Puroindoline genes introduced into durum wheat reduce milling energy and change milling behavior similar to soft common wheats

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Abbreviations: SKCS, single kernel characterisation system; NIRS, near infrared spectroscopy; PSI, particle size index; revolutions per minute, rpm.

ABSTRACT

Grain physical characteristics and milling behavior of a durum wheat line in which both wild-type puroindoline genes were translocated and stabilized after backcrossing (*Svevo-Pin*) were compared with the parent line (*Svevo*). The only observed differences between grain characteristics were the mechanical resistance and starchy endosperm porosity revealed through vitreosity measurement. A significant increase of flour and a decrease of semolina yield and break milling energy were observed from *Svevo-Pin* in comparison with the non-recombinant parent line in accordance to the lower grain mechanical resistance and higher porosity measurements. Moreover, the particle size distribution shown for *Svevo-Pin* flour appeared consistent with a lower adhesion between starch granules and the protein matrix attributed to the presence of wild-type puroindolines. Coarse bran yield was conversely increased. This appeared to be due to a lower starchy endosperm recovery as a higher proportion of grain starch was found in this bran fraction. Flour from the durum parent line was inversely enriched in phytic acid, a cellular marker of the aleurone layer. Starch damage was also lower in *Svevo-Pin* flours in comparison with *Svevo*. All of the observed differences between translocation and parent lines were confirmed independent of the culture growth conditions (n=12).

1. Introduction

Wheat grain hardness is a key parameter that influences its mechanical properties, milling behavior, and end-uses (Pena et al., 2002; Pauly et al., 2013). Grain hardness was found mainly related to the genetic background and more precisely to the *Hardness* locus, *Ha*, on the short arm of chromosome 5D, which encodes the so-called Puroindolines a and b (Bhave and Morris, 2008). The allotetraploid *Triticum durum* ($2n = 28 = AABB$), which does not contain the D genome and thus no puroindolines, displays the hardest grain texture and

essentially produces coarse semolina at milling, which is used primarily for pasta and couscous. The allohexaploid *Triticum aestivum* ($2n = 42 = AABBDD$) can be divided in two distinct grain hardness types (Morris, 2002) depending on the presence of wild-type alleles of both puroindoline genes (= soft type) or the presence of a null or mutated allele of one of the two puroindoline genes (= hard type). Differences between hard and soft common wheat texture were found to be only related to changes at the interface between starch granules and the protein network in the wheat endosperm due to the distinct nature and amount of puroindolines, which appeared to affect the adhesion between the constituent polymers (Barlow et al., 1973; Simmonds et al. 1973). Mechanical changes at the interface had only been recently demonstrated by Chichti et al. (2015) using near-isogenic lines differing only by the wild-type or mutated version of one of the puroindoline genes.

Evidence for the direct effect of both wild-type puroindolines on the mechanical properties of the endosperm also results from complementation of the null or mutated allele with the wild-type allele to restore a soft phenotype (Beecher et al., 2002; Martin et al., 2006). Conversely, silencing of puroindoline genes led to an increase in grain hardness (Xia et al., 2008; Gasparis et al., 2011). Removal of the *Hardness* locus results in grain with mechanical properties similar to durum (Morris and Beecher, 2012).

Comparison of the milling behavior of near-isogenic common wheat lines indicates that soft grain texture results in a reduction of milling energy, the production of a higher amount of small particles recovered as flour and an increase in the coarse bran fraction at the breaking stage in comparison with hard grains (Greffeuille et al., 2006). Concomitantly, the soft wheat break flours showed lower levels of ash, which likely result from the greater extensibility of the corresponding grain outer layers (Greffeuille et al., 2007a). Moreover, flours produced from soft grains can be clearly distinguished from those from hard grains as they display a bimodal particle size distribution profile rather than a unimodal one. Presence

of wild-type puroindolines was also associated with a lower level of starch damage in flours (Beecher et al. 2002; Brites et al., 2008). This difference in particle size distribution and starch damage probably results from differences in adhesion between starch granules and the protein network, which appears weaker in soft wheats. Construction of a numerical model of the endosperm structure confirms the importance of adhesion between the constituent polymers on the grain mechanical resistance differences and starch damage level (Topin et al., 2008, 2009). A lower adhesion, as expected from the presence of wild-type puroindolines, leads to a reduction of overall mechanical resistance and starch damage. Due primarily to starch damage and water absorption/batter and dough viscosity, hard common wheats are mainly used for bread making whereas soft wheats are better for cake and biscuit making (Morris and Rose, 1996).

Recently, both of the wild-type alleles of the puroindoline genes were successfully introduced in a durum wheat background providing the possibility to obtain wheat grains with expected changes in their milling behavior and product properties in comparison with the durum parent cultivar (Gazza et al., 2011; Morris et al., 2011; Morris et al., 2015; Murray et al., 2016).

The present study analyzes in detail the grain characteristics, milling behavior, and energy required to grind a recombinant Italian durum cultivar in which puroindoline genes were introduced (“*Svevo-Pin*”) in comparison with the parent cultivar (*Svevo*) grown together in distinct locations.

2. Material and Methods

2.1. Wheat samples and grain physical characteristics

Grains from the Italian durum wheat *Svevo* cultivar and a translocated wheat line, *Svevo-Pin*, where the puroindoline genes from chromosome 5D of the soft common wheat cultivar *Chinese Spring* were introduced, were collected in 2014 in one location in the south of France (Maugio) and in 11 different locations in the U.S. All U.S. locations were in Eastern Washington State, ranging between 117° 03' W to 120° 14' W Long., and 46° 0' N to 47° 40' N Lat. In each of the U.S. locations, two field plot replications of *Svevo* and *Svevo-Pin* were analyzed.

Morphological measurements (length, width, thickness) and weight of each of approximately 300 grains of *Svevo* and *Svevo-Pin* samples, grown in France, were performed with a micrometer and a micro-precision balance as described in Mabilley et al. (2003). Particle size index (PSI) was measured to evaluate hardness according to internationally approved method (AACCI N°55-30.1). At least three determinations were performed and the results presented as means and standard deviation. Grain vitreousness was evaluated after cutting the grains with a Pohl grain cutter (Versuchs- und Lehranstalt, Brauerei, Berlin, Germany) with three repetitions of 50 grains each to determine the sum of percentage of totally or partly non-vitreous grains. For the U.S. grown samples, SKCS hardness index (HI) and grain weight were determined with the Single Kernel Characterization System (SKCS) 4100 apparatus (Perten Instruments, Inc., Springfield, IL) according to approved method (AACCI N° 55-31.01). Test weight was determined with AACCI N° 55-10.01 method.

2.2. Biochemical analyses

Moisture content was determined using standardized international method (ISO 712:2009) on ground grains and milling fractions. Grain protein content was measured directly on grains using near infrared (NIR) spectroscopy (AACCI N° 39-25.01) or using the

Kjeldahl method International Standard Method (ISO20483, 2013) using a nitrogen-to-protein ratio of 5.7, with at least two measurements performed on ground grain samples.

Phytic acid content of ground grains or flours was measured at 500 nm from acidic extracts according to a colorimetric method, in comparison with a standard curve obtained with corn phytate (P-8810, Sigma-Aldrich, St. Quentin Fallavier, France) solutions of known concentrations, as detailed in Hemery et al. (2009).

Total starch concentration was determined in duplicate on ground grains or on milling fractions using a Megazyme kit “Amyloglucosidase/alpha-Amylase method” according to AACCI 76-13-01 method (K-TSTA assay kit, Megazyme International Int., Ireland). Before analysis coarse bran fractions were ground with a ball grinder (MM400, Retsch, Haan, Germany) after being frozen in liquid nitrogen. Damaged starch was also determined on milling flours with a Megazyme kit (K-SDAM starch damage assay kit, Megazyme Int., Ireland) according to method AACCI N°76-31.01.

2.3. Wheat grain processing

Two replications per U.S. location and the French samples were used to study the milling behavior of *Svevo-Pin* in comparison to *Svevo*. The French sample was analyzed twice independently.

Grain moisture content was adjusted to 16.8 ± 0.4 % by adding water to grain during a time period of 19 ± 2 h, including an initial 2 h of constant mixing. Milling was performed with around 200 g of grain using a laboratory micromill, which is instrumented to measure torque between the two rotating cylinders (Pujol et al., 2000), and thus measures the milling energy in kJ.kg^{-1} at the ‘breaking’ step (corrugated rolls, dull to dull orientation). The first break (B1) occurred with roll speeds of the fast and slow rolls adjusted to 500 and 200 revolutions per minute (rpm), respectively, a roll gap and length of 0.70 mm and 50 mm

respectively, and a grain feed rate of 2 g.s⁻¹. Milling fractions were then classified by sieving on a rotary screen (Rotex, Tripette et Renaud, Paris, France) for 5 min on square-mesh sieves (200, 500, 710, 1000, 1400, and 2500 µm). The recovered fractions with particle size superior to 1000 µm were subjected to a second break (B2) with roll speeds and gap of 1000 and 285 rpm, and 0.10 mm, respectively, at the same feed rate as B1. B2 fractions were then sieved (same sieves as B1 without the 2500 µm). Flours were the finest fractions recovered under the 200 µm sieve after B1 and B2 (FB1, FB2). Particles of fine semolina were passing through the 500 µm sieve and overs 200 µm whereas coarse semolina were passing through the 710 µm and overs 500 µm. They were recovered after B1 and B2 and pooled for further analysis. Coarse bran (CB) corresponds to fractions larger than 1000 µm obtained from B2. Energy required to produce one kg of total flour was calculated and is represented by the K' index (in kJ.kg⁻¹ of flour). The fractions were weighed at each step, and stored separately at 4°C before biochemical analyses. Flour particle size was determined by laser granulometry (Malvern Mastersizer 2000, Malvern Instruments SARL, Orsay, France).

2.4. Statistical analysis

XLSTAT (Addinsoft SARL, Paris, France) or R software (www.r-project.org) were used to perform box plots, one-way ANOVA or wilcoxon rank sum tests or comparison between means with Student t-test and linear correlation analyses. Differences between sample values were marked in the Tables as either non-significant (*n.s.*), or with asterisks depending on their level of significance 95 % (*), 99 % (**), superior to 99 % (***).

3. Results and discussion

3.1. Introduction of wild-type Puroindolines in durum wheat markedly changed the grain mechanical properties

Length, width, thickness and weight of individual grains from *Svevo* and *Svevo-Pin* grown in France were measured in order to compare their morphological and grain filling characteristics (Table 1).

Table 1

No significant differences were observed, except grain thickness which appeared slightly lower for *Svevo-Pin* in comparison with *Svevo*. Both wheat samples exhibited typical durum wheat grain features as identified by Mabilite et al. (2003), *i.e.*, a higher ratio of grain length by width compared with common wheat grains (Table 1). Similar grain weight was observed and both varieties displayed similar biochemical composition of the main compounds as starch (65%), protein (15%), and ash 1.8% (grain dry mass basis).

By contrast, the PSI value of *Svevo-Pin* was five-fold greater than that of *Svevo* (Table 1), reflecting a softer texture in accordance with the known effect of wild-type puroindoline introduction in the changes of grain's mechanical properties (Beecher et al., 2002 ; Martin et al., 2006; Morris et al., 2011). Moreover, the *Svevo-Pin* grain texture was also found to be affected as the number of non-vitreous grains was greater than that observed for *Svevo*. Indeed, the number of totally vitreous grains was less than 1%. Therefore, the presence of puroindolines also appeared to increase grain porosity as the samples were grown in the same location and cultivation conditions. These results were in agreement with previous studies undertaken on common wheat (hexaploid) near-isogenic wheat lines differing only in the allelic form of puroindoline b or presence/absence of the *Hardness* locus, and thus on grain hardness (Morris and Beecher 2012; Oury et al., 2015). Indeed, grains carrying the wild-type puroindoline alleles generally did not display vitreosity levels above 60-70 %. Therefore,

these differences in texture and mechanical resistance of grains between *Svevo* and *Svevo-Pin* must have an impact on their milling behavior.

3.2. Puroindolines in durum wheat affected the grain milling behavior

An instrumented micromill previously developed to measure the energy (E_t in kJ/kg) at grinding (Pujol et al., 2000) was used to investigate differences in milling behavior of the two wheat samples. After identical tempering, a first grinding step (B1) was undertaken on grains as described in Pujol et al. (2000) and the recovered larger fractions from 2500 to 1000 μm were subjected to a second grinding operation (B2) with a higher roll speed and narrower roll gap to mimic the grinding step of a mill processing diagram. The corresponding grinding energies are reported in Table 2.

Table 2

At each milling step, *Svevo-Pin* was shown to require significantly less grinding energy, E_t , than *Svevo* (Table 2). The second break (B2) was found to require more energy than the first break (B1) for both samples, but they differed in their response. The energy increase between first and second break was around 40% for *Svevo-Pin*, whereas it was around 71% for *Svevo*. Taking into account the milling diagram, this difference reveals greater resistance to reduce the size of the largest particles from *Svevo* grain sample, and potentially to separate the starchy endosperm from the outer bran layers. In comparison with results of Pujol et al. (2000), the energy consumption at first break for the *Svevo* sample was in the order of those found with a hard common wheat or a durum wheat, whereas the energy of *Svevo-Pin* was clearly in the order of those obtained for a soft common wheat.

Differences in flour yield at each step also clearly distinguished the two grain samples. *Svevo-Pin* produced 4 and 1.5 fold greater flour yield in comparison with *Svevo*, depending on the grinding step. Flour yield at the first break for *Svevo* was in the same range as already observed for a durum wheat, whereas that of *Svevo-Pin* was increased to a level similar to that for a softer grain, *i.e.* a hard common wheat (Pujol et al., 2000). Finally, the total flour yield for *Svevo-Pin* was found to be higher than that of *Svevo*, which is consistent with the changes observed in the grain mechanical properties estimated through the PSI measurement. Therefore, the introduction of wild-type puroindolines into a durum wheat leads to a drastic reduction of the mechanical resistance of the grain and to a significant increase (around two fold) of flour production. These results are in accordance with previous studies on *Svevo-Pin* behavior using different milling devices (Murray et al., 2016; Quayson et al., 2016). The observations are also similar to the changes in milling behavior observed with another recombinant durum wheat line possessing both wild-type puroindolines (Gazza et al., 2011). It was furthermore shown that the energy required to produce the same amount of flour was significantly reduced by a factor of 5 to 3 fold, depending on the grinding step. Indeed, the required energies to obtain 1kg of *Svevo* flour (K') were in the range of values observed previously for durum wheat, whereas those measured for *Svevo-Pin* were closer to what was observed for a soft common wheat (Pujol et al., 2000).

Additionally, a distinct particle size distribution profile of flours produced from *Svevo* and *Svevo-Pin* was observed following measurement by laser granulometry. Indeed, flour from *Svevo* appeared mainly mono-modal with a peak centered around 155-160 μm corresponding to D50, whereas flour from *Svevo-Pin* was clearly bi-modal (Fig. 1). The first observed peak, around 20-25 μm , is known to correspond to isolated starch granules (Greffeuille et al., 2007b). The other peak was observed around 110-130 μm leading to a total D50 value of 80-88 μm .

Figure 1

These differences in particle size distribution were clearly related to wheat grain hardness (Greffeuille et al., 2007b) and thus linked to the nature of the puroindoline alleles (wild-type or mutated) as demonstrated in Greffeuille et al. (2006). Moreover, introduction of wild-type puroindolines appeared thus to increase the starch granule dissociation from the protein matrix in accordance with their potential role in reducing adhesion (Morris et al., 2002).

Related to the increase of flour production during milling from *Svevo-Pin* in comparison with *Svevo*, the coarse and fine semolina amount was reduced as expected. However, this reduction was not only explained by the corresponding increase of the flour amount, but also by the three fold increase in coarse bran production from *Svevo-Pin* in comparison with *Svevo*. This milling behavior appeared to be related to differences in grain hardness, as previously observed with near-isogenic common wheats differing in the wild-type or mutated form of Puroindoline b gene (Greffeuille et al., 2006). This result could reveal differences in the starchy endosperm-outer layers separation efficiency or distinct mechanical properties of the grain outer layers as already shown with near-isogenic common wheat lines (Greffeuille et al., 2007a).

3.3. Puroindolines in durum wheat affect the grain tissue distribution at break and the level of starch damage

In order to further analyze how the introduction of wild-type puroindolines affected the durum wheat grain milling behavior, biochemical markers, *i.e.* starch and phytic acid,

already found to correctly monitor the starchy endosperm and the aleurone layer (Hemery et al., 2009), were quantified in grains and in coarse bran and flours, respectively (Table 3).

Table 3

Although starch content did not differ between the two wheat grain samples (Table 3), starch content as well as proportion of grain starch recovered in coarse bran was found to be significantly higher in *Svevo-Pin* in comparison with *Svevo*. Therefore, a higher proportion of the starchy endosperm was not efficiently isolated in the flour or semolina fractions from *Svevo-Pin* and was instead recovered in the bran fraction. This could account for the increase in the total coarse bran production from *Svevo-Pin* in comparison with *Svevo*. Concomitantly, if no difference in phytic acid content was observed between the two grain samples (around 1.7%), flour from *Svevo* displayed a higher concentration of this biochemical marker in comparison with flour from *Svevo-Pin* suggesting a difference in the separation of the aleurone layer in the harder grains as already pointed out when comparing hard and soft common wheat cultivars (Greffeuille et al., 2005). Therefore, separation between the starchy endosperm and the outer grain layers appeared to differ between the two wheat samples probably as a consequence of the observed grain mechanical property changes linked with the wild-type puroindoline alleles introduction. The aleurone content enrichment of the harder sample in comparison with the one possessing the wild-type puroindolines could be related to the higher flour ash content observed previously (Murray et al., 2016).

Examination of the percentage of damaged starch in flours from *Svevo* and *Svevo-Pin* revealed a four to five fold higher amount in the former in accordance with the differences in hardness between the corresponding grains. These differences in damaged starch content were consistent with previous data comparing starch damage from *Svevo* and *Svevo-Pin* obtained

with different milling devices (Murray et al., 2016; Quayson et al., 2016). Differences in starch damage could explain differences in flour water absorption between a different durum wheat line possessing both wild-type puroindolines and its parent line (Gazza et al., 2011). These differences in level of damaged starch are also similar to those observed between hard and soft common wheat grains, which differ in the nature of the puroindoline alleles (Pena et al., 2002), and relate well with the mechanical role of wild-type puroindolines in adhesion impairment. Indeed, reduction of the adhesion between starch granules and the protein network in a numerical model mimicking the starchy endosperm structure clearly showed an impact on the level of starch damage (Topin et al., 2008, 2009).

3.4. Milling of Svevo and Svevo-Pin grown from distinct locations strengthen the findings

Svevo and *Svevo-Pin* were also grown at 11 locations in the U.S. and two independent replicate samples of each were analyzed with the instrumented micro-mill. Even though the protein content was shown to vary from 14 to 20% depending on location, *Svevo* and *Svevo-Pin* were not found to differ significantly (Fig. 2). Grain weight and test weight were also not different with means of 43.6 ± 4.6 and 42.6 ± 5.1 mg, and 78 ± 2.9 and 77.6 ± 3.0 kg.hL⁻¹, respectively, for *Svevo* and *Svevo-Pin*. Grain hardness, measured using the SKCS, however, was shown to vary both among locations and between the two genotypes, with *Svevo-Pin* being significantly softer than *Svevo* (Fig. 2). Hardness Index values of *Svevo-Pin* varied between 2.1 and 40.8 (mean 17.2 ± 9.7), whereas *Svevo* varied between 46.3 and 84.2 (mean 66.6 ± 10.7). Similar variation across locations, but greater effect of the genotype on SKCS values was in accordance with the results obtained by Oury et al. (2015) when comparing common wheat near-isogenic lines differing only in the Puroindoline b allele (wild-type or mutated).

Figure 2

Similar significant differences in milling behavior between *Svevo* and *Svevo-Pin* wheat samples, regardless of whether they were grown in U.S. or in France, were observed. The milling energies were 13.5 ± 2.2 and 11.6 ± 1.1 kJ/kg of grains at the first break, for *Svevo* and *Svevo-Pin* grains, respectively, and 27.9 ± 3.0 and 19.1 ± 1.9 kJ/kg of grains at the second break. Differences in the K' energies needed to produce the same amount of flour from the two wheat grain samples were even greater with mean and standard deviations of 624 ± 200 and 146 ± 20 kJ/kg of flour for *Svevo* and *Svevo-Pin* grains, respectively, at the first break, and 254 ± 47 and 95 ± 12 kJ/kg of flour at second break (Fig. 2). Therefore, the introduction of wild-type puroindolines in durum wheat dramatically reduced the energy required to produce flour on the first and second break rolls. Indeed, if the specific energy to produce one kilogram of flour from *Svevo* was found to be on the order of that already observed for a durum wheat cultivar (Pujol et al., 2000), that of *Svevo-Pin* was found to be closer to that of a soft common wheat cultivar. Scattering (variation across locations) of the K' values for *Svevo-Pin* was also found to be less than for *Svevo* suggesting a lower effect of the variation in grain texture due to the different growing location when wild-type puroindolines are present. This lower variation appears consistent with the low adhesion within the starchy endosperm between the starch granules and the protein network, and the lower mechanical resistance found in these grains.

Figure 3

Moreover, differences in the yield of the different milling fractions were also found to reveal a softer texture in *Svevo-Pin* in comparison with *Svevo* with an increase in flour yield

(around 2 fold) and coarse bran yield (almost 3 fold) with a concomitant decrease (1.4 fold) in semolina yield (Fig. 3 a-c). Biochemical analyses were also in accordance with the former observations made on grains produced in France. Indeed, total starch content in the coarse bran of the overall sample set was significantly higher (Fig. 3d) in *Svevo-Pin* ($33.7\% \pm 2.0$) than in *Svevo* ($26.2\% \pm 2.4$) whereas starch damage ($1.9\% \pm 0.3$ versus $8.3\% \pm 1.8$, Fig. 3e), and phytic acid content in total flour ($3.1 \text{ mg.g}^{-1} \pm 0.7$ versus $8.9 \text{ mg.g}^{-1} \pm 1.7$, Fig. 3f) were lower. These biochemical differences were similar to those observed between soft and hard common wheats carrying wild-type or mutated puroindoline alleles (Greffeuille et al., 2005; Pena et al., 2002).

Further analysis between milling and biochemical data showed a clear correlation between the quantity of coarse bran produced and the percentage of grain starch recovered in coarse bran ($R^2 = 0.89$ and 0.71 for *Svevo* and *Svevo-Pin* samples, respectively), revealing a clear effect on the starchy endosperm difference in extraction rate between the two wheat samples, which affects the amount of coarse bran production.

Therefore, the introduction of wild-type puroindolines into durum wheat clearly leads to an increase in grain softness resulting in a higher production of flour with less damaged starch, but potentially a less efficient separation between the starchy endosperm and the outer layers. The energy required to produce flour from *Svevo-Pin* was on the order of one-quarter to one-third that of the hard durum *Svevo*, illustrating the profound and technologically transformative effect of the *Hardness* locus on durum wheat kernel characteristics and milling.

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Figure Legends

Figure 1. Particle size distribution of flours, determined by laser granulometry and obtained after milling from the first break rolls (B1, closed symbols) or the second break rolls (B2, open symbols) of *Svevo* (diamonds) and *Svevo-Pin* (squares) grains.

Figure 2. Distributions of grain protein content (a, measured with NIRS) and SKCS Hardness Index (b) for *Svevo* and *Svevo-Pin* cultivated in different U.S. locations (n=22 for each boxplot, 11 sites, two replications). Distribution of milling energy (kJ) required at first break (c, K'B1) or at second break (d, K'B2) to produce one kg of flour from *Svevo* and *Svevo-Pin* cultivated in the different French and U.S. locations.

Figure 3. Total milling flour (a), coarse bran (b), and total semolina (c) yields from *Svevo* and *Svevo-Pin* cultivated in the different French and U.S. locations. Total starch content in coarse bran (d), content of damaged starch (e), and phytic acid (f) in total flour from the same wheat grain samples.

484 Fig. 1
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487 **Fig. 2**

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490 **Fig. 3**

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Grain characteristics	<i>Svevo</i>	<i>Svevo-Pin</i>	
Length [mm]	7.9 (0.5)	7.9 (0.6)	<i>n.s.</i>
Width [mm]	2.9 (0.3)	3.0 (0.3)	<i>n.s.</i>
Thickness [mm]	3.0 (0.3)	2.9 (0.3)	*
Weight [mg]	49.7 (11.8)	48.3 (10.8)	<i>n.s.</i>
PSI hardness	3.0 (0.5)	15.0 (1.6)	***
Non vitreous grains [%]	26.0 (2.0)	99.3 (1.2)	***

Table 1. Means and standard deviations (in brackets) for grain morphological traits and weight (water content 11.5 %) of *Svevo* and *Svevo-Pin* (grown in France). Mechanical (PSI) and textural (vitreousness) characterisation of the same grain samples. Differences of significance between values for an identical parameter were marked depending on their level in the last column.

Milling data	<i>Svevo</i>	<i>Svevo-Pin</i>	
E _t B1 [kJ/kg]	17.4 (0.5)	13.9 (0.2)	***
E _t B2 [kJ/kg]	29.8 (0.2)	19.4 (0.8)	***
K'B1 [kJ/kg flour]	780 (62)	153 (6)	***
K'B2 [kJ/kg flour]	288 (2)	103 (9)	***
B1 flour [%]	2.0 (0.0)	7.9 (0.8)	***
B2 flour [%]	6.7 (0.4)	9.8 (1.3)	***
Total flour [%]	8.7 (0.4)	17.7 (2.1)	***
Total fine semolina [%]	40.2 (2.4)	29.6 (0.7)	***
Total coarse semolina [%]	44.1 (1.6)	31.8 (2.5)	***
Coarse bran [%]	7.0 (1.2)	21.0 (0.3)	***

Table 2. Milling energies at each grinding step for 1kg of *Svevo* or *Svevo-Pin* grains (E_t), or corresponding produced flour (K') and yield of each recovered milling fractions in percentages of total grain weight. Values are the means of three independent millings on two distinct tempered grain batches. The standard deviation is given in brackets. Differences of significance between values for an identical parameter were marked depending on their level in the last column.

Biochemical composition	<i>Svevo</i>	<i>Svevo-Pin</i>	
Starch content in grains [%]	64.8 (1.0)	65.6 (2.4)	<i>n.s.</i>
Starch in coarse bran [%]	28.2 (0.8)	33.9 (1.2)	*
Starch proportion in coarse bran [%]	2.9 (0.5)	10.7 (0.1)	***
Phytic acid in grains [mg/g]	15.8 (2.5)	18.8 (2.0)	<i>n.s.</i>
Phytic acid in flour [mg/g]	10.2 (1.4)	3.5 (0.0)	*
Phytic acid proportion in flour [%]	5.7 (0.2)	3.3 (0.2)	***
Damaged starch in flour [%]	11.0 (1.2)	2.4 (0.2)	**

Table 3. Starch content in grains and coarse bran and proportion of grain starch found in coarse bran, phytic acid content in grains and total break flour and proportion of grain phytic acid recovered in flour and level of damaged starch. Results were means and standard deviation from two independent milling repetitions of the same sample. All the values were reported in dry mass. Differences of significance between values for an identical parameter were marked depending on their level in the last column.