to the best of our knowledge, not published before. Both endogenous and microbial endoxylanase activity levels seemed to be inversely related to specific weight. This negative correlation has also previously been reported by Salmenkallio-Marttila and Hovinen (2005) for rye samples.

The correlations between endoxylanase activity levels and AX levels were small. No correlations could be found between TAXI- and XIP-type inhibitor levels and between inhibitor and endoxylanase activity levels, confirming earlier observations (Bonnin et al., 2005; Dornez et al., 2006b). The latter indicates that endoxylanase inhibitor genes in wheat kernels are mainly constitutively expressed, and do not depend largely on induction by wheat pathogens.

3.6. Relevance of the present findings

From this study, it is obvious that both genotype and harvest year can markedly affect the WE-AX, endoxylanase and endoxylanase inhibitor composition of wheat. Taking into account the importance of these components in wheat processing, it is possible that these variations will affect the end-use quality of wheat in biotechnological applications such as bread making, especially when wholemeal is used. The levels for total wheat-associated endoxylanase activity, reported in this study, ranged from 0.29 to 6.25 EU/g which is rather similar to the levels of endoxylanase activity often added to wheat flour for bread making applications. Indeed, based on in-house endoxylanase activity measurements on nine different commercial endoxylanase-containing bread improvers, it was demonstrated that the supplier recommended dosages ranged from 0.3 to 17 EU/g flour (Dornez et al., 2006a). It is clear that depending on the substrate selectivity of the wheat-kernel-associated endoxylanases (Courtin et al., 1999; Moers et al., 2003), these activities can either have beneficial or detrimental effects on the end-product quality. In addition, the large variability found for endoxylanase inhibitor levels, especially for the TAXI-type inhibitor levels, can seriously affect the performance of endoxylanases in commercial bread improvers added to wheat flour (Trogh et al., 2004). For example, adding the same dose of *Bacillus subtilis* endoxylanase to wheat flour will have a greater effect on Dream than on Ordéal wheat, as the levels of TAXI in Ordéal can be up to 8-fold higher than in Dream (Dornez et al., 2006b). The *B. subtilis* endoxylanase, which is only inhibited by TAXI and not by XIP, can hence be expected to be inhibited much faster and to a larger degree in Ordéal than in Dream wheat.

As WE-AX, endoxylanase activity and endoxylanase inhibitor levels are to a large extent determined by genotype, selection of appropriate wheat varieties can be an important tool in reducing the problems associated with such variability.

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