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ARTICLE

Variability in Xylanase and Xylanase Inhibition Activities in Different Cereals in the HEALTHGRAIN Diversity Screen and Contribution of Environment and Genotype to This Variability in Common Wheat†

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Endo-1,4-β-D-xylanases (EC 3.2.1.8, xylanases) and xylanase inhibitors, that is, TAXI (*Triticum aestivum* xylanase inhibitor), XIP (xylanase inhibiting protein), and TLXI (thaumatin-like xylanase inhibitor) type xylanase inhibitors, which naturally occur in cereals, are believed to be at the basis of a significant part of the variability in biotechnological functional properties of cereals. Xylanase inhibitors in particular affect grain functionality during processing and in animal feeds when xylanases are used to improve processing parameters and product quality. In the present study the variability of xylanase, TAXI, and XIP activities was quantified in different cereals, including different wheat types [common wheat (*Triticum aestivum* L.), durum wheat (*Triticum durum* Desf.), spelt wheat (*Triticum spelta* L.), einkorn wheat (*Triticum monococcum* L.), and emmer wheat (*Triticum dicoccum* Schübler)], barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and oat (*Avena sativa* L.), and the contribution of genotype and environment to this variability in common wheat was estimated. Substantial differences in xylanase, TAXI, and XIP activities exist between the different cereal types and varieties. Under the experimental conditions of this study, the durum wheat samples show very high xylanase activities compared to the other cereals. High TAXI and XIP activities were measured in, for example, common wheat, spelt wheat, and rye, whereas low activities occur in barley and oat. For wheat, a significant part of the variability in inhibitor levels can be explained by genotype, whereas xylanase activity is most strongly determined by environment. The results obtained suggest that plant breeders and industry to certain extent can select for wheat varieties with high or low xylanase inhibition activities, but the relatively high contribution of the genotype−environment interaction term to the total variability in inhibition activities indicates that TAXI and XIP activities are not very stable breeding parameters.

KEYWORDS: Cereals; wheat; xylanase; inhibitor; TAXI; XIP

INTRODUCTION

Variation in the functionality of cereals is one of the biggest issues in the cereal-processing industry. In this context, several studies have already been done to assess the impact of variety, growing site, climatological conditions, and agronomical inputs on the composition of mature cereal grains. Much research focused on the impact of these parameters on the content and properties of starch and protein [reviewed by Dupont and Altenbach (*1*)]. However, besides these two major compounds, also less abundant constituents such as lipids, nonstarch polysaccharides [e.g., arabinoxylans (AX)], enzymes [e.g., endo-1,4-β-D-xylanases (EC 3.2.1.8, xylanases)], and enzyme inhibitors (e.g., xylanase inhibitors) can have a great effect on cereal functionality (*2*).

AX are abundant cell wall nonstarch polysaccharides in cereals (*3*, *4*) and have a strong influence on the functionality of cereals in, for example, breadmaking (*3*), gluten−starch separation (*5*), and animal feeds (*6*). These polysaccharides are also very important from a nutritional point of view as they belong to the dietary fiber fraction to which several health-promoting effects have been ascribed (*7*). In the cereal-processing industry, commercially available microbial xylanases are frequently used to improve processing parameters and/or product quality (*3*, *8*, *9*). These enzymes hydrolyze the xylan backbone of AX internally and, hence, drastically affect their molecular weight, solubility, and, related herewith, physicochemical properties (*3*). However, xylanases are also naturally associated with cereal grains. Most of these grain-associated xylanases are microbial enzymes that

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mainly occur on the kernel surface, whereas a smaller fraction in sound mature grain is from plant origin that occurs inside the kernels (*10*). Although a large part of the microbial xylanases is inactivated by endogenous proteinaceous xylanase inhibitors during "wet" operations in cereal processing, the high levels at which these enzymes may be present are presumed to affect production parameters and product quality (*11*, *12*). Cereals often contain high levels of xylanase inhibitors, of which three structurally different types with different enzyme specificities are known so far: *Triticum aestivum* xylanase inhibitor (TAXI) (*13*), xylanase-inhibiting protein (XIP) (*14*), and thaumatin-like xylanase inhibitor (TLXI) (*15*). Xylanase inhibitors may affect in particular grain quality parameters for processing and feed applications when xylanases are used to improve handling and end-product quality (*9*).

In the present study, the variability of xylanase and xylanase inhibition activities is determined in different cereals, including different types of wheat [common wheat (*Triticum aestivum* L.), durum wheat (*Triticum durum* Desf.), spelt wheat (*Triticum spelta* L.), einkorn wheat (*Triticum monococcum* L.), and emmer wheat (*Triticum dicoccum* Schübler)] and nonwheat cereals [barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and oat (*Avena sativa* L.)]. Furthermore, the contribution of genotype and environment to this variability in common wheat, that is, winter and spring wheat, is estimated. For the xylanase inhibitors, this study focuses on the TAXI and XIP proteins because these are quantitatively the most important xylanase inhibitors in cereals and are active against broader spectra of xylanases than TLXI. For common wheat, similar work has been done recently, but for a more limited number of varieties (*14*) and environments (6 varieties, 1 location, 3 harvest years) (*16*). This study goes farther by analyzing an extensive sample set (153 varieties) that is much more representative for the entire wheat gene pool available for plant breeders and covers numerous environmental conditions (26 varieties, 4 locations, 3 harvest years). The work described here is part of the HEALTHGRAIN (project supported by the European Commission in the Community Sixth Framework Program) biodiversity screen, which aims at exploring the extent of variation in the levels of dietary fiber, phytochemicals, and bioactive proteins in cereal grains (*17*, *18*).

MATERIALS AND METHODS

Materials. All chemicals and reagents (at least of analytical grade) and bovine serum albumin (BSA) were obtained from a number of sources including Sigma-Aldrich (Buchs, Switzerland), VWR International (Leuven, Belgium), and Acros (Geel, Belgium). Xylazyme-AX substrate tablets were from Megazyme (Bray, Ireland). Glycoside hydrolase family (GHF) 10 *Penicillium purpurogenum* xylanase (NCBI accession AAF-71268) was a kind gift from Prof. J. Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile). GHF 11 *Bacillus subtilis* xylanase (NCBI accession AAA22897) was from Danisco (Brabrand, Denmark).

Cereal Samples. *Cereal Varieties and Growing Trials.* Supplementary Table 1 of the Supporting Information gives an overview of all samples analyzed in this study, the country in which they were grown, and the year of harvest. The sample set includes 133 winter wheat, 20 spring wheat, 10 durum wheat, 5 einkorn wheat, 5 emmer wheat, 5 spelt wheat, 10 barley, 10 rye, and 5 oat varieties (*18*).

Cereals were grown during three successive years as described in detail by Shewry et al. (*17*) and Ward et al. (*18*). In the first year (growing season 2004−2005), all above-cited cereal lines (except for the wheat varieties Crousty and Tiger) were sown in two replicate blocks in a field at the Agricultural Research Institute of the Hungarian Academy of Sciences (Martonvásár, Hungary). Harvest began in 2005 on July 20.

In the second year (growing season 2005−2006), 22 winter wheat and 2 spring wheat lines were selected from the first year sample set as explained by Ward et al. (*18*) and were cultivated again in a field at the Agricultural Research Institute in Martonvásár (Hungary) using a similar plot design and plant treatment. Also, the winter wheat varieties Crousty and Tiger were grown in this second-year trial. The selected wheats included varieties with high and low levels of xylanases and xylanase inhibitors. Harvest began in 2006 on July 18.

For the third-year trial (2006−2007), the same wheat varieties as in the second-year trial were grown in a similar plot design at four different sites across Europe, that is, Clermont Ferrand (France), Saxham (United Kingdom), Choryn (Poland), and Martonvásár (Hungary). The spring wheat varieties Chinese-Spring and Cadenza were not grown at the Polish site. Harvest began early in Hungary (July 5), about one week later in France (July 13), and two weeks later in Poland (July 20). Harvest was carried out late in the United Kingdom after a very rainy period (August 22). Because of the wide variation in weather conditions and locations across Europe in 2007 (*17*), these samples provide an ideal opportunity to study the contribution of genotype, environment, and their interaction to the total variation of xylanase and xylanase inhibition activities in wheat.

Weather Conditions during Cereal Growth. (*a*) *Temperature*. After sowing, the mean temperature in autumn was highest in France in 2006 (11 °C) and lowest in Hungary in 2004 and 2005 (6 °C). The latter temperature was comparable to that in Poland in 2006 (7 °C). For all trials, the coldest periods of the winter were the end of December and the end of January and the beginning of February. On average, the coldest winters occurred in Hungary in the years 2004−2006 (0 to −1 °C). The United Kingdom and France (2006−2007) had the mildest winters (on average 6−7 °C). The average temperature in spring was highest in Hungary in 2007 (16 °C) and lowest in the United Kingdom (12 °C) and Poland (13 °C) in 2007. The temperatures generally increased from April until July. In 2007, June was warmest in Hungary (23 °C) and coldest in the United Kingdom (15 °C).

(*b*) *Precipitation*. During autumn there was a lot of precipitation in Hungary in 2004 and in Poland and the United Kingdom in 2006 (∼17 mm/10 days). The lowest amount of rainfall was measured in Hungary in 2006 (∼6 mm/10 days). In the winter (2006−2007) especially Poland and the United Kingdom dealt with a lot of precipitation (∼20 mm/10 days), whereas the winters in Hungary in 2004−2005 and 2006−2007 and in France in 2006−2007 were relatively dry (∼10 mm/10 days). Spring was very wet in Hungary in 2005 and in the United Kingdom in 2007 (23 mm/10 days). This season was driest in Poland in 2007 (9 mm/10 days). In May 2007, there was a high amount of precipitation in Hungary (27 mm/10 days) and the United Kingdom (42 mm/10 days), whereas June 2007 was rainy in France (27 mm/10 days) and the United Kingdom (37 mm/10 days). The total amounts of precipitation in Hungary and France during the entire growing season (2006−2007) were similar and relatively low (∼315 mm). Comparable and intermediate amounts were noted for Hungary in 2004−2005 and 2005−2006 and for Poland (2006−2007) (∼426 mm). The total precipitation was highest in the United Kingdom (2006−2007) (∼689 mm).

Sample Preparation. *Milling*. Winter, spring, and durum wheat samples were conditioned to 15.5% moisture content, whereas samples of the other wheat types, barley, oat, and rye were conditioned to 14.0% moisture content. Next, the samples were milled with a Chopin CD1 laboratory mill (Chopin Technologies, Villeneuve-la-Garenne, France) to produce white flour, shorts, and bran. The spelt wheat samples were dehulled before milling by using an impact type (centrifugal) dehuller. All samples were stored in sealed plastic bags in the dark at 4 °C until analysis.

Sample Extraction. Flour (2.0 g) and bran samples (1.0 g) were extracted for 30 min at 7 °C in sodium acetate buffer (100 mM, pH 5.0, 20 mL) on a horizontal shaker (150 strokes/min). The supernatants were recuperated after centrifugation (10000*g*, 7 °C, 10 min), decantation, and filtration over MN 615 filter paper (Macherey-Nagel, Düren, Germany).

Determination of Xylanase Activity. *Apparent Xylanase Activity*. Apparent xylanase activities were determined with the Xylazyme-AX method (Megazyme). Flour or bran extracts or buffer (blank) was equilibrated for 10 min at 40 °C before the addition of a Xylazyme-AX substrate tablet. The samples were incubated further at 40 °C for up to 16 h. The extracts were appropriately diluted to ensure under these experimental conditions a linear relationship between the activity measured and the amount of xylanase(s) present. The reaction was stopped by adding

1% Trizma base solution (10.0 mL) and vigorous vortex stirring. After 10 min at room temperature, the solutions were filtered through MN 615 filter paper (Macherey-Nagel) and the extinction values at 590 nm (E_{590}) (Ultraspec III UV−visible spectrophotometer, Pharmacia Biotech, Uppsala, Sweden) were measured against a control, prepared by incubating the extracts or buffer (blank) without the Xylazyme-AX tablet. Correction was made for nonenzymic color release by the tablets. Activities are expressed here in xylanase units (XU). One XU corresponds to the amount of enzyme needed to increase the E_{590} by 1.0 per hour of incubation under the conditions of the assay. All measurements were performed in duplicate. The experimental error was typically below 3% deviation of the mean value. The assay described here provides apparent activities because of the presence of xylanase inhibitors in the extracts. During aqueous extraction, part of the cereal-associated xylanase is inactivated by complexation with xylanase inhibitors and is not measured in the assay.

Endogenous Xylanase Activity. To estimate the activity of plant xylanases in cereal grain, the kernels were first surface treated as described by Dornez et al. (*10*, *16*). Thus, intact kernels (20 g) were shaken for 15 min at room temperature with a sodium hypochlorite solution (40 mL, 3.0% active chlorine). After removal of the sodium hypochlorite, the kernels were rinsed twice with deionized water (200 mL, 10 min) and freeze-dried. The dry samples were ground on a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). Subsequently, xylanase activity was measured in whole meal as described above.

Determination of Apparent Xylanase Inhibition Activity. TAXI and XIP activities were measured with a variant of the Xylazyme-AX method as described by Dornez et al. (*19*). The measurement is based on the specificity of the xylanases used. On the one hand, TAXI activities were measured using a GHF 11 *B. subtilis* xylanase. This xylanase is inhibited only by TAXI proteins. On the other hand, XIP activities were measured using a GHF 10 *P. purpurogenum* xylanase, as GHF 10 xylanases are inhibited only by XIP proteins. So far, no enzyme has been identified that is solely inhibited by TLXI.

All enzyme solutions were prepared in sodium acetate buffer (100 mM, pH 5.0) containing BSA (0.5 g/L). The enzyme concentrations used resulted in an increase of E_{590} by 1.0 in the assay below when performed in the absence of inhibitor. The flour and bran extracts were diluted to ensure a linear response between the amount of inhibitor and the inhibition activity measured. Xylanase solutions (0.5 mL) were preincubated for 30 min at 30 °C with an equal volume of buffer (uninhibited reference) or diluted extract (inhibited sample) to allow formation of enzyme−inhibitor complexes in the latter case. After preincubation, Xylazyme-AX substrate tablets were added, and the samples were further incubated at 30 °C for 60 min, after which the reaction was stopped by adding 1% Trizma base solution (10.0 mL) and vortex mixing. After 10 min at room temperature, the tubes were shaken vigorously and the contents were filtered (MN 615 filter, Macherey-Nagel). The E_{590} values of the uninhibited reference and the inhibited samples were measured against controls, prepared by incubating the reference and sample with buffer instead of enzyme solution. Both controls allowed correction for nonenzymic color release; the second one additionally allowed correction for xylanase activity present in the flour and bran extracts. Xylanase inhibition activity is expressed here in inhibition units (IU). One IU corresponds to the amount of xylanase inhibitor needed to inhibit xylanase activity by 50% in the above-described assay. The coefficients of variation on the measurements of TAXI and XIP activities were typically 6%. This assay provides apparent xylanase inhibition activities because a fraction of the xylanase inhibitor complexes with cereal-associated xylanases during sample extraction and is not measured.

Statistical Analysis. A one-way ANOVA was performed to assess the effect of cereal type on xylanase, TAXI, and XIP activities in flour and bran. A Tukey test with a 5% family significance level was used to evaluate significant differences.

Two types of analyses were conducted to investigate the impact of environment (including impact of harvest year and growing site) and genotype on the variability of xylanase, TAXI, and XIP activities in flour and bran of common wheat. In a first analysis, the emphasis was on detecting significant differences between environments. This was done using a one-way ANOVA as described above. The aim of the second analysis was to quantify the contributions of environment, genotype, and their interaction to the total variance in xylanase and xylanase inhibition activities. Therefore, environment, 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 (*20*). Maximum likelihood was used for model selection, whereas restricted maximum likelihood was used to estimate the variance components.

To study the linear correlations between different parameters, a multivariate analysis of variance was performed. Hereby, environment was considered as a discrete covariate. The reported correlations are the partial correlations after correcting for the possible effect of environment.

All statistical analyses were performed using the Statistical Analysis System Software 9.2 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

The range of variation of xylanase and xylanase inhibition activities in different wheat types, barley, rye, and oat was assessed by analyzing an elaborate sample set. Some chemical and technical characteristics of the cereal samples analyzed here are discussed below. The climatological differences and/or the various soil properties between the growing trials (*17*) allowed estimation of the effect of genotype and environment on these activities in common wheat.

Evaluation of Cereal Samples. An overview of all samples analyzed in this study, the country in which they were grown, and the year of harvest is provided in Supplementary Table 1 of the Supporting Information. The wheat and nonwheat cereals harvested in 2005 in Hungary have already been studied earlier in terms of variability in the content of total dietary fiber and fiber components and phytochemicals (*17*, *18*). The traditional quality traits (protein, starch, amylose, free sugar, lipid, ash, and gluten contents, kernel hardness, flour yield, bran yield, Zeleny sedimentation, thousand kernel weight, and agronomical properties) of the common wheat samples of that harvest were discussed by Rakszegi et al. (*21*). The variability of the above-cited parameters for the wheat samples harvested in 2006 (Hungary) and in 2007 (Hungary, Poland, France, and United Kingdom) and the heritability of these parameters are discussed by others in this special issue of *Journal of Agricultural and Food Chemistry* (*17*). **Table 1** gives a concise overview of the thousand kernel weights, the total AX (TOTAX) and water-extractable AX (WEAX) levels in flour and bran, the protein contents in flour and whole meal, and the flour and bran yields for the wheat, barley, oat, and rye samples analyzed here (data are extracted from refs *21*−*26*).

A large variation in thousand kernel weight exists between different varieties, whereas the average thousand kernel weights of the different cereals are relatively similar. The mean TOTAX levels in the flour samples of different wheat types and barley are relatively comparable. Lower and higher TOTAX levels were measured in oat and rye flour, respectively. On average, the common wheat bran samples contain the highest TOTAX levels, whereas the lowest levels were noted for emmer wheat, barley, and oat bran. Rye flour and bran are the richest in WEAX. The lowest WEAX levels were found in emmer wheat, barley, and oat flour and bran. The protein levels in rye are low compared to the other cereals, especially for rye flour. The flour yields differ considerably for the different cereals studied. With the milling conditions used, the spelt and einkorn wheat samples have on average the highest flour yields, whereas the durum wheat, emmer wheat, and barley samples yield the lowest amount of flour. Barley, oat, and rye milling gave high bran yields under the conditions used, whereas the average bran yields of the different wheat types are more or less comparable.

Total Variability of Xylanase and Xylanase Inhibition Activities. An overview of the total variability in xylanase, TAXI, and XIP activities in flour and bran of the analyzed wheat and nonwheat cereals is provided in **Figure 1**. For each of these compounds

Table 1. Ranges and Average Values of Thousand Kernel Weights, TOTAX and WEAX Levels in Flour and Bran, Protein Contents in Flour and Whole Meal, and Flour and Bran Yields for Different Types of Wheat, Barley, Oat, and Rye

	ranges								
	thousand kernel weight (g)	TOTAX flour (% of dm)	TOTAX bran (% of dm)	WEAX flour (% of dm)	WEAX bran (% of dm)	protein flour (% of dm)	protein whole meal (% of dm)	flour yield (%)	bran yield (%)
winter wheat	27.0−60.0	1.31−2.74	13.2−22.6	0.24−1.38	0.27−0.92	9.4−19.0	11.4−19.9	30.2−65.8	20.0−36.4
spring wheat	27.1−52.7	1.64−2.73	12.1−19.2	0.29−0.77	0.28−0.57	12.1−19.0	14.1−19.2	27.7−60.3	19.9−42.4
durum wheat	34.8−48.5	1.72−2.34	10.9−13.7	0.26−0.57	0.32−0.55	11.9−15.6	14.1−16.9	20.6−53.8	24.2−30.9
spelt wheat	nd[a]	1.59−2.16	11.1−13.9	0.29−0.43	0.28−0.35	15.0−17.3	16.8−19.2	52.2−67.4	25.5−28.1
einkorn wheat	nd	1.47−2.34	9.5−10.4	0.48−0.67	0.46−0.65	14.7−19.7	16.2−20.7	60.2−66.7	21.1−26.8
emmer wheat	nd	1.42−1.94	6.1−14.4	0.17−0.54	0.20−0.45	13.8−18.5	16.1−18.5	19.5−68.5	20.8−23.9
barley	31.4−53.0	1.40−2.24	4.8−9.8	0.15−0.38	0.15−0.35	9.7−14.1	14.2−17.8	18.2−44.1	37.7−63.1
oat	nd	0.97−1.26	3.8 −13.2	0.15−0.18	0.19−0.21	11.7−13.4	12.6−17.7	35.3−42.8	46.2−51.9
rye	28.0−44.0	2.65−4.31	11.3−15.6	1.05−1.61	0.78−1.53	5.4−9.4	9.0−15.7	32.5−48.1	25.9−42.9
	averages								
	thousand kernel weight (g)	TOTAX flour (% of dm)	TOTAX bran (% of dm)	WEAX flour (% of dm)	WEAX bran (% of dm)	protein flour (% of dm)	protein whole meal (% of dm)	flour yield (%)	bran yield (%)
winter wheat	41.4	1.95	17.8	0.53	0.43	14.0	15.3	50.0	24.6
spring wheat	37.7	2.05	16.1	0.51	0.41	15.2	16.4	47.4	25.7
durum wheat	42.2	1.96	12.0	0.42	0.42	14.2	15.6	30.4	27.1
spelt wheat	nd	1.76	12.7	0.34	0.32	15.8	17.6	60.7	26.7
einkorn wheat	nd	1.94	10.0	0.58	0.54	17.4	18.3	64.2	23.9
emmer wheat	nd	1.72	8.9	0.25	0.28	15.9	16.8	31.5	22.3
barley	41.0	1.91	7.5	0.25	0.25	11.8	16.2	30.7	45.6
oat	nd	1.11	9.1	0.17	0.20	12.3	15.6	39.0	49.2
rye	37.3	3.19	12.9	1.28	1.14	6.9	11.8	40.6	37.1

[a] Not determined.

significant differences were observed between as well as within the different types of cereals. An elaborate list of all data is provided in Supplementary Table 1 of the Supporting Information.

Xylanase. In general, higher xylanase activities were measured in bran than in flour. This was also observed for wheat by Bonnin et al. (*27*), Dornez et al. (*28*), and Gys et al. (*29*), who attributed most of the xylanase activity in nonsprouted grains to xylanases from micro-organisms present on the outer grain tissues. The durum wheat samples contain unexpectedly high activities, even more then the hulled cereals, that is, barley and oat, which generally are known to have higher xylanase activities than hulless cereals, partly because of the micro-organisms that thrive between the husk and caryopsis (*30*). The spelt wheat samples were dehulled before milling, and the resulting flour and bran fractions do not contain high xylanase activities (**Figure 1A**,**B**).

The high xylanase activities in the durum wheat samples are probably mainly the result of preharvest sprouting. To support this supposition, the contribution of plant xylanases to the total xylanase activities in the durum wheat samples was estimated using the method described by Dornez et al. (*10*, *16*), according to which microbial xylanases are selectively inactivated by surface treating the intact grains with household bleach. This way, up to 70% (varieties Creso and MV-Makaroni) of the xylanase activity in the durum wheat samples can be explained by the occurrence of endogenous xylanases, which are synthesized in the grain. Dornez et al. (*16*) reported that up to 40% of the total xylanase activity in a set sprouted common wheat samples originated from endogenous xylanases. The presence of endogenous xylanases was further confirmed by immunoblot analysis of durum wheat whole meal extracts using the polyclonal antibodies produced by Debacker et al. (*31*) against recombinant barley xylanase X-1 (results not shown). These antibodies cross-react with xylanase X-1 homologues from outer cereals and not with exogenous xylanases (*31*).

TAXI. On average, the highest TAXI activities occur in rye, einkorn wheat, and common wheat. For barley and oat, very low, or even no, TAXI activity was detected in flour and bran, which is in line with the earlier reported low level and absence of TAXI in barley and oat whole meal, respectively (*32*). For rye and common, spelt, einkorn, and emmer wheat much higher TAXI activities were measured in bran than in flour. In the case of durum wheat, the activities in bran and flour are comparable (**Figure 1C**,**D**).

XIP. The lowest XIP activities occur in barley flour and bran, whereas the highest activities were noted for common and spelt wheat. Earlier studies also report very low XIP levels in barley whole meal (*32*). As observed for TAXI, for most of the cereals studied here higher XIP activities were measured in bran than in flour. For durum wheat, in analogy to TAXI, the XIP activities are comparable in flour and bran (**Figure 1E**,**F**).

The presence of higher xylanase inhibitor levels in the outer grain tissues than in the endosperm has been demonstrated earlier for wheat (*28*, *33*). Croes et al. (*33*) showed that the highest concentration of TAXI and XIP occurs in the aleurone, whereas the overall inhibitor concentration is lowest in the outer pericarp. Although wheat flour has low TAXI and XIP levels, it contains about half of the total amount of these inhibitors present in wheat grain (*33*).

Environment and Genotype Effects on Xylanase and Xylanase Inhibition Activities in Common Wheat. Comparison of the xylanase and xylanase inhibition activities in wheat samples from different locations and years provides insight into the impact of genotype and environment thereon. The data for the 24 wheat varieties common to all harvests were used in a random effects model to estimate the contribution of genotype, environment, and genotype-by-environment interaction to the observed variability of xylanase and xylanase inhibition activities.

High TAXI and XIP activities were observed for the wheat samples harvested in Hungary in 2007. These samples are also characterized by very low xylanase activities. The bran of the Polish wheat samples and the Hungarian samples harvested in 2006 contains XIP levels similar to that of the Hungarian bran samples of 2007 (**Figure 2**). The growing season of 2006−2007 in

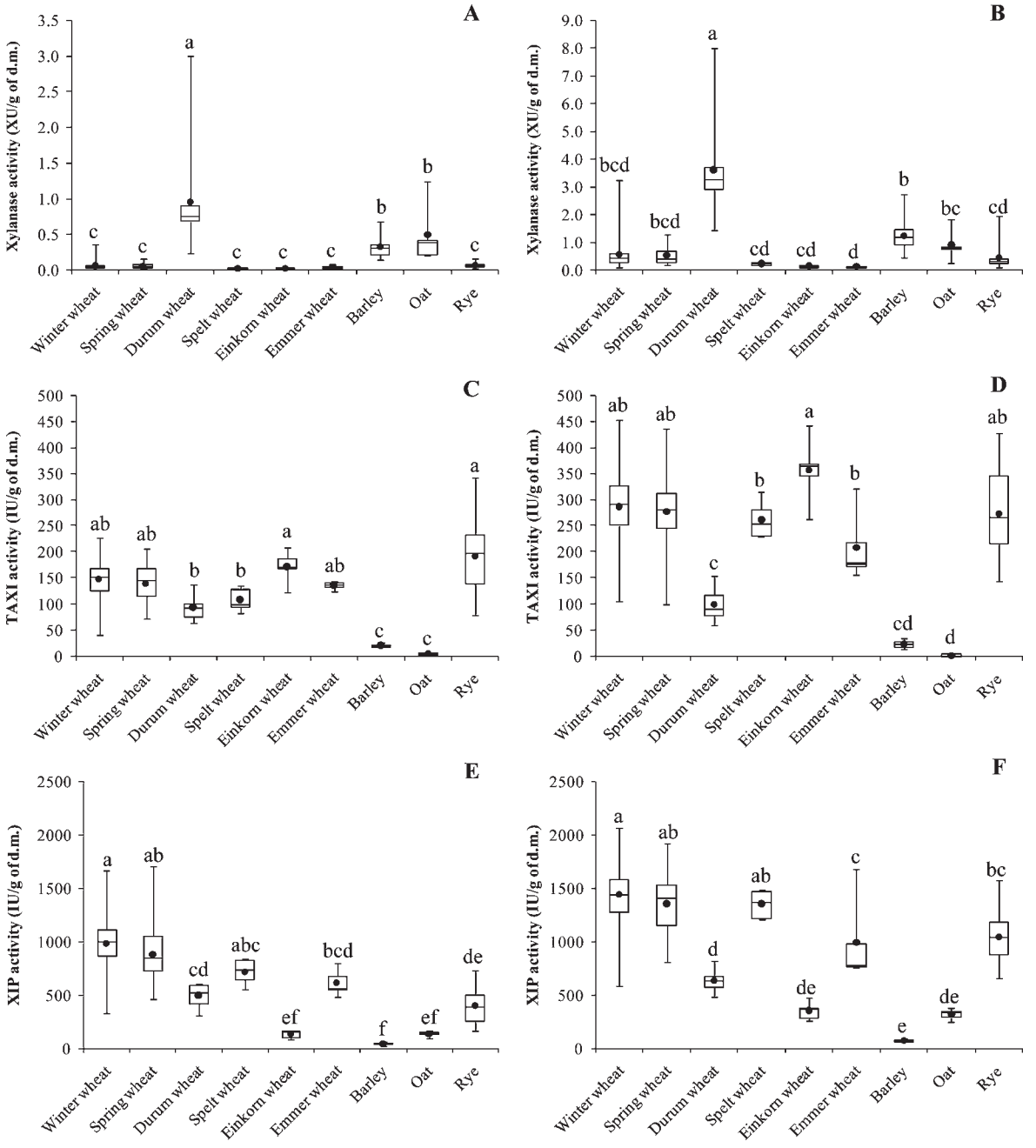

Figure 1. Box plot representation of the total variation in xylanase (**A**, **B**), TAXI (**C**, **D**), and XIP (**E**, **F**) activities in flour (**A**, **C**, **E**) and bran (**B**, **D**, **F**) of different cereals. The medians are indicated by horizontal lines in the boxes and the average values by black dots. Averages indicated by the same letter are not significantly different.

Hungary was generally the driest of all growing seasons and had a warm spring and summer (see above). The wheat samples harvested in France in 2007 have on average low inhibition activities. For the French flour samples the lowest TAXI and XIP activities were noted, whereas the inhibition activities in the bran are comparable to those in bran samples of wheat from other field trials. The French samples also contain low xylanase activities (**Figure 2**). The growing season in France in 2006−2007 had a relatively warm autumn, a mild winter, and intermediate temperatures in spring and summer. Except for the dry winter, an intermediate amount of precipitation was noted for the French growing site (see above). The flour and bran samples from the United Kingdom can be distinguished from the other ones by the occurrence of high xylanase activities, although the activities are on average much lower than those in the durum wheat samples discussed above. Also, the Hungarian flour samples of the 2006 harvest are high in xylanase activity. Although the inhibition activities in the U.K. flour samples are intermediate to high, those in the corresponding bran samples are on average the lowest compared to those in the bran samples of the other field trials (**Figure 2**). The 2006−2007 growing season in the United Kingdom had a mild winter and a cold and wet spring and summer. It had the highest total amount of precipitation. These weather conditions favor microbial growth and preharvest sprouting, which may both result in higher xylanase activities (*16*, *34*). Earlier work showed that in sound common wheat grain endogenous xylanases generally account for no more than 15% of the total xylanase activity, whereas when preharvest sprouting has occurred, their contribution can go up to 40% (*16*). As discussed above, the contribution of endogenous

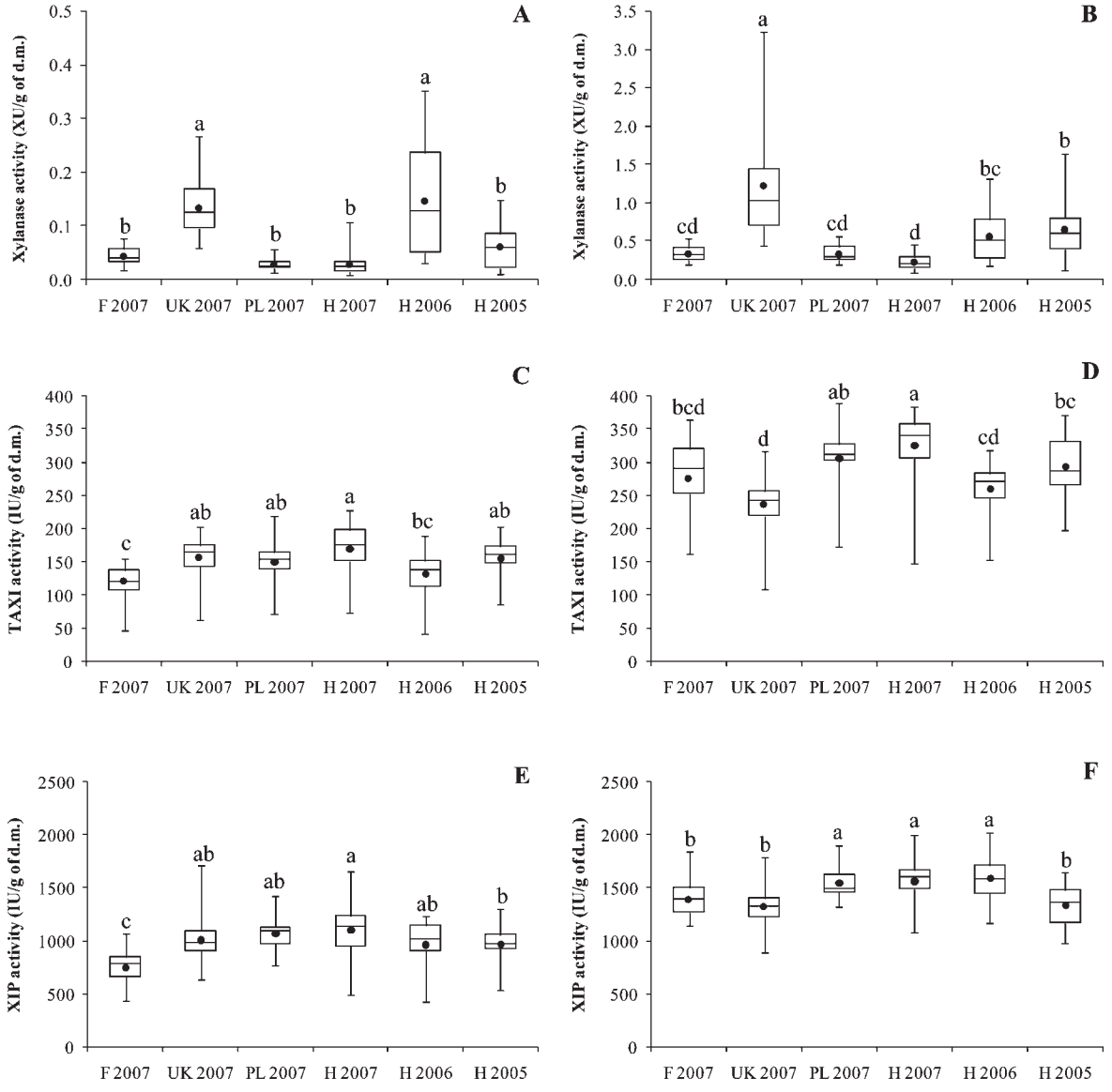

Figure 2. Box plot representation of the variation in xylanase (**A**, **B**), TAXI (**C**, **D**), and XIP (**E**, **F**) activities in flour (**A**, **C**, **E**) and bran (**B**, **D**, **F**) of common wheat grown at different locations across Europe (France, F; United Kingdom, UK; Poland, PL; Hungary, H) and harvested in different years (2005, 2006, 2007). The medians are indicated by horizontal lines in the boxes and the average values by black dots. Averages indicated by the same letter are not significantly different.

xylanases to the total activity in sprouted durum wheat can even go up to 70%.

About half of the variability in xylanase activity in the present sample set can be related to environmental factors and only 11−14% to genotype (**Figure 3**), which is the opposite of the results of Dornez et al. (*16*), who ascribed 52 and 17% of the variation in total xylanase activity to genetic and environmental factors, respectively. The interaction between genotype and environment accounts for approximately one-third of the variation in xylanase activity (**Figure 3**). Only 31−47% of the observed variation in xylanase inhibition activity can be explained by differences in genotype, whereas 22−29 and 29−39% of the variation can be attributed to variations in environment (growing site, year-to-year difference, agricultural inputs) and the interaction between environment and genotype, respectively (**Figure 3**). The portion explained by genotype is much less than described earlier by Dornez et al. (*16*), that is, 69−77%. The differences between the results discussed here and those reported by Dornez et al. (*16*) might be explained by a higher diversity of environments in which the samples were grown for the present study and the fact that here milling fractions were analyzed instead of whole meal. Indeed, the environments in the study of Dornez et al. (*16*) differ only in weather conditions, whereas those in this study differ in weather conditions and growing location (see above).

The relatively high contribution of the genotype−environment interaction term to the total variability in xylanase and xylanase inhibition activities observed here indicates that xylanase, TAXI, and XIP activities are not very stable breeding parameters. However, the levels of these enzymes and inhibitors are to a significant extent determined by heritance as some varieties tend to have consistently high and others consistently low levels of these proteins. A tendency toward high xylanase activities in flour and bran can be observed for the varieties Lynx, Rialto, and Riband. Earlier, Dornez et al. (*16*) also found relatively high xylanase activities in Rialto. This is in contrast to Atlas-66, which often contains low xylanase activities in both fractions. For most of the growing trials, the varieties Spartanka and Gloria contain low xylanase activities in flour and bran, respectively (**Table 2**). The variety Valoris often contains high TAXI levels in flour and

bran, and the variety Rialto always contains high TAXI levels in the bran for the field trials analyzed here. The varieties San-Pastore, Crousty, and Spartanka tend to have low TAXI levels in flour and bran. The Altas-66 flour samples from all six field trials have low TAXI levels (**Table 2**), whereas for only one growing trial has a low TAXI level been measured in the bran of this variety. Chinese-Spring and San-Pastore tend to have low XIP levels in flour and bran, whereas flour and bran of the variety Disponent often have high XIP levels (**Table 2**).

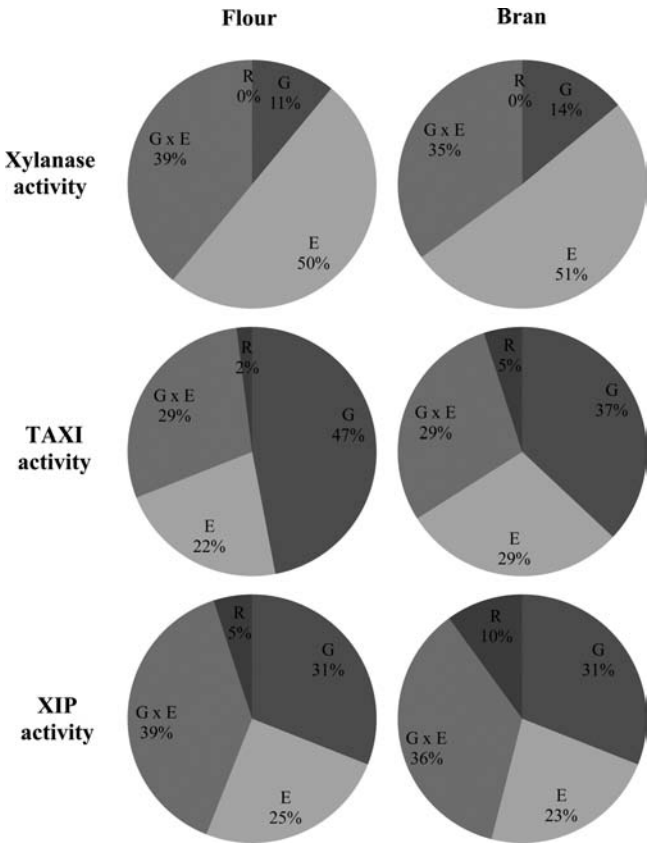

Figure 3. Contribution (%) of genotype (G), environment (E) and the interaction between G and E (G × E) to the total variability in xylanase, TAXI, and XIP activities in flour and bran of common wheat. The residual variability (R) that cannot be explained by G, E, or G × E is also presented.

Correlations between Xylanase Activity, Xylanase Inhibition Activity, AX Levels, and Other Parameters. Table 3 summarizes the results of the correlation analysis of the data on xylanase and xylanase inhibition activities and other parameters (TOTAX and WEAX contents in flour and bran, protein contents in flour and whole meal, and flour and bran yields) for common wheat with elimination of environmental effects.

When high xylanase activity was measured in the flour typically also high xylanase activity was observed in the corresponding bran. The xylanase activities in flour and/or bran are positively correlated with the WEAX contents in flour and bran, suggesting enzymic AX degradation in planta. WEAX levels in flour and bran are positively correlated.

In flour and, to a lower extent, in bran, TAXI and XIP activities are positively correlated, suggesting that wheat varieties with high TAXI levels also tend to have high XIP levels. This is in contrast to what can be expected on the basis of earlier studies, in which no such correlation between TAXI and XIP levels in whole meal of common wheat varieties was observed (*16*, *33*, *35*). More drastic differences in climatological and agronomical parameters between the different years and growing sites resulting in higher variabilities in TAXI and XIP activities and/or additional variation in TAXI and XIP activities in flour and bran introduced by milling may be at the basis of this discrepancy. Because TAXI and XIP type xylanase inhibitors are similarly distributed over the wheat kernel tissues (*33*), differences in milling performance may contribute to the covariation of both inhibitors in the resulting milling fractions. Also worth mentioning is that TAXI and XIP levels in flour are positively correlated with their respective levels in bran.

Relevance of the Present Findings. The present study demonstrates considerable variations in xylanase, TAXI, and XIP activities in flour and bran of different cereals and varieties. Variation in the level of these compounds may contribute to

Table 2. Common Wheat Varieties and Their Frequency of Occurrence in the Top Five and Bottom Five of the Lists of Varieties Ordered from High to Low Activities of Xylanase, TAXI, and XIP in Flour and Bran[a]

	xylanase flour		xylanase bran		TAXI flour		TAXI bran		XIP flour		XIP bran	
	variety	frequency	variety	frequency	variety	frequency	variety	frequency	variety	frequency	variety	frequency
top 5	Lynx	5/6	Lynx	6/6	Maris-Huntsman	4/6	Rialto	6/6	Disponent	4/6	Disponent	5/6
	Rialto	5/6	Riband	5/6	CF99105	3/6	Valoris	5/6	Lynx	4/6	Tremie	4/6
	Riband	5/6	Maris-Huntsman	3/6	Lynx	3/6	Campari	4/6	Tiger	3/5	Campari	3/6
	Campari	4/6	Rialto	3/6	Obriy	3/6	CF99105	3/6	Campari	3/6	CF99105	3/6
	Malacca	3/6	Campari	2/6	Tremie	3/6	Riband	3/6	Obriy	3/6	Tiger	2/5
	Isengrain	2/6	Estica	2/6	Valoris	3/6			Tremie	3/6	Atlas-66	2/6
	Maris-Huntsman	2/6	Herzog	2/6	Estica	2/6			Maris-Huntsman	2/6	Avalon	2/6
					Tommi	2/6			Tommi	2/6	Rialto	2/6
											Tommi	2/6
bottom 5	Spartanka	5/6	Gloria	4/6	Atlas-66	6/6	Crousty	5/5	San-Pastore	6/6	Estica	5/6
	Atlas-66	4/6	Tommi	4/6	San-Pastore	6/6	Spartanka	5/6	Chinese-Spring	4/5	Chinese-Spring	4/5
	Obriy	4/6	Atlas-66	3/6	Crousty	5/5	Estica	4/6	Gloria	4/6	Claire	4/6
	Chinese-Spring	2/5	San-Pastore	3/6	Gloria	4/6	Tiger	3/5	Atlas-66	3/6	San-Pastore	4/6
	Crousty	2/5	Cadenza	2/5	Spartanka	4/6	Gloria	3/6	MV Emese	3/6	Cadenza	3/5
	Gloria	2/6	Claire	2/6	Herzog	2/6	San-Pastore	3/6	Crousty	2/5	Malacca	3/6
	MV Emese	2/6	Disponent	2/6							Isengrain	2/6
	San-Pastore	2/6	MV Emese	2/6								
	Tommi	2/6	Tremie	2/6								

[a] Frequencies are expressed as (times of occurrence of a variety in the top or bottom 5)/(number of field trials in which the variety has been analyzed). Only varieties with frequencies of 2/6 or higher are presented.

Table 3. Partial Correlation Coefficients[a] and *P* Values (Italic) for Different Parameters and for All Common Wheat Samples from Different Harvest Years and Locations after Elimination of Environmental Effects

	TAXI flour	XIP flour	xylanase flour	TOTAX flour	WEAX flour	protein flour	flour yield	TAXI bran	XIP bran	xylanase bran	TOTAX bran	WEAX bran	bran yield	protein whole meal
TAXI flour		**0.42**	**0.21**	**0.30**	0.18	0.01	−0.17	**0.70**	0.14	0.08	−0.06	**0.28**	−0.11	−0.05
		<0.001	*<0.001*	*<0.001*	*<0.01*	*0.90*	*<0.01*	*<0.001*	*<0.05*	*0.21*	*0.32*	*<0.001*	*0.07*	*0.39*
XIP flour			**0.20**	**0.23**	0.11	**0.27**	**−0.27**	0.17	**0.40**	0.07	0.05	**0.20**	−0.17	**0.21**
			0.001	*<0.001*	*0.06*	*<0.001*	*<0.001*	*<0.01*	*<0.001*	*0.22*	*0.44*	*<0.001*	*<0.01*	*<0.001*
xylanase flour				**0.25**	**0.30**	−0.12	−0.12	0.14	0.04	**0.60**	−0.08	**0.47**	−0.01	−0.18
				<0.001	*<0.001*	*0.05*	*<0.05*	*<0.05*	*0.54*	*<0.001*	*0.18*	*<0.001*	*0.91*	*<0.01*
TOTAX flour					**0.59**	**0.20**	**−0.55**	0.19	0.13	0.11	−0.06	**0.57**	0.01	0.03
					<0.001	*<0.001*	*<0.001*	*<0.01*	*<0.05*	*0.07*	*0.30*	*<0.001*	*0.85*	*0.63*
WEAX flour						0.13	−0.12	0.17	0.11	0.17	0.11	**0.69**	0.09	0.12
						<0.05	*0.06*	*<0.01*	*0.06*	*<0.01*	*0.07*	*<0.001*	*0.16*	*<0.05*
protein flour							**−0.42**	−0.04	**0.29**	−0.02	**0.32**	0.01	**−0.25**	**0.85**
							<0.001	*0.53*	*<0.001*	*0.77*	*<0.001*	*0.91*	*<0.001*	*<0.001*
flour yield								−0.06	−0.09	0.02	0.07	−0.15	0.14	−0.18
								0.32	*0.14*	*0.80*	*0.22*	*<0.05*	*<0.05*	*<0.01*
TAXI bran									**0.26**	−0.03	−0.08	**0.24**	−0.07	−0.04
									<0.001	*0.57*	*0.21*	*<0.001*	*0.24*	*0.51*
XIP bran										0.00	0.12	**0.22**	**−0.24**	**0.24**
										0.97	*0.05*	*<0.001*	*<0.001*	*<0.001*
xylanase bran											0.14	**0.39**	−0.01	−0.03
											<0.05	*<0.001*	*0.83*	*0.63*
TOTAX bran												0.05	**−0.42**	0.16
												0.42	*<0.001*	*<0.01*
WEAX bran													−0.03	−0.08
													0.61	*0.18*
bran yield														−0.01
														0.84
protein whole meal														

[a] The bold partial correlation coefficients refer to significant correlations (*P* value < 0.001).

variability in the functionality of these cereals in biotechnological processes (*9*, *12*), for example, breadmaking (*3*, *36*) and gluten−starch separation (*8*), and in refrigerated dough (*11*, *29*) and animal feed systems (*37*, *38*).

Especially in applications in which microbial xylanases are used to improve processing parameters and/or product quality, variation in xylanase inhibitor levels may cause anomalies during production and may hamper the delivery of constant product quality by inconsistently lowering xylanase functionally (*9*). The presence of xylanase inhibitors in cereals may increase the xylanase dosage needed to achieve a certain effect. When possible, the use of uninhibited xylanases (*39*−*41*) may circumvent problems associated with xylanase inhibitors.

Recent studies showed that wheat grain-associated xylanase activity can be of the same order of magnitude as the xylanase activity levels commonly added to wheat flour as commercial bread improvers (*10*, *16*). Therefore, it is reasonable to expect that xylanases associated with cereal grains can affect the functionality of cereals during processing and product quality. The impact they have depends on the level of their occurrence and their biochemical properties. Earlier studies related wheat-associated xylanase activity with reduced shelf life of refrigerated dough as it causes the discharge of a brownish fluid during storage, a phenomenon called dough syruping (*11*). In addition, Debyser et al. (*42*) demonstrated that xylanases naturally occurring in wheat flour may have a positive impact on bread loaf volume. The results of Dornez et al. (*43*, *44*) indicate that wheat flour-associated xylanases can alter the AX population during breadmaking, thereby changing AX functionality and potentially affecting dough and end-product properties.

General Conclusion. The present study reveals substantial differences in xylanase, TAXI, and XIP activities between grains of different wheat types and nonwheat cereals. For each type of cereal large variation in the levels of these proteins occurs between different varieties. With the experimental setup used, for wheat a considerable part of the variability in inhibitor levels can be explained by genotype, whereas xylanase activity is strongly determined by environment. The results discussed above suggest that plant breeders and industry can to a certain extent select for wheat varieties with high or low xylanase inhibition activities for specific applications. However, the impact of climatological and agronomical parameters on the levels of these proteins should be kept in mind. The significant effect of genotype−environment interaction on xylanase inhibition activities observed here suggests that TAXI and XIP activities are not strong breeding parameters.

ABBREVIATIONS USED

AX, arabinoxylan; BSA, bovine serum albumin; GHF, glycoside hydrolase family; IU, inhibitor units; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; TOTAX, total AX; WEAX, water-extractable AX; XIP, xylanase-inhibiting protein; XU, xylanase unit.

Supporting Information Available: Supplementary Table 1, detailed list of all wheat varieties analyzed, the country in which they were grown, the year of harvest, and the xylanase, TAXI, and XIP activities occurring in flour and bran. This material is available free of charge via the Internet at http://pubs.acs.org.

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