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EVALUATION OF NINE PERENNIAL WHEAT DERIVATIVES GROWN IN ITALY

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ABSTRACT

As part of an international network coordinated by the Australian NSW Department of Primary Industries, nine lines of perennial wheat obtained from crosses between *Triticum aestivum* and *Thinopyrum* spp. were grown at Montelibretti (Rome) in randomized blocks with three replications during two years of testing, and compared for their agronomical, nutritional and technological properties with common wheat cultivars (cvs) Wedgetail and Enesco. All perennial genotypes were characterized by post-harvest regrowth (PHR), lateness of ear emergence, small kernels, loose spikes, variable number of seeds/spikes and high number of tillers. In addition, perennial lines had medium test weight, low percentages of hull-less kernels, high protein content, reduced sodium dodecyl sulphate (SDS) sedimentation volume and kernel texture typical of soft or medium-hard wheat. The hard-textured lines showed novel genes coding for puroindolines A and B inherited from wheatgrass (*Thinopyrum* spp.). Analysis of single seeds revealed a marked inter- and intra-line variation for gliadins and HMW-glutenin subunits (HMW-GS). The total content in bioactive compounds 5-n-alkylresorcinols and soluble polyphenols (SP) was high in perennial lines compared with their annual counterparts. Furthermore, perennial lines exhibited high yellow pigment content and resistant-starch percentage. The poor gluten quality of some perennial lines

was associated with the presence of prolamins inherited from the wheatgrass parent and the absence of high-quality, HMW-GS from the wheat parent. Evidence was obtained that chromosome substitution or allosyndetic recombination between E-genome and ABD-genome chromosomes likely occurred in some perennial lines with *Th. elongatum* in their pedigree. The perennial genotypes were found to be valuable for their PHR potential and nutritional value. However, they deserve closer attention for some negative agronomical and quality traits.

Keywords: bioactive compounds, gluten quality, perennial wheat, puroindolines, storage proteins

INTRODUCTION

In the last few decades, one third of Earth's arable land has been lost due to erosion (Pimentel *et al.* 1995) and the production systems based on annual grain crops such as wheat, maize, rice and soybean have been considered among the primary causes of this soil erosion (Glover, 2005). By contrast, the high productive potential and the efficient use of natural resources such as light, water, CO_2, nitrogen compounds and minerals by perennial plant communities (Crews, 2005) suggest that perennial grain crops could meet the increasing demands of food while reducing soil erosion. Moreover, perennial grain crops are seen as an opportunity to improve water, minerals and fertilizers management, while increasing biodiversity, underground biomass and carbon sequestration in the soil (DeHaan *et al.* 2005; Jordan *et al.* 2007). Perenniality seems to be under the control of multiple genes responsible for specific biological structures such as bulbs, rhizomes and meristems, as well as for physiological traits such as resistance to cold, drought and biotic stresses. Therefore, transformation of annual crops into perennial crops with high grain yield, coupled with superior technological and nutritional quality could turn out to be a very demanding and challenging goal. In addition, annual species supply much of the photosynthetic energy for seed development, whereas perennial species allocate a proportion of the photosynthate to their roots and green tissues late in the growing season, after the annuals have senesced. This "energy tradeoff" between grain and perennating structures would result in decreased grain production per hectare of perennial species as compared with their annual counterparts (Wagoner, 1990). However, the biological superiority of annual species in producing a high amount of seed could be the result of their evolutionary history and selection, both natural and human-oriented, rather than an integrant consequence of their annual habit (DeWet, 1981; DeHaan *et al.* 2005). On the other hand, in certain conditions, the decreased input costs of a perennial grain crop can make up the difference in profit and provide additional ecosystem services (Bell *et al.* 2008).

Common wheat (*Triticum aestivum*) is the most widespread annual grain crop grown on more than 220 million hectares. Cultivation of this cereal species is claimed to be one of the primary causes of soil erosion, with annual losses of soil as high as 31.5 tonnes/ha in the case of wheat monocultures (Reganold *et al*. 1987).

The earliest forms of perennial wheat were produced in Russia in the second decade of the last century (Tsitsin and Lubinova, 1959), whereas more recent material has been developed by The Land Institute and Washington State University in the United States (Cox *et al*. 2010; Murphy *et al*. 2010). These perennial genotypes derive from crosses between wheat and one of three species of *Thinopyrum*, namely (i) *Th*. *elongatum* (synonymous *Agropyrum elongatum*), a diploid species (2*n* = 2x = 14) with the E genome, (ii) *Th*. *intermedium* (synonyms *Ag*. *intermedium*, *Elymus hispidus* or *Elytrigia intermedia*), an hexaploid species (2*n* = 6x = 42) with the JJ^SS genome, and (iii) *Th*. *ponticum* (synonyms *Ag*. *ponticum*, *Elymus elongatus* spp. *ponticus* or *Elytrigia pontica*), a decaploid species (2*n* = 10x = 70) with three copies of the J genome and two copies of the J^s genome. In some cases, tetraploid AB-genome wheat *T. turgidum* ssp *carthlicum* was used as a bridge species with *Th*. *intermedium*, and the hybrid progeny was crossed with common wheat.

Among 176 wheat genotypes with wheatgrass (*Thinopyrum* spp.) in the pedigree, 107 lines demonstrated some ability to regrow after the sexual cycle PHR over three consecutive years of cultivation in two Australian locations (Hayes *et al*. 2012). As part of an international network of field trials coordinated by the Australian NSW Department of Primary Industries, nine of the wheat x wheatgrass derivatives with a relatively high PHR capacity mentioned above were seeded in Italy in 2011. The present study aimed to compare these lines with two commercial common wheat cultivars for their agronomical, technological and nutritional traits during two years of testing.

MATERIALS AND METHODS

Plant material

Nine perennial wheat breeding lines kindly provided by Richard Hayes (Graham Centre for Agricultural Innovation, NSW, Wagga Wagga, Australia) were grown at Montelibretti (Rome) in the Tiber valley on sandy soil, with an average annual rainfall of 954 mm. Plants were sown in December 2011 in 1 m rows, 0.5 m apart, with 30 kernels/row in a randomized block experimental design with three replications. Two annual common wheat cultivars, the Australian cv. Wedgetail and the Italian cv. Enesco, were used as controls. Perennial accessions of *Secale montanum*, *Hordeum bulbosum*, *Thinopyrum ponticum* and *Th*. *intermedium* were included in the experiment as well. The full list of entries is given in Table 1. At sowing, 31 Kg/ha nitrogen and 20 Kg/ha of phosphorus were applied in the form of urea and diammonium phosphate. The date of anthesis was recorded and maturity of each genotype was calculated as time between sowing and anthesis. At harvest, length of the main stem and total number of tillers of each plant were recorded and plants were cut at

approximately 10 cm from the soil surface. In addition, spikes from the main stems were threshed in a bench micro-thresher to determine percentage of hull-less kernels, number of kernels per spike, 1 000-kernel weight and test weight. Rows were visually monitored for signs of regrowth every four weeks for three months from harvest and each genotype received a PHR score based on the average percentage of the original plant population that was regrowing in the three replicates.

TABLE 1. PEDIGREE, CHROMOSOME NUMBER AND POST-HARVEST REGROWTH (PHR) OF NINE WHEAT X WHEATGRASS DERIVATIVES

WHEAT DERIVATIVES	PEDIGREE AND ORIGIN	NO. OF CHROMOSOMES[c]
235A	*Th. elongatum* / *T. aestivum*[a] // *T. aestivum*[b] (WSU)	42, 44, 56
236A	*Th. elongatum* / *T. aestivum*[a] // *T. aestivum*[b] (WSU)	56, 58
244B	*Th. elongatum* / *T. aestivum*[a] // *T. aestivum*[b] (WSU)	56
251B	*Th. elongatum* / *T. aestivum*[a] // *T. aestivum*[b] (WSU)	56
280B	*Th. intermedium* / *T. carthlicum* // *T. aestivum* (TLI)	56
281B	*Th. intermedium* / *T. carthlicum* // *T. aestivum* (TLI)	56
11955	*Triticum* spp./ *Thinopyrum* spp.(USA)	56
OK72	*T. aestivum*/*Th. ponticum* (USA)	56
OT38	*T. aestivum*/*Th. intermedium* (ex USSR)	56

a Cv. Chinese Spring
b Cv. Madsen
c According to Hayes *et al.* (2012)
WSU = Washington State University
TLI = The Land Institute

Technological analyses

Analyses were performed on wholemeal from mature kernels of each replication ground with a laboratory mill (Cyclotec, mod. 1093-Tecator/Hoganas, Sweden) equipped with a 1.0 mm sieve. Protein content was determined by micro-Kjeldhal nitrogen analysis (N x 5.7), whereas gluten quality was evaluated by the SDS sedimentation test using a solution of 2 percent sodium dodecyl sulfate as described by the standard method 56-70 (AACC, 1995), and the sedimentation volumes were expressed in milliliters. The sequential extraction of protein in the wholemeal was carried out according to Wang *et al.* (2007). Kernel hardness was evaluated on 50 hull-less kernels by the Perten Single Kernel Characterization System (SKCS) 4100 (Springfield, IL, USA) following the manufacturer's operating procedure. The instrument was set in a range of hardness between -40 and +120.

Puroindoline and storage protein analysis

DNA was extracted from leaves by the cetyltrimethyl ammonium bromide (CTAB) method and puroindoline genes were amplified by PCR as described by Gautier *et al.* (1994). Puroindolines were extracted with 50 mM NaCl and 50 percent (v/v) propan-2-ol from 50 mg of air-dried starch

granules as described previously (Corona *et al.* 2001). Fractionation of puroindolines by acidic poly-acrylamide gel electrophoresis (A-PAGE) at pH 3.1 was carried out as described by Corona *et al.* (2001). Gliadins and total proteins were extracted and fractionated by A-PAGE and SDS-PAGE, respectively, as described by Pogna *et al.* (1990).

Extraction of phenolic compounds and alkylresorcinols

Immediately after harvest, grain samples from two replicates were milled with a laboratory cyclone mill (Cyclotec 1093, Foss, Italy) to pass through a 0.5 mm sieve and kept at 4°C until extraction and analysis. All determinations were carried out in triplicate on two independent aliquots of each composite sample. Moisture content was determined at 120°C with a thermobalance (Sartorius MA 40, Gottingen, Germany).

Samples (1 g) of wholemeal were extracted to determine SP compounds and 5-n-alkylresorcinol (AR) content. Samples were placed in 50 mL tubes and extracted with 40 mL acetone for 24 hours by continuous mechanical shaking at room temperature. The extracts were then filtered through a Whatman n.42 filter paper and evaporated to dryness at 60°C in a rotary evaporator (Buchi R-114, Switzerland). The dry residues were then dissolved in pure methanol (1 mL) and immediately analysed. All reagents were of analytical spectrophotometric grade (Carlo Erba, Rome, Italy).

Determination of total soluble phenolic compounds (TSPCs)

SP of wholemeal extracts were determined using the Folin–Ciocalteu (F-C) method as reported by Moore and Yu (2008). SP content was calculated from a calibration curve, using (+) catechin as standard. Results were expressed as micrograms of catechin equivalents per gram of wholemeal sample.

Gas chromatography-mass spectrometry (GC-MS) analysis of alkylresorcinols

Total AR content in wholemeal extracts was determined by GC-MS analysis according to Landberg *et al.* (2009) using methyl behenate as internal standard. The AR extract (10 mL) of each sample was dried under nitrogen and a mixture (400 µL) of pyridine and TMCS (9:1, v/v) was added. The mixture was then shaken and heated at 70°C for 60 min. GC-MS analysis was performed with a Perkin Elmer gas chromatograph GC Clarus 600 series coupled to the mass spectrometer Clarus 580D (Perkin Elmer, Milan, Italy) equipped with a split/splitless injector, a RTX-5MS column (0.25 mm 30 m, 0.25 mm film thickness, Restek, Milan, Italy) and a quadrupole mass spectrometer (Clarus 580D, Perkin Elmer, Milan, Italy) operating in electronic impact (EI) ionisation mode (70 eV). The chromatographic conditions employed were reported by Bellato *et al.* (2013). AR content was determined by comparing the relative retention times with those obtained for a mix of the AR homologue standards C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0. Chromatographic peak areas of the AR homologues in each sample were summed to yield total AR content.

Total dietary fibre, resistant starch and yellow pigments

Total dietary fibre (DF) content was determined using an enzymatic-gravimetric method (AOAC, 1995) and an automatic filtration of the hydrolysed products (Fibertec system, FossItalia, Italy). Total and resistant starch (RS) contents were evaluated by enzymatic methods using Megazyme kits, K-TSTA and K-RSTAR (Mc Cleary *et al.* 1997; Mc Cleary and Monaghan, 2002; Mc Cleary *et al.* 2002). Total yellow pigment (YP) content was determined following the AOAC 14045 (1975) method and expressed as ppm of β-carotene.

Statistical analysis

As reported by Li *et al.* (2009), two independent aliquots of composite wholemeal sample were considered as statistical replicates of each genotype. Analysis of variance was performed with the Microcomputer Program for the Design, Management, and Analysis of Agronomic Research Experiments (MSTATC) program (Michigan State University, East Lansing, MI). Simple correlation coefficients were calculated as well.

RESULTS

Post-Harvest Regrowth

Three months after the first harvest in 2012, the PHR of the nine wheat x wheatgrass derivatives varied between 5.6 and 42.1 percent, without any evident association between PHR score and genetic origin (Table 2). In some genotypes, new plants arose at the level of the ground surface or immediately below, while in others they emerged at the level of the first or the second internode. The accessions of *Thinopyrum intermedium*, *Th. ponticum* and *Hordeum bulbosum* showed PHR scores between 60.0 and 87.8 percent, approximately double that of the best perennial wheat derivatives. *Secale cereale* had a moderate (23.8 percent) PHR score, whereas annual common wheat cvs Wedgetail and Enesco did not exhibit any sign of regrowth. In 2013, the perennial wheat derivatives in the three months following the second harvest revealed a modest decline in their PHR scores with respect to the those recorded in 2012 (Table 2).

Morphological and physiological traits

The perennial wheat derivatives proved to be significantly different for all the morpho-phisiological traits analysed with respect to cvs Wedgetail and Enesco (Table 2). On average, the nine lines were characterized by lateness of ear emergence (20-30 days later compared with annual varieties), high number of tillers (13.4 *vs* 7.3, except line 236A), tall plants (88.6 *vs* 70.2 cm), loose spikes (1.13 *vs* 1.79 spikelets/cm), reduced number of kernels per spike (39.8 *vs*

63.0) and small kernels (21-33 mg *vs* 40-41 mg). However, lines 280B and 281B, which have in their pedigree the bridge tetraploid species *Triticum turgidum* ssp. *carthlicum*, were comparable with cvs Wedgetail and Enesco for plant height and ear length. In addition, the perennial wheat derivatives revealed mean test weights intermediate between those of annual controls Wedgetail (66.6 kg/hL) and Enesco 74.2(kg/hL), with the only exception of lines 244B and 280B, which showed test weights lower than 58.5 Kg/hL. Upon a single passage through a micro-thresher, spikes of perennial wheat genotypes released a low percentage of hull-less kernels (20.7 to 63.0 percent) compared with control cultivars (84.9 and 91.8 percent). Leaves and stems of perennial wheat derivatives showed no damage due to powdery mildew and rusts (*Puccinia* spp.), with the only exception being lines 251B and 236A, which revealed slight symptoms of stem rust (*Puccinia graminis tritici*). By contrast, line 244B was heavily attacked by *Helminthosporium* spp.

TABLE 2. AGRONOMIC TRAITS AND POST-HARVEST REGROWTH (PHR) OF NINE PERENNIAL WHEAT DERIVATIVES, TWO COMMON WHEAT CULTIVARS AND FOUR PERENNIAL CEREAL SPECIES[a]

GENOTYPE	HEADING TIME*	NO. OF TILLERS	PLANT HEIGHT (CM)		SPIKE LENGTH (CM)	NO. OF SPIKELETS/ SPIKE	NO. OF SPIKELETS/ (CM)	NO. OF KERNELS/ SPIKE	KERNEL WT (MG)	TEST WT (KG/ HL)	HULL-LESS KERNELS (%)	PHR (%)	
			2012	2013								2012	2013
235A	155.0c	10.6d	94.6b	139.3a	15.7a	18.5b	1.18b	59.8b	24.5d	71.2bc	46.0ef	28.1d	25.0d
236A	150.0d	7.1e	85.7c	138.5a	15.3ab	16.5c	1.08b	43.9e	24.8d	68.9c	50.3de	11.1f	9.2f
244B	157.7b	11.2d	80.5d	90.0f	14.0b	15.7c	1.13b	13.1g	23.2de	58.3e	38.8g	5.6g	5.6g
251B	150.0d	11.4d	95.8b	133.0b	16.7a	16.5c	0.98b	58.4b	25.2d	70.2c	41.7fg	36.1b	33.3c
280B	147.0e	12.1cd	64.5f	114.3e	9.8d	11.7e	1.19b	18.5f	21.4e	58.2e	25.0h	20.6e	17.6e
281B	142.3f	18.7a	79.1d	119.3d	10.3cd	13.3de	1.29b	9.2h	32.9b	70.0c	20.7h	40.5a	37.8b
11955	146.3e	15.6b	102.3a	130.5b	15.7a	15.6c	0.99b	53.5c	30.1c	68.7c	48.3de	33.3c	33.3c
OK72	141.3f	19.6a	98.4b	126.2c	15.2ab	14.8cd	0.98b	53.2c	28.3c	74.0a	51.7d	42.1a	42.1a
OT38	160.0a	14.4bc	96.8b	127.7c	15.9a	20.8a	1.31b	48.7d	23.5de	72.0b	63.0c	35.0b	30.0c
Mean	150.0	13.4	88.6	124.3	14.3	15.9	1.13	39.8	26.0	68.0	42.8	27.8	25.9
Wedgetail	129.3g	7.5e	70.3e	-	11.5c	20.7a	1.79a	63.2a	39.9a	66.6d	84.9b	0.0	0.0
Enesco	120.1h	7.0e	70.0e	-	11.1c	21.0a	1.80a	62.8a	41.0a	74.2a	91.8a	0.0	0.0
Th. intermedium	171.2	20.3	129.8	167.7	33.1	26.1	0.79	nd	nd	nd	nd	66.7	60.0
Th. ponticum	193.4	22.1	166.7	194.0	29.7	22.8	0.77	nd	nd	nd	nd	80.0	80.0
S. montanum	133.4	7.7	130.1	142.3	12.0	18.8	1.57	nd	nd	nd	nd	23.8	19.0
H. bulbosum	120.1	14.3	160.8	170.7	11.3	17.6	1.56	nd	nd	nd	nd	87.8	87.8

a Agronomic traits of plants harvested in 2012 (first harvest); plant height and PHR were recorded in 2013 (second harvest) as well.
*Number of days from sowing.

nd, not determined. In each column, means followed by the same letter do not differ significantly from one another (Duncan test at P<0.05).

Quality traits

Protein contents as high as 19.7 to 23.7 percent were observed in the perennial wheat derivatives, with an average value of 20.6 percent, 3.3 percentage units higher than those of their annual counterparts (Table 3). On average, the perennial wheat derivatives revealed a high proportion of gliadins (37.2 percent of total proteins *vs* 33.0 percent in cv. Wedgetail) coupled with a significantly low proportion of HMW-GS (on average 9.3 percent of total protein *vs* 10.7 percent in cv. Wedgetail). This was particularly evident in lines 235A, 236A, 244B and 251B developed at the Washington State University, and was associated with poor gluten quality as determined by the SDS sedimentation test, line 235A being unique in showing an SDS sedimentation volume as high as 58 ml. The contrasting behaviour of these lines was likely due to their HMW-GS, which are known to play an important role in the visco-elastic properties of dough.

TABLE 3. PROTEIN CONTENT, PROPORTION OF FOUR PROTEIN FRACTIONS AND SDS SEDIMENTATION VOLUME OF NINE PERENNIAL WHEAT DERIVATIVES AND TWO COMMON WHEAT CULTIVARS

GENOTYPE	PROTEIN CONTENT %	ALBUMIN & GLOBULIN %	GLIADIN %	HMW-GS %	LMW-GS %	SEDIMENTATION VOLUME (ml)
235A	19.7	11.1	39.8	9.7	19.4	58c
236A	19.4	15.1	41.9	7.4	15.4	45f
244B	23.7	15.1	42.7	8.0	13.7	30i
251B	19.9	9.3	41.8	9.7	18.8	43g
280B	20.8	14.3	24.8	12.0	27.1	50d
281B	21.5	10.4	35.1	11.2	22.4	41h
11955	19.7	24.3	31.5	7.9	18.5	50d
OK72	20.6	10.9	38.1	10.1	20.0	50d
OT38	20.7	17.3	38.2	8.1	16.3	47e
Mean	20.6	14.2	37.2	9.3	19.1	46
Wedgetail	17.3	14.2	33.0	10.7	18.8	67b
Enesco	17.4	nd	nd	nd	nd	79a
F value	**	ns	***	**	ns	

**, ***Significant at P<0.05 and P<0.01 respectively; ns, not significant. In the last column, means followed by the same letter do not differ significantly from one another (P<0.05).

The HMW-GS of the perennial material were fractionated by SDS-PAGE and classified according to the nomenclature described by Payne and Lawrence (1983) and Pogna *et al.* (1989) (Figure 1). Six perennial lines (11955, OK72, OT38, 235A, 280B and 281B) showed no trace of HMW-GS inherited from the wheatgrass parent and exhibited the commonly occurring subunits 1 or 2* encoded by the *Glu-A1* locus on the long arm of chromosome 1A together with subunits 20, 7*+ 8 or 7+9 encoded by the *Glu-B1* locus (chromosome 1BL) and subunit pairs 2+12 or 5+10 encoded by the *Glu-D1* locus (chromosome 1DL) (Table 4). By contrast, lines 236A, 244B and

251B exhibited unusual HMW-GS, likely inherited from the wheatgrass parent (Figure 1, arrows) and lacked HMW-GS encoded by the *Glu-D1* locus on chromosome 1DL. In addition, line 244B did not show any subunit encoded by the *Glu-B1* locus. SDS-PAGE fractionation of 10 single kernels from each genotype revealed that the perennial wheat derivatives were homogeneous for their HWM-GS patterns, with the only exception being line 281B, which turned out to be a mixture of three different genotypes (biotypes) with contrasting HMW-GS compositions at *Glu-A1* (subunit 1 or Null) and *Glu-D1* (subunit pair 2+12 or 5+10) (Figure 2 and Table 4).

FIGURE 1. SDS-PAGE FRACTIONATION OF TOTAL PROTEINS FROM NINE PERENNIAL WHEAT DERIVATIVES

(1) line 235A, (2) line 236A, (3) line 244B, (4) line 251B, (5) line 280B, (6) line 281B, (7) line 11955, (8) line OK72 and (9) line OT38. HMW-GS are numbered. Arrowheads indicate HMW-GS inherited from wheatgrass (*Thinopyrum* ssp).

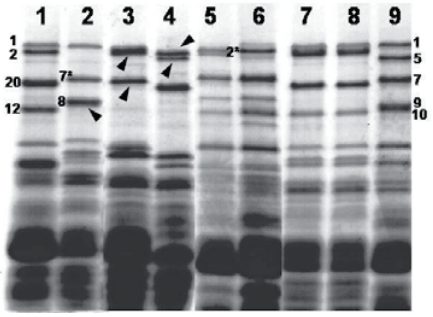


FIGURE 2. SDS-PAGE PATTERN OF TOTAL PROTEINS FROM THREE SINGLE SEEDS OF PERENNIAL WHEAT LINE 281B

HMW-GS are numbered.

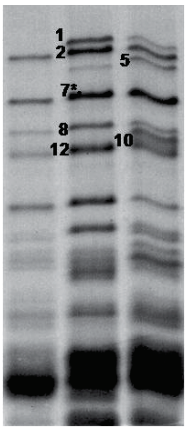


TABLE 4. HMW-GS COMPOSITION OF NINE PERENNIAL WHEAT DERIVATIVES

WHEAT GENOTYPE	WHEAT LOCUS			WHEATGRASS LOCUS
	GLU-A1	*GLU-B1*	*GLU-D1*	
11955	1	20	2+12	absent
OK72	1	20	2+12	absent
OT38	1	7+9	5+10	absent
235A	1	20	2+12	absent
236A	1	7+8	absent	1 subunit
244B	1	absent	absent	2 subunits
251B	Null	20	absent	2 subunits
280B	2*	7+8	2+12	absent
281B-1*	1	7+8	2+12	absent
281B-2	Null	7+8	2+12	absent
281B-3	1	7+8	5+10	absent

*Line 281B contains 3 biotypes with contrasting HMW-GS compositions

Gliadin patterns of the perennial wheat derivatives fractionated by A-PAGE were comparable with those of annual wheat cvs. Bolero and Chinese Spring. However, lines 236A, 244B, 251B and OT38 revealed some ω- or γ-gliadins inherited from the wheatgrass parent (Figure 3, arrowhead). Upon A-PAGE fractionation of gliadins from single seeds, line 236A (Figure 4), 235A and 281B turned out to be a mixture of two or more biotypes with contrasting gliadin bands encoded by homoeologous group 1 chromosomes of common wheat.

FIGURE 3. A-PAGE FRACTIONATION OF GLIADINS FROM NINE PERENNIAL WHEAT DERIVATIVES

(1) Line 235A, (2) line 236A, (3) common wheat cv. Enesco,(4) line 244B, (5) line 251B, (6) line 280B, (7) line 281B, (8) line 11955, (9) line OK72 and (10) line OT38. Arrowheads indicated gliadin inherited from wheatgrass (*Thinopyrum* spp.).

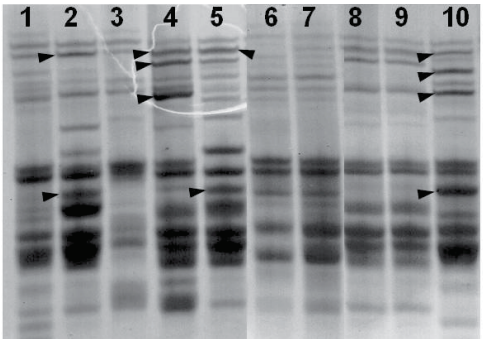
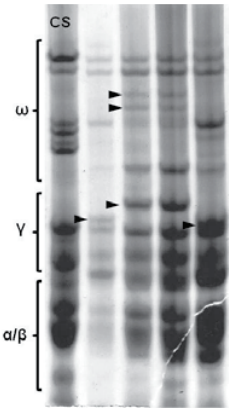


FIGURE 4. A-PAGE FRACTIONATION OF GLIADINS FROM COMMON WHEAT CV. CHINESE SPRING (CS) AND FOUR SINGLE SEEDS OF PERENNIAL WHEAT LINE 236A

Arrowheads indicate differential gliadin bands.



Kernel hardness was determined by the SKCS method using 50 grains for each line and found to be typical of soft-textured (mean SKCS index=30) or medium-hard common wheat (mean SKCS index =68) (Table 5).

TABLE 5. MEAN SKCS VALUE AND ALLELE COMPOSITION AT THE PUROINDOLINE LOCI IN NINE PERENNIAL WHEAT DERIVATIVES

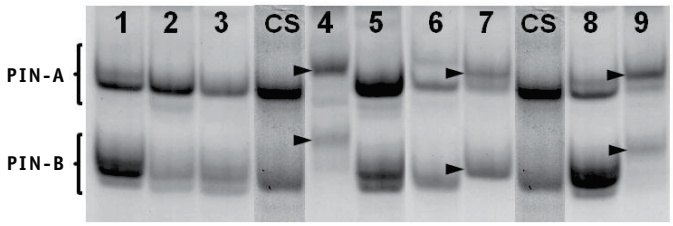
PERENNIAL WHEAT LINE	WHEAT LOCUS		WHEATGRASS LOCUS		SKCS
	PIN-A	*PIN-B*	*PIN-A*	*PIN-B*	
235A			Novel allele	Novel allele	55.4b
236A	*Pina-D1a*	*Pinb-D1a*			34.5c
244B	*Pina-D1a*	*Pinb-D1a*			46.7bc
251B	*Pina-D1a*	*Pinb-D1a*			35.4c
280B			FJ898232.1*	nd	69.1b
281B			FJ898232.1*	nd	60.0b
11955	*Pina-D1a*	*Pinb-D1a*			29.5c
OK72	*Pina-D1a*	*Pinb-D1a*			32.5c
OT38	*Pina-D1a*	nd			37.7c
Wedgetail	*Pina-D1a*	*Pinb-D1b*			61.5b
Enesco	*Pina-D1b*	*Pinb-D1a*			79.8a

nd, not determined ; * GenBank number. In the last column, means followed by the same letter do not differ significantly from one another (P<0.05).

Upon A-PAGE fractionation, the perennial wheat derivatives exhibited puroindoline-A (PIN-A) and puroindoline-B (PIN-B) inherited from either wheatgrass or common wheat. Novel, slow-moving PIN-A and PIN-B likely inherited from wheatgrass (*Thinopyrum spp.*) occurred in the medium-hard kernels produced by lines 235A, 280B and 281B (Figure 5, arrowheads). By contrast soft-textured perennial lines 236A, 244B, 251B, 11955, OK72 and OT38 exhibited wild-type PIN-A and PIN-B. When submitted to PCR amplification and sequencing, these latter soft lines revealed alleles *Pina-D1a* (coding for PIN-A) and *Pinb-D1a* (PIN-B) inherited from common wheat, whereas the medium-hard lines showed three unusual alleles (Table 5). In particular, the PIN-A allele in lines 280B and 281B was found to be identical to that amplified in *Aegilops tauschii* isolate TT52 (GenBank reference no. FJ898232.1), whereas the PIN-A and PIN-B alleles in line 235A were novel sequences never described before.

FIGURE 5. A-PAGE FRACTIONATION OF PUROINDOLINES A (PIN-A) AND B (PIN-B) IN WHEAT CV. CHINESE SPRING (CS) AND PERENNIAL WHEAT LINES (1) OK72, (2) 244B, (3) 251B, (4) 280B, (5) 236A, (6) OT38, (7) 235A, (8) 11955 AND (9) 281B

Arrowheads indicate puroindolines inherited from wheatgrass (*Thinopyrum* spp.).



Phytochemical profile of perennial wheat derivatives

Wholemeals from perennial wheat derivatives and cv. Wedgetail harvested in 2012 (first harvest) were compared for their content in resistant starch (RS), AR, soluble polyphenols (SP), and DF. The YP content of the representatives of perennial wheat varied in the ranges 5.12 to 11.37 ppm, their average content being 43 percent higher than that of cv. Wedgetail (Table 6).

TABLE 6. YELLOW PIGMENT (YP), DIETARY FIBRE (DF), 5-N-ALKYLRESORCINOLS (AR), SOLUBLE POLYPHENOLS (SP), TOTAL STARCH (TS), RESISTANT STARCH (RS) AND RS/TS RATIO IN 9 PERENNIAL WHEAT DERIVATIVES AND ANNUAL WHEAT CV. WEDGETAIL HARVESTED IN 2012 (FIRST HARVEST)

GENOTYPE	YP (PPM)	DF (%)	AR (µg/g)	SP (mg/g)	TS (%)	RS (%)	RS/TS (X100)
235A	5.1 e	15.9 bc	384 d	206 h	57.8 b	0.71 ab	1.2
236A	6.8 c	15.1 d	266 h	287 e	46.4 f	0.47ab	1.0
244B	6.0 d	16.9 a	329 g	231 g	49.9 e	0.74 a	1.5
251B	7.0 c	13.9 e	391 c	255 f	57.9 b	0.59 ab	1.0
280B	11.4 a	15.3 cd	500 b	640 a	57.4 bc	0.49 ab	0.9
281B	9.4 b	13.6 e	372 e	309 d	55.4 c	0.46 b	0.8
11955	6.9 c	16.3 ab	522 a	406 b	51.4 de	0.63 ab	1.2
OK72	6.2 d	16.3 ab	346 f	193 i	68.3 a	0.58 ab	0.8
OT38	7.1 c	12.8 f	182 i	340 c	53.3 d	0.45 b	0.9
MEAN	7.3±1.9	15.1±1.4	366±105	319±280	55.3±6.2	0.6±0.1	1.0±0.22
cv. Wedgetail	5.1	13.4	308	280	73.7	0.4	0.5

In each column, means followed by the same letter do not differ significantly from one another (P<0.05).

On average, the total content in 5-alkylresorcinol (AR), soluble polyphenols (SP) and DF was high in the perennial wheat derivatives compared with cv. Wedgetail. However, there was a considerable variability for these bioactive compounds among the germplasm in this study. For instance, the lowest and highest SP values of 193 mg and 600 mg were determined in lines OK72 and 280B, the range of variation among these lines being as high as 407 mg. Another example of variability includes lines OT38 and 11955, which showed 5-n-alkylresorcinol contents of 182 mg/g and 522 mg/g, respectively. As expected, the high protein content of the perennial wheat lines was associated with a reduced amount of total starch (TS) compared with the annual wheat control. Interestingly, all perennial wheat lines exhibited a high concentration of RS, which resulted in a high RS/TS ratio (Table 6). No significant correlation was found between seed weight and the amount of phytochemicals AR, SP, DF and RS.

GC-MS analysis was used to determine the alkylresorcinol homologue composition of grain harvested in 2012. Compared with common wheat cv. Wedgetail, perennial wheat lines 236A,

OK72 and OT38 revealed an unusual AR pattern, with a prevalence (> 50 percent) of C19:0 homologue and a relatively high percentage (11-16 percent) of C17: 0 (Table 7). In addition, the nine perennial wheat representatives were found to belong to two groups based on the C17/C21 ratio, which is peculiar of the different *Triticum* species. The first group includes six lines with a C17/C21 ratio of 0.09 to 0.22, comparable to that of the control cv. Wedgetail (0.11), while the second group includes three lines (236A, OK72 and OT38) with a C17/C21 ratio of 0.34 to 0.76.

TABLE 7. HOMOLOGUE PROFILES (%) OF 5-N-ALKYLRESORCINOLS IN NINE PERENNIAL WHEAT DERIVATIVES

GENOTYPE	HOMOLOGUE						C17/C21 RATIO
	C15:0	C17:0	C19:0	C21:0	C23:0	C25:0	
235A	0.54±0.03	5.00±0.23	29.80±1.25	43.21±1.90	13.49±0.86	7.97±0.28	0.12
236A	0.21±0.13	12.61±1.63	50.89±0.50	31.77±1.69	3.98±0.22	0.54±0.24	0.40
244B	0.77±0.12	7.19±0.13	43.05±1.66	36.85±0.89	8.73±0.83	3.41±0.54	0.20
251B	0.46±0.19	6.52±0.63	39.62±1.65	40.66±2.13	8.72±0.19	4.02±0.24	0.16
280B	0.64±0.15	8.06±0.21	38.04±1.18	36.87±0.34	10.22±0.70	6.16±0.65	0.22
281B	0.51±0.19	4.21±0.22	30.72±1.09	47.80±1.27	11.67±0.32	5.09±0.85	0.09
11955	0.44±0.09	6.70±0.24	43.75±0.75	39.78±0.63	6.82±0.19	2.52±0.12	0.17
OK72	0.43±0.11	11.20±0.89	51.02±1.45	32.91±1.60	3.71±0.67	0.73±0.17	0.34
OT38	0.24±0.16	16.33±2.39	60.73±3.17	21.57±0.95	0.99±0.12	0.14±0.11	0.76
MEAN	0.47±0.16	8.65±0.99	43.07±1.35	36.82±1.11	7.59±0.23	3.40±0.25	
LSD (0.05)	0.38	1.20	1.40	1.25	0.78	0.57	
Cv. Wedgetail	0.68±0.31	4.84±0.26	36.72±0.33	45.23±0.27	9.22±0.19	3.30±0.04	0.11

DISCUSSION

Agronomic traits and PHR

The significant differences in the mean PHR value and the wide variation of this trait (5.6 to 42.0 percent) in the nine perennial wheat derivatives developed by the Land Institute and Washington State University indicate that several genes interacting with environment and climatic conditions play an important role in modulating regrowth after harvest. Survival of regrowing plants through the 2012 winter following the first harvest was very high, approaching 100 percent in most lines and leading to a small decline of PHR in 2013. This was likely due to the mild temperatures registered during autumn 2012-winter 2013 in the Tiber valley. The mean PHR scores of 27.8 percent in 2012 and 25.9 percent in 2013 and the performance of

lines OK72, 251B, 281B and 11955 which showed regrowth scores greater than 33 percent indicates that the present germplasm is valuable for its regrowth potential. These results confirm the recent conclusions by Hayes *et al.* (2012), who pointed out the strong relationship between PHR and the presence of at least one whole genome equivalent of 14 chromosomes from the wheatgrass parent. According to these authors, lines with 2*n* = 56 chromosomes in Table 1 likely contain 42 wheat chromosomes plus 14 wheatgrass chromosomes. However, wheat derivatives with a relatively high regrowth score may contain a reduced number of chromosomes due to substitution of one or more wheat chromosomes by wheatgrass homoeologous (Hayes *et al.* 2012). Here, evidence has been obtained that substitution or recombination involving E-genome and ABD-genome chromosomes likely occurred in lines 236A, 244B and 251B, which have 2*n*=56 and *Th. elongatum* in their pedigree. In addition, all perennial wheat derivatives showed endosperm proteins inherited from either wheat or wheatgrass parents. These aspects will be discussed later. The current study identified some negative agronomic attributes, mainly tenacious glumes and reduced kernel weight, together with many desirable traits such as reduced plant height, high tiller number and disease resistance, which can contribute to increase the agronomic potential of perennial wheat.

Quality traits

The HMW-GS account for only about 1 percent of the dry weight of wheat kernel (Payne *et al.* 1987) Nevertheless the results presented in this paper are consistent with accumulated evidence that they are the principal subunits that impart elasticity to gluten. Variation in composition of HMW-GS among the perennial material was found to make a large contribution to the gluten quality of these genotypes. In particular, the absence of HMW-GS encoded by the *Glu-D1* locus on the long arm of chromosome 1D in lines 236A, 244B and 251B (Figure 1, lanes 2-4) proved to be deleterious to gluten quality as determined by the SDS sedimentation volume (Table 3). In these lines, the presence of HMW-GS inherited from wheatgrass (Figure 1, arrowheads) could additionally affect gluten quality. Furthermore, lines 236A, 244B and 251B contain 1-3 w-gliadins plus one prominent g-gliadin arrowed in Figure 3, which have been likely inherited from their wheatgrass parent. In common wheat, all ω-gliadins and most γ-gliadins are encoded by genes on the short arms the homoeologous group 1 chromosomes (Payne *et al.* 1984). Moreover, a comparative study showed that all species in the genera *Triticum*, *Aegilops*, *Secale* and *Hordeum* contain genes coding for prolamins HMW-GS, gliadins or gliadin-type proteins such as secalins and hordeins) on homoeologous chromosome 1, suggesting that wheatgrass prolamins in lines 236A, 244B and 251B are likely encoded by chromosome 1E from *Thinopyrum elongatum*. As introgression of wheatgrass prolamin genes into perennial wheat lines 236A, 244B and 251B has been accompanied by concomitant removal of the *Glu-D1* locus, it is not clear whether it is the presence of wheatgrass prolamins or the absence of wheat HMW-GS that negatively impact the

bread-making quality. Line OT30 could offer an opportunity to elucidate this aspect. According to the quality score assigned to each HMW subunit or subunit pair based on its effect on gluten quality (Payne *et al.* 1987), the HMW-GS composition of line OT38 (subunit 1 of chromosome 1A, subunits 7+9 of 1B and subunits 5+10 of 1D, Table 4) has the high *Glu-1* quality score of nine, the maximum score being ten and the minimum three. The finding that the presence of wheatgrass gliadins in line OT38 (Figure 10, arrowheads) is associated with a SDS sedimentation volume as low as 47 ml (Table 3) suggests a direct negative effect of these proteins on gluten quality. In this context it is worth noting that some European-grown wheat cultivars contain the short arm of chromosome 1R from rye combined with the long arm of chromosome 1B (1BL/1RS). This translocated chromosome causes a decrease in gluten quality due to the presence of 1RS-encoded secalins, which increases dough stickiness (Zeller *et al.* 1982). The negative effects of rye prolamins on bread making quality of hexaploid AABBRR triticale are documented as well, and substitution of chromosome 1D for chromosome 1R dramatically improved such parameters of bread-making quality of triticale as SDS-sedimentation, mixing time, mixing tolerance, and loaf volume (Kazman and Lelley, 1996).

Kernel texture, a major determinant of flour quality and end-use quality of wheat, is mainly modulated by allele variation at the *Pina-D1* and *Pinb-D1* loci on chromosome 5DS coding for PIN-A PIN-B, respectively. In the present work, the molecular analysis of puroindoline genes from perennial wheat lines of different genetic origins identified novel alleles coding for PIN-A and PIN-B in line 235A with *Th. elongatum* in the pedigree. When compared with wild-type *Pinb-D1a* allele, the gene coding for PIN-B in this line shows 19 SNPs, whereas the encoded PIN-B protein contains nine amino acid substitutions in its mature form. The novel *Pina-E1a* and *Pinb-E1a* alleles in the homoeologous chromosome 5ES inherited from of *Th. elongatum* conferred a medium hard texture to line 235A (SKCS value = 55.4, Table 5). Interestingly, the absence of *Pina-D1* and *Pinb-D1* sequences from chromosome 5DS suggests that 5E (5D) chromosome substitution or homoeologous recombination between wheatgrass chromosome 5E and 5D of wheat occurred in line 235A.

Medium-hard kernel texture in lines 280B and 281B was associated with the presence of two unusual puroindolines with reduced mobility with respect to wild-tipe PIN-A and PIN-B (Figure 5, lanes 4 and 9). As *T. carthlicum* does not contain *Pina* and *Pinb* genes, puroindolines in lines 280B and 281B have been likely inherited from *Th. intermedium*. The 100 percent similarity between the DNA sequence coding for PIN-A in lines 280B and 281B and the *Pina-D1* allele in *Aegilops tauschii* isolate TT52 (GenBank reference no. FJ898232.1) suggests a strong phylogenetic relationship between D genome and J, J^S or S genome of *Th. intermedium*. As observed in line 235A, the absence of *Pina-D1* and *Pinb-D1* sequences from chromosome 5DS as determined by PCR amplification suggests chromosome substitution or allosyndetic recombination involving chromosome 5D of wheat and a homoeologous chromosome of *Th. intermedium*.

Nutritional traits

Based on the genotypes studied here, there is a great variation in the phytochemical composition among wheat cv. Wedgetail and the nine representatives of perennial wheat. In particular, there is an increased amount of yellow pigments, dietary fibre and RS in most of the perennial wheat derivatives analysed. In addition, unique bioactive phytochemical patterns with high levels of both 5-alkylresorcinol (AR) and soluble polyphenols (SP) were detected in lines 280B and 11955. Among the nine perennial wheat genotypes, line 11955 is characterized by relatively high values for kernel weight (30.1 mg), number of kernels/spike and PHR (33.3 percent). In addition, line 11955 showed the *Glu-1* quality score of six coupled with an above-average SDS sedimentation volume of 50 ml, suggesting its use as a component of a range of traditional and specialty products naturally enriched with health-promoting compounds.

The C17:0/C21:0 ratio of AR homologues has been used to distinguish between different cereal species. This ratio ranges between 0.01 in *Triticum monococcum*, 0.02 in *Triticum turgidum* ssp *dicoccum*, 0.05 in T. *turgidum* ssp *durum*, 0.06 in *T. timopheevii*, 0.11 to 0.18 in common wheat and *T. turgidum* ssp *turanicum,* and 0.25 in *T. zhukovskyi* (Ross *et al.* 2003; Ciccoritti *et al.* 2013). On average, the nine perennial wheats showed a high proportion of C17:0 and a low proportion of C21:0 homologues compared with cv. Wedgetail. This was mainly due to lines OT38, 236A and OK72, which were unique in having C17/C21 ratios as high as 0.34 to 0.76 (Table 7). By contrast, the remaining six perennial lines exhibited C17/C21 ratios of 0.09 to 0.22 comparable to those observed in the different species of the genus *Triticum*. Interestingly, lines 235A and 281 showed unusual AR homologue compositions with a high proportion of C21:0, C23:0 and C25:0 homologues (complessively about 64.5 percent as compared with 55.8 percent in cv. Wedgetail). As long-chain resorcinolic lipids affect protein structure and activity (Stasiuk *et al.* 2008), these lines may have some potential as a source of cereal foods for prevention of cardiovascular diseases and cancer.

The successful development of perennial wheat cultivars and their widespread adoption by millers, bakers and consumers will be facilitated by improvement of kernel threshability, milling and bread making quality, and nutritional characteristics including gluten digestibility (tolerance). Therefore, in addition to addressing the major agronomic traits (grain yield, PHR and disease resistance), good milling and baking quality and superior nutritional quality seem to be key traits to target for genetic improvement. The wide variation in storage protein composition and bioactive compounds detected in the germplasm analysed here can be easily exploited by breeders in the development of new perennial wheat genotypes with improved end-use qualities.

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