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Grain Quality Traits and Bread-Making Characteristics of Old and Modern Italian Durum Wheat Varieties Grown Under Low Input Conditions in a Mediterranean Environment

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CICLO XXXII

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INTRODUCTION

Old durum wheat (*Triticum turgidum* L. spp. *durum* Desf.) cultivars were grown in Italy until the beginning of the 20th century. They are characterized by being tall (up to 180 cm), prone to lodging and late in flowering. By 1950, Italian breeders started to adopt intraspecific crosses techniques for durum improvement, from which they selected shorter pure lines, earlier in flowering, called intermediate varieties, which were still prone to lodging. Yet some studies do not differentiate between old and intermediate varieties and include them all under the umbrella of 'old varieties'. In the 1960s, the introduction of the *Rht-1* dwarfing genes, responsible for a drastic reduction in plant height and an increase in the harvest index for similar biomass levels generated the so-called Green Revolution. Semi-dwarf cultivars or modern varieties are still grown today and their performance is conditioned by a high nitrogen fertilization. (Bozzini, 1970 and Giunta et al, 2007). The production of durum wheat in 2016/2017 was almost 40 million tons which makes durum wheat the second most produced wheat type, after common wheat, and the tenth most produced crop worldwide. Durum wheats kernel is generally considered the hardest kernel of all wheats, it is large, golden amber, and translucent (Elias, 1995, Taylor and Koo, 2015, De Vita and Pecchioni 2016).

Lately, a global interest in old wheats end-products is arising again. This was consequently to the re-introduction of low-input management practices for sustainable and resilient agricultural production systems (FAO 2017). In contrast with modern varieties, old cultivars cannot benefit from high density of sowing rates and high nitrogen fertilization rates due to their susceptibility to lodging and they are generally later in flowering than modern ones (Dexter, 2008 and Giunta et al, 2017) which make from them a crop more adapted to low fertilization agriculture system where the higher yield potential of modern cultivars cannot be achieved. Another reason behind the big attention taken by old wheats is the increase in consumer's demand for healthy products and their desire to revert back to traditional food (Guarda et al, 2004). Through a dilution mechanism, the higher grain yield feature given to modern varieties caused a decrease in grain nitrogen concentration and hence in proteins percentage (Motzo et al, 2004).

Wheat grain protein (accounting for 10-15% of grain dry weight) is composed of the structural proteins albumin and globulin (15-25%) and the storage proteins gluten (75-85%). This latter is divided into gliadin classified into four groups (alfa/beta, gamma and omega) and glutenin and its subunits (high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits (GS)) (Dexter et al, 1977 and Shewry et al, 2016). The amount of proteins in the grain and its partitioning into the different protein fractions and subunits depend on the amount of nitrogen available in each grain which is derived from the non-structural nitrogen accumulated by the crop at anthesis (Martre et al., 2003), and the uptake of soil nitrogen during the grain filling period.

Grain protein content, as well as grain yield, depend also on the plant grain number per unit area and the nitrogen available at anthesis to the mature grain which are all affected by the type of genotypes (especially when comparing old and modern varieties) studied, environment, management and/or the interaction of these factors.

Studies showed that a hot environment affects negatively the durum wheat crop yield and high temperatures (>35ºC) can shorten grain filling period and hence affecting the production of gliadin and glutenin and especially lowering the amount of glutenin and consequently increasing the ratio of gliadin

over glutenin (Fois et al., 2011 and Nuttall et al., 2015). Nitrogen fertilization could have different effects and this depends on the time when N is introduced. The later N is applied, the greater is the effect on protein content and the lower is the effect on grain yield (Sander et al, 1987).
The quality of durum wheat end products depends mainly on the grain total protein content and the amounts and composition of gluten proteins. Together, they determine the specific combination of dough elasticity, extensibility and viscosity and define the biophysical and functional properties of quality of the end-product. Gliadin is mainly responsible for the extensibility of dough while glutenin is responsible of the dough's tenacity or resistance to extension and its elasticity. When pasta is the final product, strong and tenacious gluten is needed for a firm and less sticky dough. In the case of bread-making, strong and extensible gluten is the prerequisite for obtaining an extensive viscoelastic matrix with good physical and handling properties (Dexter and Matsuo 1977; Mastromoteo et al, 2014 and Shewry and Tatham 2016).
The improvement of technological quality of durum wheat semolina was the second priority of breeding programs focusing mainly on pasta as end product and taking into account the amelioration of the following traits: protein content, gluten viscoelastic properties, semolina colour, kernel vitreousness and red colour and pasta firmness. The decrease in protein percentage caused by breeding programs triggered the work on improving gluten strength of modern durum wheat cultivars through the incorporation of more favorable combination of gliadin and glutenin (gluten fractions) alleles and especially the glutenin alleles HMW and LMW-GS. Subsequently, gluten strength improved as well as the ratio of tenacity over extensibility. Breeding for strong gluten and high quality of pasta negatively affected the genetic variability of gluten characteristics. Therefore it is believed that old varieties represent a potential source of genetic variation that could be useful for identifying the best gluten composition and level of strength suitable for each specific end-product (Edward et al., 2007; De Vita et al., 2007 and Subira et al., 2014).
Durum wheat is used in bread production in the Near East, Middle East, and Italy (Williams et al., 1984 and Williams, 1985). Several types of bread are made in Italy from durum wheat, depending the region (Quaglia, 1988). Bread made from durum wheat has an exceptional flavour and color, prolonged shelf life and high nutritional quality (Liu et al., 1996 and Fadda et al., 2014). The interest in developing a durum wheat with adequate bread making characteristics and pasta quality should not be disregarded, considering the potential benefit in the international market. A good bread-making process requires a balanced ratio of tenacity over extensibility. However, modern durum wheat varieties possess a standardized strong gluten leading most researchers to consider these varieties responsible for a tight and inextensible dough, resulting in dense bread (Quaglia et al., 1988; Ammar et al., 2000 and Guzman et al., 2016). In consideration of the increased awareness on environmental and biodiversity preservation, we believe that old varieties constitute an interesting pool to find quality traits suitable for bread making.

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OBJECTIVES

My PhD thesis is divided into three independent chapters and the objectives of each work were:

The first chapter is a published review, in the Journal of the Science of Food and Agriculture, entitled, 'From Ancient to Old and Modern Durum Wheat Varieties: Interaction among Cultivar Traits, Management and Technological Quality' and discusses thoroughly the evolution of ancient to old and then modern durum wheat varieties in terms of agronomy, genetics, technological and end-product qualities and the role that could play old durum wheats in modern agriculture in terms of agriculture systems and types of end products. The second and third chapters of my thesis are experimental studies.

The second chapter, is a published article, in the Journal of the Science of Food and Agriculture, entitled, 'From seed to bread: variation in quality in a set of old durum wheat cultivars' and had as objectives to analyze the morphological, phenological, productive and qualitative traits of a set of fourteen old Italian durum wheat genotypes, and one modern cultivar (used as a reference point) chosen from diverse origins and eras of breeding to represent the different phases of durum wheat evolution in Italy with the aim of analyzing their bread-making potential under two low rates of N fertilization and to examine whether and if so, which quality traits of the grain influence the quality of the semolina, the dough and the end-product (i.e. the bread). The use of the modern variety was crucial in order to investigate the genetic variability that exists within old cultivars.

The third chapter tackled in my thesis with the title: 'The key role of grain number in the determination of grain nitrogen content and composition in durum wheat cultivars grown under low input conditions in a Mediterranean environment', aimed at examining the role of grain number and nitrogen absorbed and translocated by the crops in the variation of grain protein content of an Italian set of twelve old, two intermediate and two modern durum wheat cultivars specifically selected for their good to high grain quality and grown in a low input environment and assessing the role of genotypes in the accumulation of nitrogen in the grain and its partitioning to the different wheat proteins fractions and subunits.

CHAPTER 1

From Ancient to Old and Modern Durum Wheat Varieties: Interaction among Cultivar Traits, Management and Technological Quality

This chapter is a published review in the Journal of the Science of Food and Agriculture; J Sci Food Agric 99:2059–2067 (2018) - DOI 10.1002/jsfa.9388

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Abstract
Following the boom in durum wheat breeding, ancient wheat disappeared from the human diet and old durum wheat varieties were replaced by what is believed to be their better versions: higher yielding modern varieties grown in high input systems. Although breeders have worked intensely ever since to improve the quality of durum wheat traits, mainly gluten subunit alleles, in order to obtain a superior technological quality of the main durum wheat end products (first pasta and then bread), conflicts about predicting their quality still exist; this is because quality is neither governed by one trait alone nor conditioned by a single controllable factor. What is also fascinating is the obsession of today's population for ancient wheat varieties, fueled by eating healthy trends and increased environmental preservation awareness suggesting that consumers are not satisfied with what modern agriculture is offering. The evolution of ancient to old and then modern durum wheat varieties, in terms of agronomy, genetics, technological and end-product qualities, is tackled in this review. The environmental effects will not be discussed.

Keywords: emmer, breeding, evolution, pasta quality, bread quality

INTRODUCTION

Nearly 40 million tons of durum wheat (*Triticum turgidum* L. spp. *durum* Desf.) were produced in 2016 around the globe, making it the second most important wheat type, after common bread wheat, and the tenth most important crop worldwide[1]. The Mediterranean region forms more than half of the wordwide durum wheat growing area.[2,3,4] Durum wheat is always allocated to lower-yielding environments, whereas bread wheat is sown in higher-yielding areas under the assumption that durum is more tolerant than bread wheat to environmental stresses.[5,6,7] Recently,[8] reversed this assumption and demostrated that durum wheat is more efficient than bread wheat under high yielding conditions, opening up the possibility of enlarging the area devoted to this species. Italy is the second biggest producer of durum wheat in the world, after Canada, generating 4.4 million tons from an area amounting to half the total EU area dedicated to durum wheat cultivation.[9] The leading role of Italy in durum wheat production is partly attributable to the economic importance of the pasta industry in this country, which has fueled the intense breeding work carried out in Italy since the beginning of the 20th century.

Despite their lower grain yield compared with modern cultivars, reasons for the renewed consumer and market interest in old varieties include their supposed greater sustainability and better nutritional profiles. In this review we will address how the management and the agronomical (grain characteristics) and technological qualities of the main durum wheat end-products have changed over time, from the ancient to the modern durum wheat cultivars, taking into account the complex interactions between cultivar traits, management practices and technological processes. The role of ancient and old durum wheat cultivars in modern agriculture, in terms of agricultural systems and types of end products, will also be discussed.

FROM ANCIENT TO MODERN DURUM WHEAT: NOT ONLY A CHANGE IN GENOTYPES, BUT ALSO IN THE TYPES OF CULTIVARS GROWN AND THEIR MANAGEMENT

The populations of wheat plants (cultivars) grown nowadays are very different from their wild progenitors and from domesticated and cultivated populations. Over the centuries, wheat populations have been subjected to continuous

genetic selection; either unconscious selection, as a consequence of the actions of the first farmers who simply chose the best seeds for the following season, or conscious selection, as carried out by breeders by applying the genetic laws of inheritance and modern techniques to guide wheat evolution towards populations that are better adapted to certain environments and management techniques, more productive and of better quality.[10]

The first step was the domestication of wild progenitors. The modification of certain morphological as well as physiological traits to meet human needs and to adapt to agricultural practices occurred and distinguished domesticated wheats from their respective wild progenitors.[11] The traits modified, together referred to as the 'domestication syndrome', were: loss of spike shattering at maturity to prevent seed loss at harvesting; conversion of hulled kernels into a free-threshing form through the loss of tough glumes; increase in seed size; and reduction in tiller number.[4,12]

Wheat is an autogamous species, whose natural population are constituted by a mixture of several different pure lines. When cultivated, these genetically heterogeneous populations evolve under the pressure of farmer and natural selection in the specific environments in which they are grown, giving rise to the so-called ***'landraces'***.[10]

Ancient wheats

The term ***'ancient wheat'*** is generally used in reference to the wheats cultivated by ancient civilizations following their domestication. *Triticum turgidum* L. spp. *dicoccum* Schrank ex Schubler (emmer wheat) is the ancient durum wheat which represented the transition from the wild tetraploid spp. *dicoccoides* (wild emmer wheat) to durum wheat.[13]

Emmer wheat was one of the first crops to be domesticated.[14] Its cultivation started approximately 8000-10000 years ago during the 'Neolithic Revolution' in the Fertile Crescent zone, an area in the Middle-East that stretches from Palestine, Jordan, and Lebanon to Syria, Iraq, and Iran, where its wild ancestors can still be found; from there, it later spread on to Asia, Europe (Italy in particular) and Africa.[12,13,15,16,17] Emmer was the most widespread wheat species during the Neolithic and Bronze Age periods and was a staple food for the Babylonians, Assyrians and Egyptians, who were the first to make oven-baked bread. Its cultivation started to diminish at the end of the Bronze Age, and by the beginning of the 20th century it had been almost completely substituted by the derived free-threshing species, durum and bread wheat. In Italy, emmer was still the main food for the Romans and it was the most cultivated of the three ancient species (emmer, einkorn and spelt).[17,18,19]

The first landraces of emmer were replaced by cultivars based on single pure lines with the aim of improving adaption to marginal areas and low-input systems.[17] More recently, emmer breeding programs have been implemented aimed at the introgression of certain durum wheat traits into emmer by interspecific hybridization: emmer × durum wheat which generated a new set of modern emmer cultivars.[20] The cultivars grown in Italy today are: landraces (Garfagnana, Amatrice, Molisana, Prometeo, etc.); pure lines selected from landraces (Farvento, Lucanica, Molise selection Colli, ecc.); and, most recently, modern emmer cultivars (Davide, Mosè, Padre Pio).[20,21,22] In 2016, nine emmer cultivars were present in the Italian National Register of Cultivars.[23]

Old durum wheat cultivars

Until the beginning of the 20th century, landraces were the only types of emmer and durum wheat cultivars being grown. In Italy, a plethora of durum wheat landraces could be found, mainly in the South and on the Italian islands (Sardinia and Sicily). The most diffuse landraces before 1925, according to De Cillis (1927),[24] were *Rossia, Biancuccia, Sicilianu, Saragolla siciliana, Trigu arrubiu, Trigu biancu, Duro di Puglia, Realforte, Sammartinara, Russello, Scorzonera and Trigu murru.* Most of them belonged to the Mediterraneum type[25] and were tall (up to 180 cm), prone to lodging and late in flowering. Subsequently, other landraces arrived in Italy from the Near-East and North Africa, including some Syriacum types, which were shorter and earlier in flowering,[25] such as *Azizah,* and *Eiti*.[26]

Italy was the first country within the Mediterranean basin to begin 'conscious' durum wheat breeding. The first step was extraction of the single best pure lines from the heterogeneous landraces. This led to an increase in genetic uniformity within the new types of cultivars, which were constituted by a single highly homozygous genotype (pure line). These new cultivars gradually replaced the landraces in Italy, while other countries continued to grow durum wheat landraces until the advent of the Green Revolution in the late 1960s.[27] The cultivar Senatore Cappelli, a pure line belonging to the Mediterraneum type extracted from the North African population Jean Retifah in 1915, was the most outstanding of this period. It rapidly became diffuse, not only in Italy, where it was cultivated on 60% of the total durum wheat-growing area, but also in other Mediterranean countries (e.g., Spain and Turkey) thanks to its higher yields and better quality compared with the other cultivars available in that period (*Dauno, Timilia, Russello, Triminia, Biancale*).[28,29]

By 1950, Italian breeders began to utilize intraspecific crosses, mainly mediterraneum × syriacum[25,26] for durum improvement, from which they selected earlier and shorter pure lines, which were, nevertheless, still prone to lodging (*Capeiti, Patrizio, Casale, Castelporziano, Ichnusa, Maristella, Trinakria Appulo*). Senatore Cappelli was widely used in these crosses, as well as in later ones, such that more than 80% of the cultivars present in the Italian Register of durum wheat cultivars in 1987 had this cultivar in their pedigree.[30]

Modern durum wheat cultivars

The progressive release of more productive cultivars with improved qualities occurred in synchrony with developments in agriculture and management practices. It was the synergy between breeding and management which generated the most remarkable results with the introduction of the *Rht-1* dwarfing genes, responsible for a drastic reduction in plant height and an increase in the harvest index for similar biomass levels.[31] The new semi-dwarf cultivars, shorter and more resistant to lodging, were able to exploit high nitrogen rates – in the same period, industrially produced ammonia (via the Haber-Bosch reaction) replaced animal waste as the primary source of fertilizer – and were better suited to the changes coming about in agricultural practices through increased mechanization. In the 1960s, the substantial increase in grain yield obtained with the semi-dwarf cultivars generated the so-called Green Revolution, which spread in the following years from developed to developing countries. CIMMYT® (www.cimmyt.org, International Maize and Wheat Improvement Center), an internationally funded, not-for-profit organization that conducts research and training related to maize and wheat throughout the developing world, was the main actor in this phase. In 2002, about 90% of the total area planted with durum wheat in developing countries was occupied by semi-dwarf cultivars, and 95% of these cultivars contained CIMMYT germplasm.[32]

Semi-dwarf cultivars are still grown today and the discontinuity between them and the preceding cultivars justifies the distinction between ***'old'*** and ***'modern'*** durum wheat cultivars.[33] The initial richness in landraces, their use in breeding programs in the first half of the 20th century, and the intense breeding work carried out in that period make the Italian durum wheat gene pool the most outstanding and diverse in the Mediterranean basin.[34] This is also reflected in the high number of durum wheat cultivars present in the Italian National Register (235 in 2016). The main goal of the breeding programs involving modern cultivars was to increase grain yield. Preserving or improving the grain quality traits was their second priority, with a prime focus on pasta-making.[35]

Cultivar, management and grain protein content

Emmer landraces, old and modern durum cultivars differ in certain traits that have a profound impact on their management and on grain quality. Landraces and old cultivars are characterized by their lower yield potentials compared with modern ones in both low and in high input systems.[31] They cannot benefit from high sowing rates and high nitrogen fertilization rates due to their susceptibility to lodging[31] and they are generally later in flowering than modern ones.[36] These traits make them suitable to low input systems and marginal areas where the higher yield potential of modern cultivars cannot be fully expressed.

As far as quality is concerned, all grain quality evaluations begin with the characteristics of the raw material, including grain hardness, yellow colour and protein content, composition and aggregation levels, which influence the dough characteristics and the quality of the final product. Grain protein percentage contributes the most (40%) to the EU Quality index for durum wheat (European Commission Regulation No. 2237/2003, 23 Dec 2003) and can influence the amount of money paid to wheat farmers due to the importance of this trait for the quality of both pasta and bread. Grain protein percentage can vary as a consequence of genotype, environment or management, or an interaction of these factors. Although 'environment' represents the main source of variation when modern cultivars are considered[37,38,39] the aim of this review does not include a discussion of the environmental effects; it will instead only consider genotypic and management effects.

Genotypic variation in protein percentage is usually high when old and modern cultivars are compared[31,40,41,42] this is due to the constantly lower protein percentage of modern cultivars. Depending on the environmental conditions and genetic composition of the groups tested, protein percentage has been found to be about 1 to 1.5% lower in modern cultivars compared with landraces[31,40,41,42] corresponding to a decrease of annual rate ranging from -0.14 to -0.19% year^{-1}.[40,42]

The decrease in grain protein percentage observed in modern durum wheat cultivars compared with older constitutions cannot be analysed, as is often done, without addressing the corresponding variation in productivity, as the two traits are generally negatively associated.[10,31] Hence, the negative association between protein percentage and year of cultivar release should be considered as a consequence of the improvement in grain yield of durum wheat brought about via the introgression of *Rht* genes and the consequent increase in harvest index and in grain number per unit surface as already discussed in Giunta et al. (2007).[31]

Management practices can modify grain protein percentage and the most influential factors are nitrogen and sulphur fertilization and sowing date.[43]

Nitrogen fertilization has different impacts depending on the type of cultivar. Modern cultivars need a higher fertilization rate than old ones to reach their maximum grain yield[44] (up to 100-120 kg N ha^{-1}). Even higher – and often anti-economic – fertilization rates are sometimes needed for these cultivars to increase their grain protein percentages, because maximum grain protein percentages are achieved with higher nitrogen availabilities than maximum grain yields.[45] The lower grain protein percentage of modern compared with old cultivars therefore derives, at least in part, from inadequate nitrogen fertilization. Old cultivars, on the other hand, are able to realize high protein percentages even at low nitrogen inputs thanks to their low productivity, and excessive nitrogen availability can be even deleterious to them as it causes lodging. In both cases, late N applications are expected to improve grain protein percentage more than grain yield because the later the application of N, the greater its effect on grain protein content and the lower its effect on grain yield.[46]

Sulphur plays a key role in the technological quality of wheat[43,47,48,49] mainly associated with changes in the quantitative composition of sulphur-rich *vs*. low-sulphur subunits of gluten proteins[50] and the number and distribution of the disulphide bonds responsible for the aggregation level of gluten protein subunits.[51,52] Over the last decades, several factors have contributed to the increase in the area affected by sulphur deficiency in agricultural soils,[53,54,55] which can be alleviated by proper fertilization. Since the high grain yield of modern cultivars is one of the causes of sulphur deficiency, old cultivars are expected to contribute less and be less affected than modern ones by this problem.

In general, delayed sowing has adverse effects on grain yield, but positive effects on grain protein content. For example, delaying the sowing of modern Italian durum wheat cultivars from October to March reduced grain yield, but increased protein percentage from 10.7 to 14.7%.[36] In a comparison of old *vs*. modern cultivars[56], moving the sowing date from November to March caused the protein percentage in the old cultivar Senatore Cappelli to increase by 2.7%, but the increase was only 1.3% in the modern cultivar Svevo.

TECHNOLOGICAL QUALITY OF DURUM WHEAT: THE ROLE OF PROTEINS AND THE CHANGES INDUCED BY BREEDING

End-use and technological quality

The economic value of durum wheat cultivation is strongly supported by its wide range of end-products.[57] The determining factors of the overall quality of each durum product is explicitly customized and depends on the transformation status of the grain (cracked grain, milled semolina, etc.), the cooking or baking process and the interaction with other ingredients required, if any.[27] When wheat grains only undergo dry processing (de-branning, milling) to obtain the end-product, as is the case for many emmer end-products, the quality depends on the hardness and nutritional profile of the grain and on the level of yellow colour of the cooked grain. When grains undergo wet processing (dough fermentation, extrusion, baking, etc.), as in the production of pasta and bread, the chemical and rheological properties of semolina are the key quality determinants, measured using dough rheology instruments, such as the farinograph, extensograph, mixograph and alveograph.[37,58,59,60]

Gluten and its breeding-induced changes

Wheat gluten proteins – the major group of grain storage proteins found in the starchy endosperm - are responsible for the unique properties of wheat exploited in bread making; although gluten is also found in other cereals, like barley and rye, it does not confer the strength and elasticity needed for stretching dough and trapping the CO_2 bubbles formed during fermentation in leavened bread-making as occurs in wheat flour dough.[51,52,61,62,63] Wheat gluten proteins are classified into alcohol-soluble monomeric gliadins and alcohol-insoluble polymeric glutenins (high molecular mass polymers with subunits assembled by disulphide bonds). [52]

When wheat flour is mixed with water to obtain dough, gluten proteins form a continuous network, with gliadin responsible for the extensibility of dough and glutenins responsible for the dough's tenacity or resistance to extension and its elasticity.[54] Total protein content (accounting for 10-15% of grain dry weight) and the amounts and composition of the gluten proteins (which form up to 80% of the total proteins) determine the specific combination of elasticity, extensibility and viscosity, which define the biophysical and functional properties of dough and the quality of the end products. [52,65,66]

A high protein and gluten content or a medium protein content with strong gluten is needed for optimal baking properties.[42] **Gluten strength** is an indicator of the gluten viscosity and elasticity and describes the ability of the proteins present in the grain to form a satisfactory network in terms of continuity and strength and governs its suitability for end-use production.[62] When pasta is the final product, strong gluten is needed for a firm and less sticky

dough. In the case of bread-making, strong and extensible gluten is the prerequisite for obtaining an extensive viscoelastic matrix with good physical and handling properties.[62]

Given similar protein percentages, gluten strength depends on the types of glutenin and gliadin proteins present (genetically determined) and on their ratio, the ratio of high molecular weight (HMW) to low molecular weight (LMW) glutenin subunits (GS), and the amount of unextractable polymeric proteins (UPP).[38,62]

Gluten strength can be evaluated using a Glutomatic analyzer, which measures the gluten index (GI) and the wet and dry gluten content of a flour sample independently of protein content, or using the SDS-sedimentation volume test, the results of which, however, is affected by protein content and is thus only considered as reliable when comparing varieties with the same protein percentage.[37,67] Gluten strength is also strongly dependent on the percentage of UPP in the total polymeric protein fraction, which measures the amount of the largest glutenin polymers, which are likely to shift the balance of the molecular-weight distribution towards stronger dough properties.[51] GI is only strongly associated with UPP% when the latter exceeds 35%; hence, GI cannot be used as a reliable index of strength for genotypes with lower UPP%, corresponding with GI values less than 10.[37]

The improvement in gluten strength in the modern durum wheat cultivars through the incorporation of more favourable alleles for both HMW and LMW-GS[37,38,40,42,68,69,70] counterbalanced the above mentioned significant decrease in grain nitrogen and protein percentage brought about by breeding for higher yields. More precisely, gluten quality was changed by incorporating specific combinations of alleles, as no single allele, no matter how strength-enhancing, is absolutely necessary for adequate strength.[71] Modern durum wheat cultivars are characterized by greater expression of LMW-GS contributing to a higher glutenin to gliadin ratio.[56,72,73] As a consequence, gluten strength as assessed by means of the GI rose from the very low values typical of most landraces (6-32%) to as high as 55-87% in modern cultivars.[41,56,73] Gluten strength as evaluated using the SDS sedimentation test also rose, increasing by 30% and 26% in modern Italian and Spanish durum cultivars, respectively, in comparison with their old equivalents.[40]

The gluten in emmer wheat is considered weak because it lacks the necessary allele combination for strong gluten subunits. The main goal more recent emmer breeding programs, was to improve protein composition and the rheological properties of dough.[20] De Vita et al, (2006)[20] showed the resulting modern emmer varieties Mosè and Padre Pio to have a satisfactory GI and alveograph parameters.

Although gluten strength is a major contributor to the quality of durum wheat, it is just one of several quality attributes, and its impact on the overall quality of the various end-products obtained from this species is highly dependent on the specific end-product considered, which may require different levels of gluten strength.[71]

Breeding for quality has also affected the genetic variability of gluten characteristics. A study comparing modern durum wheat cultivars with a large set of landraces representative of the genetic diversity of ancient local durum populations from the Mediterranean Basin was able to detect 76% of the 173 theoretical different allelic/banding pattern combinations for glutenin composition in the landraces. This confirmed a level of genetic variability in the landraces that is much larger than that in the set of modern cultivars with regard to gluten strength.[71] In the same study, the various allelic combinations present in the group of modern cultivars did not affect gluten strength, probably due to the fixing of this trait to high levels through selection. Therefore, landraces represent a unique source of genetic variation that is potentially useful for identifying the best glutenin composition for each specific end-product. More recently, Vita et al. (2016)[2] highlighted the existence of high genetic variability both within and between Mediterranean landraces and modern cultivars of durum wheat in both gliadin and glutenin composition and identified distinctive profiles within both groups of cultivars.

PASTA-MAKING QUALITY: IS THE COMBINATION OF STRONGER GLUTEN AND A LOWER PROTEIN PERCENTAGE (MODERN CULTIVARS) ALWAYS BETTER THAN THE OPPOSITE COMBINATION FOUND IN OLD CULTIVARS?

The art of pasta cuisine was first developed in the 14th century and during the Renaissance Period pasta became a staple food in the Italian diet.[74] Pasta is considered a healthy food as it is low in fat, has a good protein content and is high in slow digestible carbohydrates and thus has a low glycemic index.[75,76,77] Being the best ingredient for pasta-making, only durum wheat can be used, by law, for pasta production in Italy, France and Greece.[75,77] The estimated production of pasta worldwide is 14.3 MT per year, with Italy being the largest producer (3.2 MT), followed by the US (2 MT).[78] Italy is renowned worldwide as the leader in top quality pasta production and export[2] and, not surprisingly, Italians are the major consumers of pasta worldwide, with consumption in 2014 reaching 25.3 kg per capita.[79]

Starting from semolina, pasta is produced through hydration and mixing, sheeting or extruding, and drying.[80] Cooking is the ultimate step left to the consumer. A minimum of 12-15% of protein content is required in

manufactured pasta as it secures a semolina with uniform particle size producing an elastic, resilient, non-sticky, and firm cooked pasta, offering an 'al dente' texture. [64,65,75,77,81] However when protein content is intermediate to low, strong gluten is needed for preserving the quality of pasta. Indeed, a strong gluten durum variety confers a less sticky dough with superior texture properties compared with a variety with the same protein level but weak gluten; this is because gluten confers the tenacity needed to retain gelatinized starch granules during cooking.[62,64,81,82]

Most studies comparing the technological behaviour of old and modern durum wheat varieties for pasta-making confirm that gluten strength and dough tenacity improves following the introgression of the superior quality gluten alleles into modern varieties.[40,42,73] On the contrary, in the few studies that have directly compared the end-product spaghetti made from old *vs*. modern cultivar semolina, the lower protein percentages of modern durum wheat cultivars result in a lower or similar pasta cooking quality, despite their higher gluten strength.[56,77] This discrepancy could be due to the fact that the relative importance derived from the quantity and quality of proteins also depends on the drying temperature. One of the main developments in pasta technology has been the introduction of high temperature (HT) drying (60°C to 80°C) and ultra-high temperature (UHT) drying (80°C to 110°C), [83] which have influenced durum wheat quality specifications. HT and UHT drying produce pasta of acceptable or even superior cooking quality as it is governed by protein content only while the quality of pasta dried at low temperatures (<60°C) is governed by both protein content and gluten content quality; [84] this is because a high drying temperature induces the creation of a protein coagulation network, which traps starch molecules during cooking.[85]

Enhancing the carotenoid content of modern cultivars through the introgression of a yellow pigment gene (Yp) was an important objective for breeders as pasta colour is a very important parameter in the competitive pasta market.[35] Several studies have also confirmed that modern durum wheat varieties have a higher yellow index than the old ones; [40,86] conversely, De Vita et al. (2007)[42] did not find any significant difference between the carotenoid content of old *vs*. modern durum varieties.

According to De Vita et al. (2006),[20] when dried at high temperature, emmer wheat – with its high protein content – could be a suitable material for producing a good quality of cooked pasta. Modern emmer have a better yellow index than their respective landraces, whilst conserving a high protein percentage; some, such as Padre Pio, also possess one of the strong HMW-GS allele found in today's modern durum wheat varieties and associated with its good technological quality for pasta-making.

BREAD-MAKING: HAS BREEDING FOR BETTER PASTA HAD A NEGATIVE IMPACT ON THE BREAD-MAKING QUALITY OF DURUM WHEAT?

The high protein content, yellow colour and long shelf-life of durum wheat make it an appealing ingredient for preparing bread, a food that has been indispensable throughout the ages and a major source of our daily energy and protein requirements today. [17,64,87,88,89] Emmer bread was consumed by ancient Egyptians and is still the main end-product of this ancient durum wheat still diffuse in Italy ('*pane di farro*') and Switzerland. Indeed, old cultivars were used for bread-making since the beginning of its practice dating back to 500 BC and durum breads are still diffuse throughout the Mediterranean region. In the south of Italy,[90,91,92] a vast number of different breads are found. Sardinia for instance, offers dozens of delicious traditional durum breads, leavened (Moddizzosu, Civraxu, Coccoi, Tunda, etc.) or flat (Pane Carasau, Pane Fresa, Pistoccu, Spianadda, Zichi, etc.) sold at high prices. Durum wheat semolina is also used to make certain speciality breads that combine dough with other ingredients that may be savoury (oil, potatoes, olives, etc.) or sweet (honey, saffron, almonds, etc.), depending on the season.[93]

The search for good durum wheat cultivars for the production of the classical leavened breads started in the beginning of the 20th century, with no major success until 1950, when durum wheat started to be blended with common wheat hoping to achieve a good loaf volume.[94] Again, gluten strength was the main objective of the breeders since the elasticity required to support CO_2 bubble formation during dough fermentation is provided by gluten.[51,61,62,63] The main problem encountered when durum wheat is used for bread-making, however, is the unsatisfactory loaf volume obtained (in comparison with bread wheat) because durum gluten is more tenacious but less strong and elastic and it lacks the glutenin-D genome that confers extensibility and strength to common wheat dough.[65,94,95,96] Studies aimed at identifying the best allelic combinations for obtaining satisfactory durum breads.[37,42,70,97,98,99,100,101,102] Edward et al, (2007b)[38] gave contrasting results, but they did not exclude the potential for obtaining bread of good quality.

Breeding programs were, and still are, interested in finding a dual purpose durum variety in an attempt to ameliorate the quality of flour blends. Geneticists worked on transferring the glutenin-D genes from bread wheat into durum wheat in order to improve gluten and dough strength and the resulting bread quality; this approach was more successful than substituting the glutenin alleles of durum wheat.[64,70,96] Another attempt to ameliorate durum wheat bread-making characteristics was made by crossing strong durum wheat with a weak emmer wheat characterized by

its high extensibility. The new born varieties improved extensibility and baking quality, while preserving their characteristics for good pasta.[37]

A concern that arose about the breeding carried out on durum for bread-making is that it always aims at producing the most typical form of leavened bread, the one commonly made with bread or common wheat. This is probably justified by the fact that the majority of cultivated wheat is common wheat, and the majority of bread types worldwide are produced from common wheat, which therefore represent a 'reference bread'. On the other hand, durum wheat was and still is used to produce innumerable traditional local breads of high quality, although their quality criteria are different from those considered for common bread. As a consequence, whilst breeding has standardized the bread quality definition, it has also reduced the value of old durum biodiversity available and its economic potential. Diversity is also important for another quality trait relevant in bread-making: bread aroma. The analysis of aromatic profiles is very complex and is often influenced by compounds present at very low concentrations and hence difficult to detect. The latest improvements in the analytical technology available for the characterization of volatile organic compounds (VOC) enabled Vita et al. (2016)[2] to highlight quantitative differences in the VOC profiles of bread produced with old compared with modern durum wheat cultivars, and to hypothesized a possible genetic control of these differences.

BACK TO THE FUTURE: ANCIENT AND OLD WHEATS IN MODERN AGRICULTURE

Given their low productivity, any possible role of ancient and old durum wheats in modern agriculture should be based on the greater sustainability of their cultivation[103] on the quality of their end-products and on their rich genetic variability. Their low nitrogen requirements make them a valid choice for rotation with legumes in small-areas of rainfed cereal systems in Mediterranean environments when low-input or organic farming methods are adopted as a consequence of either low soil fertility and/or the conservative attitude of the farmers.[104] Many of such areas, once extensively cultivated, are at risk of abandonment since they cannot be adapted to exploit the high yield potential of modern cultivars; indeed, old and ancient cultivars may offer the possibility of recuperating them.[103]

The limited productivity and diversity of these grains produced under these circumstances should be valorized through the production of traditional and/or innovative products. Traditional breads made with traditional technologies (e.g., stone milling) seem to be the best choice for old durum wheats, whereas the range of possible emmer end-products is more ample. Emmer is nowadays used for making bread, pasta, biscuits, cakes and cookies (mixed with other flours, such as durum or common wheat flour, oat flour, etc.), as whole or cracked grains for salads and soups, and even in beer production in Germany.[18] The three ancient wheat species (Einkorn, Emmer, Spelt) mixed together are known under the name 'Farro', and sold as whole or pearled grains.[4,19] The marked demand for these types of products is high as a consequence of the increased awareness of consumers about traditional and sustainable agricultural products. Indeed, it is hoped that the high demand for ancient wheat products will guarantee favourable prices for these crops and thus farmers' incomes and safeguard the precious large genetic diversity of these populations.

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CHAPTER 2

From seed to bread: variation in quality in a set of old durum wheat cultivars

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Abstract
Old durum wheat varieties are being appreciated again because of their interesting genetic diversity and low fertilizer needs. The agronomic and bread-making performances of fourteen old Italian durum wheat varieties grown under two low nitrogen inputs (46 and 86 kg ha^{-1}) were determined and the relationships among grain, semolina, dough and bread quality parameters were established. The old varieties yielded similarly to the check modern variety Svevo under both nitrogen levels. Increasing nitrogen fertilization from 46 to 86 kg ha^{-1} did not increase grain yield or the mg of N in the grain, although grain protein percentage increased due to a decrease in grain weight and increase in gliadin content. Despite a resulting decrease in the gluten index, dough and bread quality improved at the higher N rate, highlighting the influential role of protein percentage and gliadin in bread quality. The genotypic variation in grain protein percentage among old varieties was more strongly associated with glutenin than with gliadin content. Variation in the gluten index was high (4-54), indeed it was the most variable semolina parameter, and proved to contribute the most to variation in bread quality. This variation was independent of the glutenin alleles (HMW 20, 20*, 7, 13+16, 6+8) and was linked to the quality of the grain in terms of grain weight and the associated mg of N per grain. Remarkably, two old varieties, namely Calabria and Cappelli, were able to produce both a good yield and high quality bread. Old Italian durum wheats continue to boast significant biodiversity and are worth exploring in low-input production systems.

Keywords: gluten; gliadin/glutenin; dough rheology; old durum wheats; bread

Introduction

The use of durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.) in bread-making has a long history in many Mediterranean countries, where the art of baking durum bread constitutes an integral part of the culture[1,2] due to its attractive features: unique color and nutty taste, uniform crumb structure [3,4], and low staling characteristics.[5,6]
The evolution of durum wheat, via the replacement of old varieties with modern ones, was driven by the goal of producing high yielding cultivars with strong gluten suitable for the pasta industry, but led to a decline in the bread-making features of durum wheat since the key determinant of bread-making quality is a balanced ratio of tenacity over extensibility.[7-9] This led to a consensus among most researchers in considering modern durum wheat varieties unsuitable for making-bread as a consequence of the tenacity of their gluten that produces tight, inextensible dough and low loaf volumes, resulting in dense bread.[1,7,10] Only Edwards et al. (2007)[11] have sustained that modern durum varieties can generate dough with good extensibility suitable for baking.
Recently, old varieties have attracted renewed attention due to the re-introduction of low-input management practices for sustainable and resilient agricultural production systems.[12] This is because old varieties have lower nitrogen requirements than modern ones. Consumer interest in old varieties is also growing due to the desire to revert back to traditional and organic products perceived to be 'safer' and

'healthier' foods.[13] Today consumers are even changing their perception of what 'tasty' foods are. Consequently, the popularity of traditional home-made durum bread is once again spreading across the globe.[14]

The quality of bread is determined by the protein content and protein composition of semolina, specifically the content and ratios of the two gluten fractions, the gliadins and glutenins, and of their high molecular weight (HMW) and low molecular weight (LMW) subunits. In certain environmental conditions, protein content varies significantly depending on the cultivar grown and the management of the crop, particularly the rate and time of nitrogen (N) application. As a general rule, grain protein (GP) content increases with increasing N fertilization rate. The later N is applied, the lower its effect on grain yield (GY) and the greater the increase in GP percentage.[15] The change in GP percentage by rate and time of N application may also affect the relative proportions of the gluten fractions, as globulins and albumins are scarcely influenced by nitrogen nutrition[16-18], and gliadins are more influenced than glutenins.[19-21] The choice of cultivar can also impact both GP concentration and composition, with old cultivars and landraces usually showing higher protein percentages than modern ones at the same or even lower level of N fertilization [22,23], and different alleles at the key loci for HMW and LMW glutenin subunits.[24]

Old durum varieties, which have not been subjected to modern breeding have preserved wide genetic diversity in protein content and composition. Indeed, only a part of the genetic diversity of durum wheat has been captured in modern varieties generated through breeding over the last century.[25] Old varieties therefore provide an important source of biodiversity for investigation and identifying the durum cultivars most suitable for the production of bread or other typical bakery products.[26,27]

In this study, we investigated the morphological, phenological, productive, and qualitative traits of fourteen old Italian durum wheat genotypes from diverse origins and eras of breeding to analyze their bread-making potential under two rates of nitrogen fertilization. We also examined whether and, if so, which quality traits of the raw material, i.e. the grain, influence the quality of the semolina, the dough and the end-product, i.e. the bread. A modern cultivar with a good reputation for pasta-making ('Svevo') was also included in the trial.

Materials and methods

Site, soil, and management

The experiment was carried out in Ottava (Sardinia, Italy, 41°N; 8°E; 80 m asl) over 2 growing seasons ('2014', sown on 10/12/2013 and '2015', sown on 7/1/2015). The environment is typically Mediterranean with a long-term mean annual rainfall of 553±29 mm, concentrated between October and April. The soil is a sandy-clay-loam with a depth of about 0.6 m due to underlying layers of limestone (typic Xerochrepts). In both seasons the preceding crop was a leguminous species. The sowing bed was prepared by ploughing to a depth of 0.25 m, followed by surface cultivation. In both seasons, the materials were sown with an 8-row planter at a density of 250 viable seeds m^{-2}. Each 10 m^2 plot consisted of eight 8.4 m rows, separated from one another by 0.15 m. Phosphoric fertilizer (92 kg P_2O_5 ha^{-1}) was applied before sowing in the form of ammonium bi-phosphate and N fertilizer was applied as described below. Weeds, pests, and diseases were chemically controlled.

Treatments and experimental design

The experiment compared fourteen old Italian durum wheat genotypes from different breeding eras. One *Rht1* semi-dwarf modern cultivar (Svevo) was sown in the same field, representing a modern high-quality cultivar. It was chosen among the many durum wheat cultivars available because of its reputation as a good quality wheat for pasta production.[28] The names, geographic or genetic origin, and year of release of the 14 old cultivars are shown in **Table 1**.

The cultivars were compared at two levels of nitrogen fertilization: 46 kg N ha^{-1} applied at sowing as urea ('N46'), and a higher rate obtained thanks to a supplementary application of N as ammonium nitrate at the onset of stem elongation ('N86'). The plots were arranged in a split-plot design with 4 replications, with the nitrogen treatment as main plot and the cultivars as sub-plots.

Field measurements

Anthesis date was recorded as the time at which 50 % of the ears in a plot had visible anthers. Physiological maturity was set as stage 90 in the Zadoks' Scale.[29] Plant height was defined as the distance from the ground to the tip of the spike (awn excluded), and was assessed pre-harvest on four randomly chosen plants per plot. Grain yield was calculated on a per plot basis, edge rows excluded.
Grain weight (GW), grain moisture content, absolute weight (AW), and yellow berry incidence (YB) were obtained from the mean of four 250 grain sub-samples per plot. The number of grains per m^2 (GNO) was calculated by dividing GY by GW. The grain moisture content was used to express both GY and GW on a 0% moisture basis. Grain nitrogen percentage was estimated using the Carbon/Hydrogen/Nitrogen Determinator (CHN 628 Series, Leco Corporation, St. Joseph, MI, USA). Nitrogen data were used to calculate: GP percentage as N percentage x 5.7, and the amount of nitrogen in each grain (GNmg) as GW at 0% humidity x N percentage. During the course of the experiment, weather conditions (rainfall, solar radiation, temperature) were recorded at a meteorological station located in an adjacent field.

Bread-making process

Dough was prepared by mixing semolina (100%), water, salt (1.8%), and yeast (2%) in a 10 kg spiral mixer (Sigma srl, Italy) for 10 min at low speed. The amount of water used to prepare each sample was calculated according to the consistographic HYDHA values, as previously reported by Secchi et al. (2018).[30] Bulk fermentation was carried out for 30 min at 30°C, 85 % RH. The fermented doughs were then divided (500 g), molded into baking pans, placed in a proofer (30°C, 80 % RH) until they rose to double their original size, and baked for 35 min at 230°C in an electric oven (Europa, Molina di Malo; VI, Italy). After baking, the loaves were cooled at room temperature, packaged into plastic bags, and stored at 20 °C.

Semolina, dough and bread quality measurements

Raw material was ground using an industrial mill and sifted to collect the resulting semolina. Semolina protein content was determined in the same way as grain protein content. The gluten index (GI) and dry gluten (DG) content were determined using the Glutomatic system 2200 according to AACC Method No. 38-12.02[31]. The yellow color index (YI) of semolina was determined using a model CR 300 Minolta colorimeter.
The evaluation of dough rheological properties was carried out using the Kieffer dough and gluten extensibility rig developed by Stable Micro Systems for the TA-XTplus Texture Analyser. Extensibility (E) and resistance to extension (RE) were determined in tension mode by recording the peak force and the distance at the maximum and the extension limits.[32] The ratio of 'extensibility' to 'resistance to extension' (E/RE), a measure of the balance between extensibility and resistance to extension, was also calculated.
Bread loaf volume was measured using the small seeds displacement method (AACC Standard 10-05.01[33]). The specific volume was calculated as bread volume (ml) over bread weight (g). Bread mechanical properties (hardness, cohesiveness, springiness, and chewiness) were recorded in a texture analyzer TA-XTplus (Stable Micro Systems, UK) using a 36 mm cylindrical probe, a speed rate of 1 mm/s, 40% penetration depth, and a 30 s gap between compressions on three central slices (20 mm thickness) of two loaves.

Sequential extraction of gliadin and glutenin for RP-HPLC analysis

The sequential extraction of protein from wheat semolina was done as described by Marchylo et al. (1989)[34] using a Agilent 1100 Series HPLC and ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The gliadin to glutenin (Gli/Glu) ratio was estimated from the ratio of the total chromatogram peak area for 50% propan-1-ol extracts to the total chromatogram peak area for propan-1-ol+ DTT extracts. For each protein fraction and peak, the protein content fractions (gliadin and glutenin) were expressed as amounts (area, mV/min) per mg of semolina, as described by Triboi et al. (2000)[35].

SDS and A-PAGE for gliadin and glutenin subunits separation

For A-PAGE, gliadin extraction from 30 mg of durum wheat flour was performed as described by Clement (1988)[36]. While for the SDS-PAGE, extraction of protein fractions from 20 mg of wheat durum flour was performed as described by Singh. et al (1991)[37]. Electrophoresis was performed in an SE 600 Ruby Hoefer vertical unit.

The identification of high-molecular-weight subunits of glutenins (HMW-GS) and alleles was based on the classification put forward by Payne & Lawrence (1983)[38].

Statistical Analysis

All data were subjected to appropriate statistical analyses (ANOVA, PCA) using R software (R Development Core Team, 2008),[39] with the year and block considered as the random effects and nitrogen rate and cultivar as the fixed effects. Different ANOVA tests were performed: one limited to the 14 old cultivars to detect N and cultivar effect and their interaction; and another using the 14 old cultivars plus the modern cultivar Svevo in order to identify any old cultivars that differed from Svevo using a Dunnett Test performed at the 0.05 probability level. ANOVA was performed considering the whole set of varieties, except in relation to the bread analysis, for which only a subset of 11 cultivars was available due to the insufficient quantity of semolina obtained in the remaining 3 cultivars.

Results

Protein characterization

Based on their mobility on a SDS-PAGE and comparison with data in the literature[24,28,40,41], six high molecular weight (HMW) GS patterns were revealed: 20, 20*, 7, 13+16, 6+8, and 7+8. All genotypes studied had the superior LMW 2 associated with gliadin γ- 45, except for Saragolla, which had the unusual combination of LMW2/gliadin γ- 42 (**Table 1**).

Table 1. List of the investigated genotypes with the relative year of release, geographic or genetic origin and details of the gluten sub-units allelic composition

Name	Geographic or genetic origin	Year of release	HMW-GS – B1	Glu - A1	LMW-GS – B3	Gliadin-A1
Calabria	Calabria	<1915	20	Null	2	45
Dauno[25]	Apulia	1900	6+8	Null	2	45
Ichnusa[41]	Biancale x Capeiti 8	1968	20	Null	2	45
Maristella	DAUNO-III/CAPEITI-8	1969	20	Null	2	45
Russello[28]	Indigenous landraces from sicily	1910	13+16	Null	2	45

Saragolla*	Apulia	1910	6+8;20	Null	2	42
Scorzonera	Indigenous landraces from sicily	<1915	20	Null	2	45
Senatore Cappelli (Cappelli)[24,28,40,41]	Nord-african landrace Jean Retifah	1920	20	Null	2	45
Svevo[28,41]	Sel. CIMMYT x Zenit sib	1996	7+8	Null	2	45
Taganrog	Russia	1908	20*	Null	2	45
Timilia	Sicily	1930	20	2*	2	45
Trigu Biancu	Sardinia	<1915	20	Null	2	45
Trigu Murru[41]	Sardinia	1910	20*	2+	2	45
Triminia	Sicily	1920	7	2+	2	45
Trinakria [24,40,41]	B14 × Capeiti 8	1973	20	Null	2	45

*Saragolla seeds were heterogeneous regarding HMW-GS pattern

Weather

The weather conditions of both seasons were typical for the Mediterranean area explored (all weather parameters fell within the $\mu \pm \delta$ interval obtained from 58 years of data), although some differences were detected between the two seasons. Rainfall in the October-May period of 2014/2015 amounted to 545 mm, which was 42% greater than that for the 2015/2016 season, although the same amount of rain fell in the two seasons between anthesis and maturity (13 mm). Temperatures were also higher in 2014/2015 than in 2015/2016 from the end of December onwards (**Figure 1**).

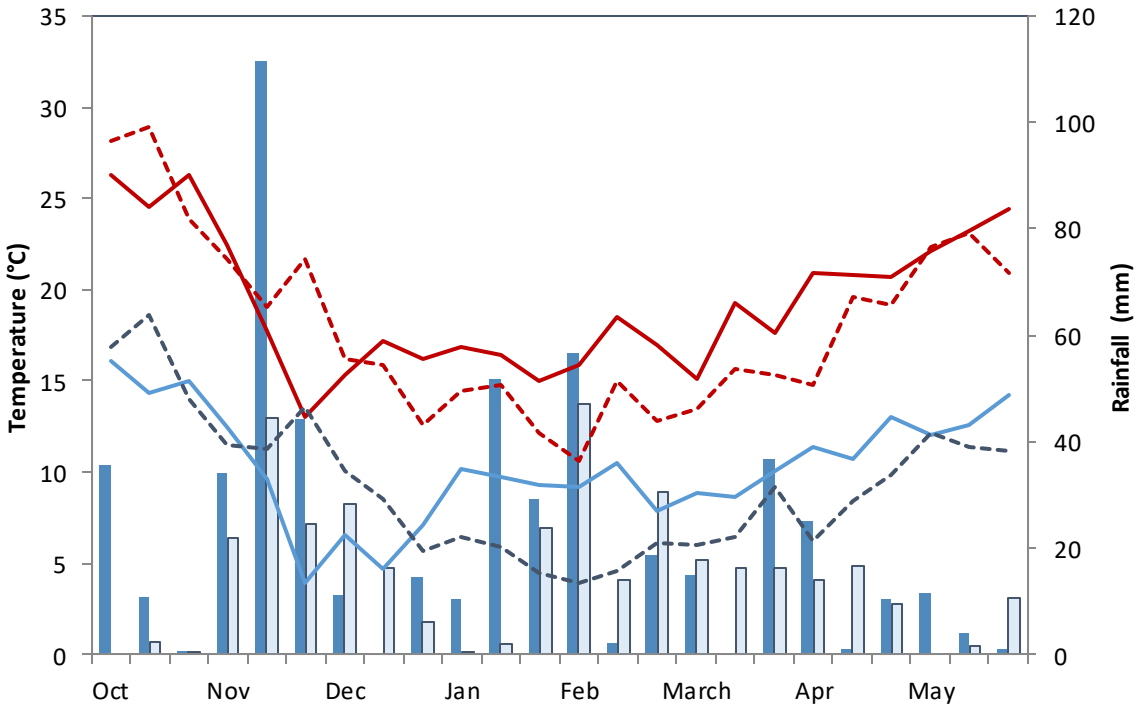

Figure 1. Rainfall (bars), maximum (red lines) and minimum (blue lines) temperatures from October to May of 2014/2015 (continuous lines, blue bars) and 2015/2016 (dotted lines, light blue bars).

Agronomic traits

No agronomic trait showed significant effect (by ANOVA) of the interaction 'genotype by nitrogen' (GxN). The same was true for the semolina, dough, and bread traits. Only the mean effects of the N treatments and the cultivars are therefore shown in the tables.

The N treatment did not affect GY, an expected result given the late application of the second amount, whereas GP was higher in N86 in spite of no change in the mg of N accumulated in each grain (**Table 2**). The reason for this increase in GP was therefore the lower GW observed in the N86 treatment (3 mg less), accompanied by a 13% higher GNO. The lower GW of N86 was mirrored in a lower AW, whereas YB was strongly reduced at the higher N rate. As expected, lodging incidence in the N86 treatment was almost two-fold that of the N46 treatment despite the unchanged plant height.

Old cultivars all flowered later than Svevo, with a flowering date that ranged from the April 30 to May 16 (cultivar Russello). GY ranged from 2 t ha^{-1} in the least productive genotype (Timilia) to about 4 t ha^{-1} in the two most productive genotypes (Maristella and Calabria). No difference in GY was observed between the 8 best performing old cultivars and the modern cultivar Svevo (Dunnett test, $P<0.05$). The genotypic variation in GY was associated with the genotypic variation in GNO ($r = 0.86$, $P<0.001$), but not with GW. The low number of grains m^{-2} (ranging from 4044 to 7883) was the likely reason behind both the lack of any relationship between GNO and GW and the high GWs observed (up to a maximum of 63 mg).
All except three old cultivars (Triminia, Taganrog, and Maristella) had a GW superior than that of Svevo. The cultivars with the higher GW where able to accumulate more mg of N in their grains ($r = 0.93$, $P<0.01$). GP was explained by the corresponding variation in GNmg ($r = 0.93$, $P<0.01$), in contrast with what was observed with the N treatment, which determined a variation in GP due to a corresponding variation in GW.
GP ranged from 13.0% (Scorzonera) to 15.6% (Cappelli) and was higher in all old varieties except three cultivars (Maristella, Scorzonera, and Triminia) than in the modern Svevo variety. No relationship was apparent between GP and GY. The cultivars most affected by YB were those with the lower GP ($r = -0.56$, $P<0.05$) and no difference was detected between the old cultivars and Svevo in this trait. Timilia was the cultivar most affected by lodging.

Table 2. Genotypes and Nitrogen fertilization effect on different agronomic traits: anthesis day, grain yield, grain weight, number of grain per m^2 (Grain number), grain protein, mg of nitrogen per grain (N per grain), absolute weight, yellow berry, plant height, lodging percentage

	Anthesis	**Grain yield**	**Grain weight**	**Grain number**	**Grain protein**	**N per grain**	**Absolute weight**	**Yellow berry**	**Plant height**	**Lodging**
	doy	t ha^{-1}	mg	n m^{-2}	%	mg $grain^{-1}$	kg hl^{-1}	%	cm	%
Nitrogen	Ns	*ns*	***	***	***	*ns*	***	***	*ns*	***
46	124	2.81	56.0	5096	13.9	1.4	80.5	28.9	124	14
86	125	3.01	53.0	5765	15.3	1.4	79.6	11.7	126	32
Cultivars	***	***	***	***	***	***	***	***	***	***
Calabria	125 *bd*	3.92 *ab*	62.7 *a*	6259 *ac*	14.08 *ac*	1.63 *a*	81.0 *ab*	13.2 *ac*	139 *ab*	15 *b*
Cappelli	124 bd	3.21 *ad*	59.1 *ab*	5439 *bd*	15.6 *a*	1.62 *a*	80.8 *ab*	7.30 *c*	133 *bc*	31 *b*
Dauno	127 *b*	2.27 *de*	55.4 *be*	4106 *d*	14.1 *ac*	1.45 *ae*	81.5 *a*	9.30 *bc*	138 *ab*	24 *b*
Ichnusa	120 *d*	3.31 *ac*	54.2 *bf*	6110 *ac*	14.5 *ad*	1.36 *bf*	80.4 *ac*	36.6 *a*	102 *f*	6 *b*
Maristella	121 *d*	3.95 *a*	50.1 *ef*	7883 *a*	13.3 *de*	1.18 *fg*	79.4 *ac*	33.2 *ab*	101 *f*	14 *b*
Russello	136 *a*	2.89 *ce*	52.5 *df*	5487 *bd*	14.1 *ac*	1.36 *cf*	78.9 *bc*	16.8 *ac*	121 *cd*	28 *b*
Saragolla	126 *bc*	2.96 *be*	54.7 *bf*	5406 *bd*	15.1 *ac*	1.46 *ad*	80.7 *ab*	14.8 *ac*	149 *a*	28 *b*
Scorzonera	130 b	2.67 *ce*	55.5 *be*	4803 *cd*	13.0 *e*	1.27 *df*	79.3 *ac*	19.5 *ac*	122 *cd*	23 *b*
Taganrog	128 *b*	2.64 *ce*	49.4 *f*	5351 *bd*	14.3 *ae*	1.24 *eg*	80.0 *ac*	21.0 *ac*	140 *ab*	26 *b*

Timilia	121 *cd*	2.84 *ce*	52.9 *cf*	5411 *bd*	15.2 *ac*	1.40 *be*	80.5 *ab*	12.0 *bc*	121 *cd*	67 *a*
TriguBiancu	122 *cd*	2.05 *e*	56.9 *bd*	3862 *d*	14.3 *be*	1.44 *ae*	80.0 *ac*	24.4 *ac*	104 *ef*	11 *b*
TriguMurru	125 *bd*	2.99 be	58.6 *ab*	5116 *bd*	15.4 *ab*	1.58 *ab*	79.5 *ac*	14.5 *ac*	133 *bc*	19 *b*
Triminia	129 *b*	2.86 *ce*	42.4 *g*	6749 *ab*	13.9 *ce*	1.04 *g*	80.4 *ab*	32.6 *ab*	118 *de*	24 *b*
Trinakria	*125 bd*	*2.17 e*	58.3 *ac*	4044 *d*	15.3 *ac*	1.56 *ac*	78.1 *c*	29.0 *ac*	132 *bd*	10 *b*
Svevo	115	3.68	45.6	8061	12.9	1.0	78.3	26.4	76	3

Level of significance: *$P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; ns: not significant.
Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$

Semolina characteristics

The percentage of semolina obtained with milling was not affected by the N treatment but differed among cultivars (**Table 3**). The lowest values (52%) were observed in the cultivars Calabria and Cappelli which combined the highest GY and GW, while the highest values (60%) were observed in the low-yielding cultivars Taganrog and Triminia, which had as well the lowest GWs. No one of the old cultivars differed from Svevo in the semolina yield. Indeed GY was the unique traits associated with semolina percentage and the association was negative (r = -0.79, P<0.01). This association was the reason why the semolina yield expressed on a unit surface basis was not affected by the cultivar.

Additional N fertilization improved semolina protein percentage by 1.3%, i.e. the same increase observed in grain protein percentage. This increase was accompanied, as expected, by an increase in DG due only to the gliadin fraction (+12.4%) – the glutenin fraction being unaffected. The gliadin-to-glutenin (Gli/Glu) ratio, which was 3.95 for the N46 treatment, was, therefore, higher for N86, at 4.30. GI decreased with the additional N application from a mean of 32 to 27. YI was not affected by N treatment.

Semolina protein percentage varied from 11.8% in Scorzonera to 15.2% in Cappelli and was strongly associated with GP (r = 0.91, P<0.001) and DG (r = 0.71, P<0.01). The protein percentage of the modern cultivar Svevo (12.5%) was lower than that of most old cultivars. According to the RP-HPLC chromatograms, the gliadin area varied among varieties from 373 to 493 (mV/min) per mg of semolina, with Calabria Ichnusa, Trigu Biancu, and Trinakria having the highest values and Taganrog the lowest. Dauno had the highest glutenin area, followed by Cappelli, Trigu Murru, and Calabria, while Scorzonera, Maristella, and Triminia had the lowest. The gliadin area of Svevo was lower than in most of the old cultivars, but its glutenin area was similar to all old varieties except Dauno.
The variation in gliadin and glutenin areas was associated with the variation in total grain N content (**Figure 2**), but the slope was significantly higher for the gliadins (P<0.001), indicating that the increase in grain N content due to genotype is accompanied by a genotypic variation in gliadins that exceeds that for the glutenins.

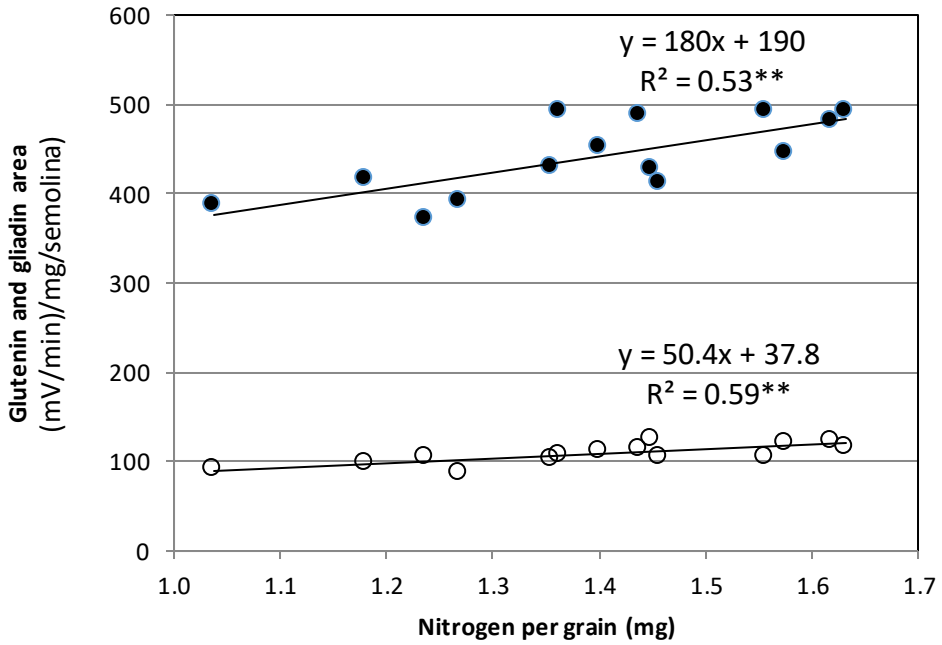

Figure 2. Variation in gliadin (filled symbols) and glutenin (empty symbols) as a function of the corresponding variation in the grain nitrogen content. Data include means of the 14 old cultivars.

Gli/Glu ratio varied from 3.46 to 4.74 among old varieties and was not different to that observed in the modern Svevo, in spite of its lower gliadin content (**Table 3**).

The genotypic variability in GI was very large, extending from the low (<30) to the medium range (30 – 60). Maristella, Trigu Biancu, Taganrog, Trigu Murru, Calabria, and Dauno were the only cultivars with a GI in the middle range. As expected, the modern cultivar Svevo had a GI in the upper range (83), significantly higher than that of all the old cultivars except for Dauno.

YI varied between 12 (Triminia) and 18.2 (Ichnusa). The YI of Svevo was far higher than that of the old varieties.

Table 3. Semolina yield and quality parameters: semolina yield as percentage (%) and t/ha^{-1}, protein percentage (%), dry gluten (DG), gluten index (GI), gliadin area, glutenin area, gliadin-to-glutenin (Gli/Glu) ratio and yellow color index (YI) for the various treatments (cultivars and N rates).

	Semolina yield (%)	Semolina yield (t ha-1)	Protein (%)	DG (%)	GI	Gliadin area (mV/min)/ mg /semolina	Glutenin area (mV/min) /mg /semolina	Gli/Glu	YI
Nitrogen	*ns*	*ns*	***	***	*	***	*ns*	***	*ns*
46	56.6	1.59	13	12.1	32	404	104.26	3.95	15.5
86	55.8	1.68	15	13	26.6	454	108.61	4.30	16.3
Cultivars	***	*ns*	***	***	***	***	***	***	***
Calabria	52.2 *b*	2.05	14.5 *ab*	13.5 *ab*	53.6 *ab*	492 *a*	117 *ac*	4.24 *ad*	17.1 *ac*
Cappelli	52.1 *b*	1.67	15.2 *a*	14.3 *a*	26.6 *cd*	481 *ab*	124 *ab*	3.95 *ad*	16.5 *ac*

Dauno	57.9 ab	1.33	14.3 *ab*	12.3 *ad*	60.6 *a*	427 *ac*	125 a	3.46 *d*	17.5 *ab*
Ichnusa	53.4 *ab*	1.78	13.5 *ab*	13.1 *ac*	14.3 *de*	493 *a*	107 *ae*	4.62 *ab*	18.2 *a*
Maristella	56.9 *ab*	2.25	12.8 *ab*	12.1 *ad*	36.5 *bc*	416 *ac*	98 *ce*	4.28 *ad*	17.3 *ab*
Russello	57.7 *ab*	1.68	14.0 *ab*	12.4 *ad*	24.1 *ce*	430 *ac*	102 *be*	4.31 *ad*	16.1 *ac*
Saragolla	57.6 *ab*	1.71	13.8 *ab*	11.9 *bd*	9.1 *de*	412 *ac*	106 *ae*	3.91 *bd*	15.7 *ac*
Scorzonera	57.7 ab	1.54	11.8 *b*	10.7 *d*	11.6 *de*	391 *bc*	88 *e*	4.45 *ac*	13.9 *cd*
Taganrog	60.3 *a*	1.59	13.3 *ab*	12.5 *ad*	49.0 *ab*	373 *c*	105 *ae*	3.58 *cd*	15.6 *ac*
Timilia	55.6 *ab*	1.58	15.1 *a*	13.5 *ac*	14.6 *de*	453 *ac*	111 *ad*	4.07 *ad*	13.9 *cd*
TriguBiancu	54.6 *ab*	1.12	13.7 *ab*	13.7 *ab*	43.3 *ac*	489 *a*	115 *ac*	4.26 *ad*	17.7 *ab*
TriguMurru	55.1 *ab*	1.65	14.4 *ab*	12.9 *ad*	50.8 *ab*	447 *ac*	121 *ab*	3.72 *cd*	14.8 *bd*
Triminia	60.4 *a*	1.74	13.1 *ab*	10.8 *d*	4.50 *e*	387 *bc*	92 *de*	4.21 *ad*	12.0 *d*
Trinakria	55.5 *ab*	1.18	14.1 *ab*	11.3 *cd*	11.0 *de*	492 *a*	104 *ae*	4.74 *a*	16.0 *ac*
Svevo	55.9	2.06	12.5	10.6	83.0	363	105	3.44	22.2
CV (%)	4.67	18.37	6.6	8.7	65.8	9.7	10.5	9.1	11

Level of significance: **P* < 0.05; ** *P* < 0.01, *** *P* < 0.001; ns: not significant.
Means with the same letter are not statistically different at the Tuckey Test for *P*<0.05
CV: coefficient of variation

Dough and Bread quality

As shown in **Table 4,** nitrogen treatment improved the quality of the dough and bread: dough extensibility increased by 21% and bread specific volume increased by 8.3%. Favorable textural properties of the bread (springiness and cohesiveness) also increased and the dough's resistance to extension decreased as did the level of bread hardness (by 16%). Only chewiness did not respond to N fertilization.

Among the old varieties, Calabria combined the best quality traits for both dough and bread. It had the highest dough E (56.5 mm; the lowest was for Scorzonera at 10.6 mm) and E/RE ratio, the highest bread volume (2.79 ml g^{-1}; the lowest was for Triminia at 2.15 ml g^{-1}) and cohesiveness (0.83), and the lowest measure of crumb hardness (12.8 N; the highest was for Triminia at 35.7 N).
Triminia, on the other hand, combined several negative traits, including the highest dough RE (29.7 g), the highest crumb hardness (35.7 N), and the lowest bread volume. The modern variety Svevo was similar to Triminia with regards to the above mentioned negative traits, but with an even higher dough RE (61.7 g), and produced one of the poorest bread qualities.

Table 4. Dough and bread quality parameters: extensibility (E), resistance to extension (RE), extensibility-to-resistance to extension (E/RE) ratio, bread specific volume (V), hardness, springiness, cohesiveness and chewiness for the various treatments (cultivars and N rates).

	E (mm)	RE (g)	E/RE (mm/g)	V (ml/g)	Hardness (N)	Springiness	Cohesiveness	Chewiness (N)
Nitrogen	*	**	**	***	***	*	***	*ns*
46	23.5	21.6	1.25	2.4	21.2	0.96	0.79	15
86	28.4	18.1	1.71	2.6	18	0.97	0.8	13.7

Cultivars	***	***	***	***	***	***	***	***
Calabria	56.5 *a*	18.2 *bc*	3.45 *a*	2.79 *a*	12.8 *d*	0.97 *ab*	0.83 *a*	10.3 *c*
Cappelli	36.9 *bc*	19.1 *bc*	2.02 *bc*	2.68 *ab*	15.3 *cd*	0.97 *ab*	0.81 *ab*	11.8 *c*
Ichnusa	16.7 *ef*	13.7 *c*	1.08 *cd*	2.62 *ab*	14.2 *cd*	0.97 *ab*	0.81 *ab*	11.2 *c*
Maristella	28 *ce*	19.8 *ac*	1.4 c*d*	2.45 *cd*	18.5 *cd*	0.97 *ab*	0.79 *cd*	14.2 *bc*
Russello	17.4 *df*	16.2 *bc*	1.02 c*d*	2.6 *ac*	15.3 *cd*	0.96 *ac*	0.78 *d*	11.5 *c*
Saragolla	13.6 *ef*	17.4 *bc*	0.79 *cd*	2.48 *bc*	19.9 *c*	0.97 *ab*	0.79	15.2 *bc*
Scorzonera	10.6 *f*	18.6 *bc*	0.60 *d*	2.26 *de*	27.6 *b*	0.95 *bc*	0.76 *e*	15.5 *bc*
Taganrog	33.9 *bd*	18.2 *bc*	2.03 *bc*	2.78 *a*	13.1 *cd*	0.98 *a*	0.80 *bc*	10.4 *c*
Timilia	11.9 e*f*	24.6 *ab*	0.55 *d*	2.24 *e*	27.8 *b*	0.94 *c*	0.76 *e*	19.7 *bc*
TriguMurru	47.4 *ab*	23.2 *ac*	2.85 *ab*	2.65 *ac*	15.8 *cd*	0.97 *ab*	0.81 *ab*	12.4 *c*
Triminia	12.8 e*f*	29.7 *a*	0.49 *d*	2.15 *e*	35.7 *a*	0.95 *bc*	0.76 *e*	25.7 *a*
Svevo	21.0	61.7	0.37	2.27	26.9	0.96	0.79	20.4
CV (%)	60.8	22.2	67.0	8.7	38.1	1.20	3.00	32.8

Level of significance: *$P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; ns: not significant.
Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$
CV: coefficient of variation

From agronomy to end-product

The relationships between all the quality traits analyzed was assessed by PCA.

The biplot in **Figure 3** illustrates the first two axes (70% of variation explained) of a principal component analysis (PCA) based on agronomic (AW, anthesis date, GW, and mg of N per grain), semolina (GI, DG, gliadin and glutenin areas and Gli/Glu ratio), dough (E, RE, and E/RE), and bread (specific volume, cohesiveness, springiness, hardness, and chewiness) traits. PC1, representing 53.6% of the variability, was positively related to all the favorable bread quality parameters and negatively associated with hardness and chewiness. The semolina and dough traits more tightly linked to the favorable bread characteristics were GI and E/RE and, to a lesser extent, DG and glutenin area. These latter parameters were, in turn, strongly associated with two agronomic traits: GW and mg of N per grain. Semolina protein percentage and gliadin area were less important than glutenin area in determining the bread volume. Gli/Glu and RE appeared to be strongly but negatively associated with bread specific volume, whereas flowering date was totally irrelevant. Note that Gli/Glu was the trait that contributed the least to the variation explored.

The biplot was able to differentiate contrasting wheat groups, highlighting the diversity in the various quality traits between genotypes. Calabria and Trigu Murru were grouped together with the best bread quality traits, whereas Calabria, Cappelli, and Ichnusa were demonstrated to be the best for protein percentage, DG, and glutenin area, as well as GW and mg of N per grain. Triminia, Scorzonera, and Timilia had the poorest performance. Note that, of the old cultivars, Calabria had the highest GI (53.6), since Dauno was not included in the by-product analysis, whereas Timilia had the lowest GI (4.5) (**Table 3**).

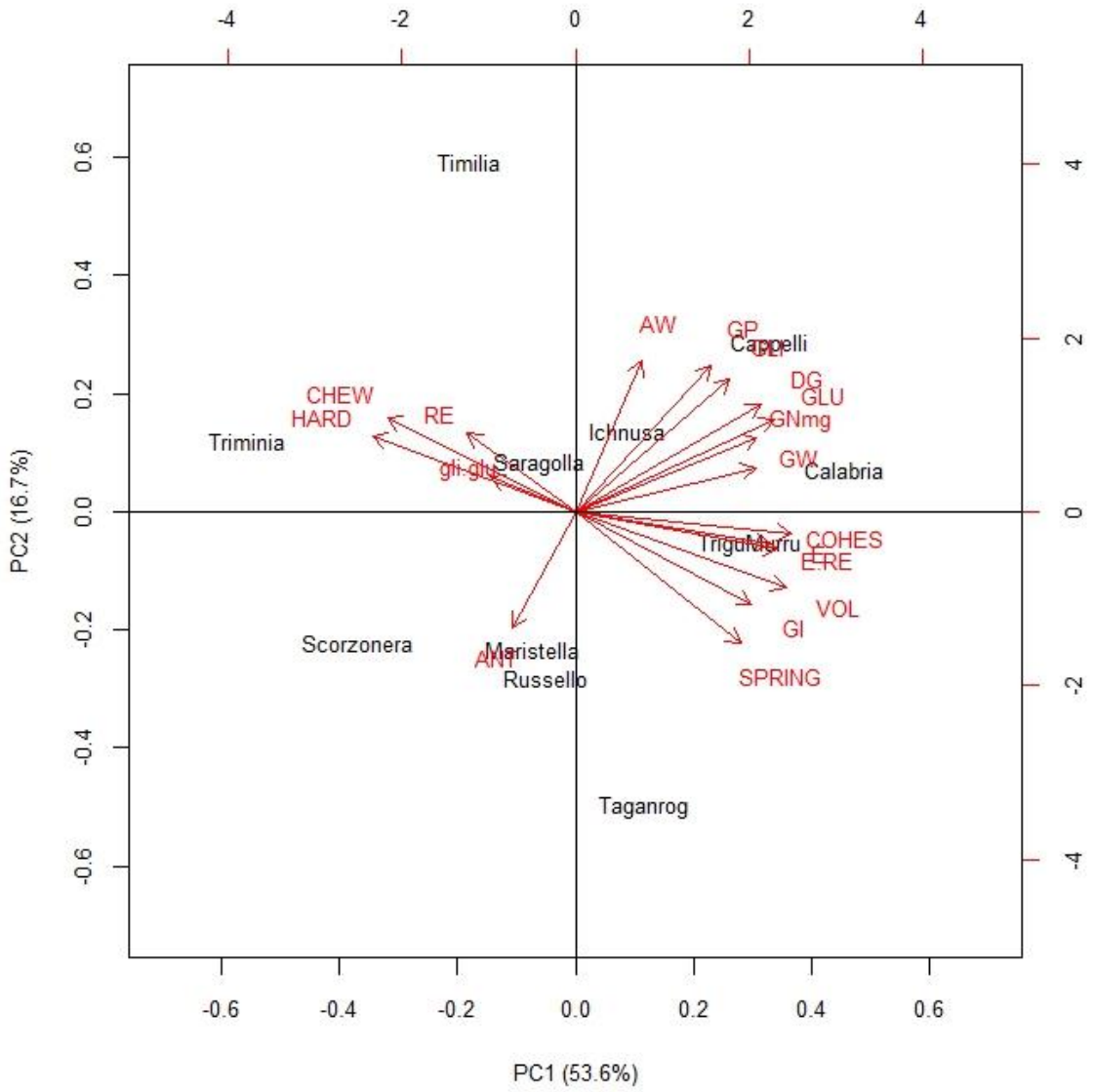

Figure 3. Biplot of agronomic, semolina, dough and bread quality characteristics obtained from the eleven cultivar means across nitrogen treatments: anthesis date (ANT), absolute weight (AW), grain weight (GW) and mg of N per grain (GNmg), grain protein percentage (GP), dry gluten (DG), gluten index (GI), gliadins (GLI), glutenins (GLU), gliadin-to-glutenin (gli-glu) ratio, resistance to extension (RE), extensibility (E), extensibility-to-resistance to extension (E/RE) ratio, specific volume (VOL), hardness (HARD), springiness (SPRING), cohesiveness (COHES) and chewiness (CHEW) for the old durum wheat varieties.

Discussion

Bread quality partly derives from the quality of the raw material, i.e., the grain. Our experiment allowed us to highlight the relationships between the principal grain quality traits and the resulting bread quality, whilst also considering both semolina and dough properties. To the best of our knowledge, no other papers have assessed the whole durum wheat product production chain in this way.

The lack of any effect of N fertilization on grain yield has already been reported by Giunta et al. (2019)[42] for the same rainfed agricultural system adopted in this experiment, known to be well suited to old cultivars. It is likely that the low N rate applied at sowing plus the N residual from the preceding leguminous crop was enough to let the old cultivars express their water-limited yield potential.[41] On the other hand, the increase in lodging observed at the higher N rate negatively affected the productive performance.

Modern durum wheat cultivars are more productive than old ones even at low N fertilization rates.[22] In our study, the grain yield of the modern cultivar included in the experiment was comparable to that of several old cultivars. Indeed, the specific modern cultivar chosen, i.e. Svevo, was selected because of its high protein percentage[28], which is known to be negatively correlated with the yield level.[42] and papers cited therein

Nitrogen fertilization represents the most influential management option for increasing grain N percentage, although the susceptibility of old cultivars to lodging limits its use.[43] In this experiment, the increase in N rate from 46 to 86 kg ha^{-1} did not increase the mg of N per grain, but resulted in a higher grain protein percentage as an indirect effect of the decrease in grain weight. This negative response of grain weight to N fertilization has already been noted for old cultivars[22] and attributed to the corresponding increase in the plant population density. The higher lodging incidence could have contributed to the lower grain weight of crops grown with N86.[43] The sole increase in the gliadin fraction in response to N fertilization has been previously noted,[19,21] although not in all genotypes.[16,17]

At the semolina and dough level, the change in grain protein percentage and composition deriving from N treatment led to a decrease in GI, but dough properties and bread quality traits improved nevertheless, although to a limited extent compared with the genotypic effect, due to the increase in both gliadins (and hence dough extensibility) and grain protein percentage. This confirms the role of gliadins and grain protein percentage in determining the bread-making quality of durum wheat, at least when the change in protein percentage is in response to N fertilization.

In contrast with what was observed for N fertilization, the variation in protein percentage induced by genotype was due to the corresponding variation in mg of N per grain and was positively associated with grain weight. By analyzing the entire chain, from seed to the baked product, we are able to confirm the strength of the role of these two raw material traits in determining bread quality. The relationship between grain N content and composition was highlighted by the observed different slopes of the association between the variation in mg of N per grain and the variation in gliadin and glutenin content. These relationships have already been observed by Triboi et al. (2003)[44], although, in their case, the variation in mg of N was due to environmental and management variations. Here, we demonstrate that the same relationships hold when grain N content varies with genotype.

GI seems to be a reliable index for predicting the quality of old durum cultivars with respect to bread-making despite the contrasting opinions in the literature.[45] The use of Svevo in our study as a reference for modern varieties was crucial in order to deduce that the rules that can be generated for old varieties do not necessarily apply to modern ones and vice-versa. The GI of the modern cultivar Svevo was in the high range and produced tough bread of limited loaf volume due to its tenacious and inextensible dough, confirming the results of Quaglia (1988)[1] and Ammar et al. (2000)[10]. This result contrasts those reported by Edwards et al. (2007)[11] and Ćurić et al. (2001)[46]. The latter showed that a 75<GI<90 provides a grain with the optimal bread-making quality for Central European wheat cultivars. But what is the best range of GI for an optimal durum bread-making quality is not known. As Svevo is the leading cultivar for pasta-making, our data confirm that the requirements for good pasta quality are different from those for good bread-making quality, and that the genetic "improvements" of modern cultivars have actually lowered their potential to make good bread.

From the genetic perspective, our results contradict two assumptions: 1) that varieties with HMW-GS 20 produce a weak dough and low-quality bread, and 2) that the alleles HMW GS 7+8 and 6+8 are associated with high baking quality.[10,11,47,48] In fact, Svevo, with its HMW 7+8, did not outperform 11 of the 14 old genotypes that possessed the HMW-GS 20 allele. Old varieties sharing the same allelic composition (gliadin 45 associated with LMW-GS 2; HMW-GS 20) resulted in diverse dough performances and bread

qualities, again highlighting that quality cannot be defined by a single allele or trait,[9,26] but by the combination of allele composition and the quantitative ratios of their products determined by their level of expression.
Grown under a low to medium nitrogen input, cultivars Calabria and Cappelli combined good grain yield, similar to that of the modern cultivar Svevo, with high quality semolina and bread, confirming that in a low-input system it is possible to find good yielding varieties producing good quality bread.

Conclusion

New genotypic variability is needed to face the challenges of increasing the quantity and quality of production in low-fertility areas. Old durum wheat cultivars grown for bread-making are an interesting option to consider since they have been demonstrated to couple good productivity levels with good bread quality.
By considering the entire productive chain of a durum wheat product, from seed (cultivar choice) to bread, it was possible to link genotypic related variations in bread quality to specific grain quality traits (N content per grain). Old cultivars are able to produce grains with high N content even in low fertile soils and with low N inputs.
Finally, Italian durum wheat cultivars deserve more attention as they can provide new market opportunities and improve the environmental and economic sustainability of the durum wheat chain.

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CHAPTER 3

The key role of grain number in the determination of grain nitrogen content and composition in durum wheat cultivars grown under low input conditions in a Mediterranean environment

Abstract
Old durum wheat cultivars are attracting renewed attention due to their suitability to low input agricultural systems. Fourteen old and two modern durum wheat cultivars were analyzed in two field trials at two nitrogen (N) levels (46 and 86 Kg N ha^{-1}) to assess the effect of grain number and N absorbed and translocated by the crops on grain protein percentage and whether the genotypic variation in grain N was associated with a variation in the quantitative ratios between the various protein fractions.
Mean grain yield was below 3 t ha^{-1} and strongly associated with the corresponding variation in the number of grains m^{-2} (GNO) (r = 0.93***). The greater amount of N present in the biomass of old cultivars at anthesis, due to their greater biomass (r = 0.87***), resulted in a greater N source for the growing grains (15-23 g m^{-2}) compared with modern cultivars (13-16 g m^{-2}) despite the greater post-anthesis N uptake of modern cultivars. In spite of this larger source, most old cultivars generally delivered a lower amount of N m^{-2} (4.1 - 8.5 g m^{-2}) to their mature grains compared with modern cultivars (8.1-10.3 g m^{-2}). Nevertheless, their lower GNO resulted in a greater amount of N in each grain, which was the main determinant of their higher grain protein percentage (r = 0.81***).
Genotypic variation in grain N content correlated with a variation in the content of all three protein fractions (albumins-globulins, gliadins and glutenins) but the strength of the correlation with gliadin and albumin-globulin was higher than that with the glutenins. Genotypic variation in gliadin and glutenin content was more tightly correlated with the variation in the sulphur-rich protein groups and subunits (alpha/beta, gamma and low molecular weight glutenin subunits) than with the sulphur-poor protein groups and subunits. The significant genotypic differences in the ratios GLI/GLU, Srich/Spoor and HMW/LMW were not influenced by the corresponding variation in grain N content, even when the slope of the regressions for the two terms of the ratios against the total, as in the case of HMW and LWM, were different. The final N content can only explain part of the variation in the quantitative ratios between fractions and components since genotypic differences other than grain N content also contribute to these variations.

INTRODUCTION

Old durum wheat cultivars have been proposed as a sustainable management option for low input cultural systems in the less fertile areas of the Mediterranean region (Giunta et al., 2019). In these cultural systems, the grain yield achieved by old cultivars is comparable to that of modern durum wheat cultivars specifically selected for their high grain protein content grown under the same conditions, but old cultivars are characterized by an even higher grain nitrogen (N) content and are hence better suited for the production of traditional wheat products such as bread (Mefleh et al., 2019b).
The effect of nitrogen application on grain quality andgrain yield of old durum wheat cultivars have been explored by several authors (Cossani et al., 2012 and Albrizio et al., 2010), but little has been done in terms of physiological analyses to explain the variation in grain protein content and composition (i.e., the types of proteins and the quantitative ratios between the different fractions) in a framework where the role of grain number per unit area (the N sink) and the nitrogen available to the mature grain (the N source) are considered.
The grain number per unit area (GNO) establishes a link between the supply of nitrogen and dry matter and the grains' demand for them (Martre et al., 2006). GNO depends on the amount of biomass produced by anthesis, the fraction of this biomass allocated to the ear and the number of grains per unit of biomass of the ear (Fischer, 1985; Weir et al., 1984; Jamieson et al., 1988). All the components of GNO are modulated by the environment and by its interaction with genotype. In their modelling approach to GNO determination, Weir et al. (1984) assumed that a fixed percentage of biomass produced by the crop 2.5 phyllochron before anthesis would be allocated to the ear on a daily basis and that a fixed number of grains would be produced per dry matter unit of the ear at anthesis. However, old and modern durum wheat cultivars present different features in relation to Weir's framework. Tall old wheat cultivars partition a lower amount of biomass to the growing spike compared with the short modern ones (Brooking and Kirby, 1981). Consequently, and according to the relationship between the spike dry weight at anthesis and floret survival (Fischer and Stockman, 1980;

Brooking and Kirby, 1981; Fischer, 2011), modern cultivars have the potential to set a higher grain number per ear.
Differences in partitioning at anthesis could also be relevant in creating a different balance between the number of grains and the amount of nitrogen available for each grain. The latter was shown to be responsible for regulating the accumulation of the various metabolic (albumins-globulins) and storage protein (glutens, which form up to 80% of total protein) fractions in the grain (Martre et al., 2003). Gluten proteins are composed of gliadins that are classified into the following groups: alpha/beta, omega and gamma gliadins; and glutenins, which comprise the high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits (GS) (Wieser 2007). Total protein content (accounting for 10–15% of grain dry weight) and the amount and composition of the various storage proteins partly determine the levels of dough elasticity, extensibility and viscosity, all of which contribute to the biophysical and functional properties of dough and end product quality (Mefleh et al., 2019a).
Wheat grain nitrogen is mostly derived from the non-structural nitrogen accumulated by the crop by anthesis, although a significant contribution can also be derived from the uptake of soil nitrogen during grain filling (Martre et al., 2003). The amount of nitrogen absorbed by the crop is related to its biomass (Lemaire et al., 2008); therefore, differences in biomass at anthesis could be responsible for differences in the quantity of nitrogen source available to the grain, provided that the number of grains has not changed.
In this study we tested the role of grain number and nitrogen absorbed and translocated by the crops in varying grain protein content of durum wheat cultivars (from different breeding eras) grown in a Mediterranean environment on poor soils and with relatively low N inputs. We also assessed whether and to what extent the genotypic variation in the final quantity of N in the grains was associated with a variation in the quantitative ratios between the various protein fractions.

MATERIALS AND METHODS

Site, soil and management

The experiment was carried out during seasons 2015/16 ('2016') and 2016/17 ('2017') at the experimental station of the University of Sassari located at Ottava (41°N; 8°E; 80 m above sea level). The environment is typically Mediterranean, with a long-term mean annual rainfall of 539 mm – the majority of which occurs between October and April.

Treatments and experimental design

A total of 16 cultivars of durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.) from different eras of wheat breeding were compared (Table 1). Most of them were old tall cultivars diffused in Italy in the first half of the 20th century. Two cultivars, Ichnusa and Maristella deriving from crosses between Mediterraneum and Syriacum types (Ali Dib et al., 1992) and widely grown in the 1960s, are generally shorter and earlier than the old cultivars. Two modern , semi-dwarf varieties, Svevo and Aureo, were also investigated. They were chosen from the many semi-dwarf Italian durum wheat cultivars because of their reputation for producing good quality pasta (De Santis et al, 2017).
Cultivars were sown on 10th December, 2015 (2016 season), and 15th November, 2016 (2017 season), at a sowing rate of 250 viable seeds m^{-2} and at two fertilization rates: a low N level ('N46') with a single nitrogen application at the time of sowing (46 kg ha^{-1} in the form of urea); and a high N level ('N86'), which benefited from a second application of 40 kg ha^{-1} at the onset of stem elongation in the form of ammonium nitrate.

The soils, just 0.4-0.5 m deep due to an underlying layer of limestone, have an organic matter content of 1.4±0.3%, 45 kg ha^{-1} of mineral nitrogen, 8.4 ± 0.5 ppm of available phosphorus and high total $CaCO_3$ (40±4.4%). The available water amounted to 61 mm. In both seasons, the preceding crop was fava bean and the sowing bed was prepared by ploughing to a depth of 0.25 m followed by surface cultivation. Weeds, pests and diseases were chemically controlled.
Each plot consisted of six 1.2 m rows with an inter-row distance of 0.18 m. The plots were set out as a split-plot design with 3 replications. Cultivars were assigned to the main plots and nitrogen rates to the sub plots.

Table 1. The investigated genotypes with the relative year of release, geographic or genetic origin, and details of the allelic composition of gluten genes

Name	Geographic or genetic origin	Year of release	HMW-GS – B1	Glu - A1	LMW-GS – B3	Gliadin-A1
AUREO	Kofa × Svevo	2009	6+8	Null	2	45
CALABRIA	Calabria	<1915	20	Null	2	45
DAUNO	Apulia	1900	6+8	Null	2	45
ICHNUSA	Biancale x Capeiti 8	1968	20	Null	2	45
MARISTELLA	Dauno-III/Capeiti-8	1969	20	Null	2	45
RUSSELLO	Indigenous landraces from sicily	1910	13+16	Null	2	45
SARAGOLLA*	Apulia	1910	6+8;20	Null	2	42
SCORZONERA	Indigenous landraces from sicily	<1915	20	Null	2	45
SENATORE CAPPELLI (CAPPELLI)	Nord-african landrace Jean Retifah	1920	20	Null	2	45
SVEVO	Sel. CIMMYT x Zenit sib	1996	7+8	Null	2	45
TAGANROG	Russia	1908	20*	Null	2	45
TIMILIA	Sicily	1930	20	2*	2	45
TRIGU BIANCU	Sardinia	<1915	20	Null	2	45
TRIGU MURRU	Sardinia	1910	20*	2^{+}	2	45
TRIMINIA	Sicily	1920	7	2^{+}	2	45
TRINAKRIA	B14 × Capeiti 8	1973	20	Null	2	45

*Saragolla seeds were heterogeneous regarding the HMW-GS pattern.

Measurements and derived data

The time to anthesis and to physiological maturity were determined by the periodical inspections of plots and attributed to the plot when more than 50% of plants had reached these phenological stages. Grain filling duration was roughly estimated as the difference between the time to anthesis and time to maturity. Plant height, defined as the distance from the ground to the tip of the spike (awn excluded), was assessed pre-harvest for four randomly chosen plants per plot.

Dry matter production was evaluated at both anthesis and maturity on samples of 0.21 m^2 of uprooted plants per plot, roots excluded. Ears were separated from the rest of the sample at anthesis, whereas at maturity samples were subdivided into grains, chaff and the remaining culms plus leaves (straw). All biomass samples were oven-dried at 80°C for 48 hours before weighing. Nitrogen percentage was determined on each sub-sample by means of a Carbon/Hydrogen/Nitrogen Analyzer (628 Series, LECO Corporation, St. Joseph, MI, USA).

The harvest index (HI) determined on the maturity sample by dividing grain weight by the total sample biomass was used to estimate the plot grain yield (GY). Grain moisture content and grain weight (GW) were obtained from four 250 grain sub-samples per plot. The number of grains per m^2 (GNO) was calculated by dividing GY by GW. Fruiting efficiency was calculated as the ratio of GNO to ear dry matter at anthesis. GY and GW were expressed on a 0% moisture basis. Nitrogen data were used to calculate: grain protein

percentage as N percentage x 5.7; the amount of nitrogen (in μg) per grain (GNμg) as GW at 0% humidity x N percentage; the amount of N allocated to ears and vegetative tissues at anthesis and to grains, chaff and vegetative tissues at maturity. The Nitrogen Harvest Index (NHI) was obtained as the ratio between N in the grains and total N in the biomass. The amount of N taken up after anthesis was evaluated as the difference between total N per unit surface at maturity and total N per unit surface at anthesis. Critical N was calculated using the critical N dilution curve for wheat described by Justes et al. (1994) and used to determine the Nitrogen Nutrition Index (NNI) as the ratio between the actual above-ground crop N percentage at anthesis and critical N (Gaju et al., 2014). Nitrogen relocation efficiency from ears (NREears) and from the vegetative tissues (stems plus leaves) (NREveg) were calculated as the difference between the amount of N present in ears/vegetative tissue at anthesis minus that present at maturity and expressed as percentage of the N content at anthesis (Gaju et al., 2014). According to Pask et al. (2012), the structural nitrogen of the vegetative tissues is assumed to correspond to the minimum N percentage observed at maturity in the straw.
Weather data (maximum and minimum temperatures, rainfall, solar radiation, wind speed and air humidity) were recorded at the agro-meteorological station on the experimental farm where the fields were located and used to calculate mean temperature, reference evapotranspiration (ETo, Allen et al., 1998) and the rainfall deficit (RD) as the difference between rainfall and ETo.

Sequential extraction of gliadin and glutenin for reverse phase-high performance liquid chromatography (RP-HPLC) analysis

Albumins-globulins, gliadins and glutenins were sequentially extracted from 50 mg of ground wholemeal grain as described by Wieser and Seilmeier (1998) and separated at 50°C on a ACE C18 column (250x2.1 mm, 5μm, 300Å) using an Alliance HPLC system (Waters). Flow rate was 0.2ml min^{-1} and absorbance was recorded at 210 nm.
Following RP-HPLC separation, the area under the protein curve was calculated, corresponding to the amount of protein extracted in each fraction. For each grain sample, the protein extracted was then expressed as an amount per grain. Gluten amount was obtained by summing the gliadin and glutenin peaks. Total protein content was estimated by adding the albumin-globulin, gliadin and glutenin peaks together. Areas corresponding to gliadin groups (alpha/beta, omega and gamma) were calculated by cutting the whole gliadin chromatogram as follows: 7 to 14 min (omega); 14 to 22 min (alpha/beta); and 22 min until the last peak (gamma). The same procedure was applied to glutenin chromatograms: 7 to 17 min (HMW-GS); and 17 min to the last glutenin peak (LMW-GS).
Total protein content was estimated by adding the albumin-globulin, gliadin and glutenin peaks together. The relative percentage of each protein fraction (albumin-globulin, gliadin and glutenin) was calculated from total protein, and the relative percentage of gliadin groups alpha/beta, omega and gamma and glutenin subunits (HMW and LMW) were calculated from total gluten. Gluten percentage was considered as the sum of the gliadin and glutenin percentages.

Extraction of extractable and unextractable polymeric proteins for size exclusion-high performance liquid chromatography (SE-HPLC) analysis

Extraction of polymeric protein and separation by SE-HPLC was conducted as described by Dachkevitch, T. et al. (1989) and Morel et al. (2000) with minor modifications. Briefly, SDS-soluble proteins were extracted from 160 mg wholemeal flour by the addition of 20 mL 1% (w/v) SDS in 0.1M sodium phosphate buffer (pH 6.9) and incubation on a rotary shaker (60 rpm at 60°C for 80 min). Following centrifugation (37,000g, 30 min, 20°C), the supernatant was set aside for further SE-HPLC analysis. The un-extractable proteins (or SDS insoluble proteins) were obtained by adding 5 mL SDS-phosphate buffer to the pellet, vortexing, and sonicating for 3 min at 7.5 watts, then centrifuging at 37,000g at 20°C for 30 min. The supernatant was subjected to SE-HPLC analysis and the pellet discarded. SE-HPLC separations were performed on an Alliance system (Waters) equipped with a TOSOBIOSCIENCE TSKgel G4000SWXL column (8×300 mm, 8μm, 300Å) protected by a guard column TOSOBIOSCIENCE TSKgel SWXL guard (6×40 mm, 7μm mm). Proteins were eluted at ambient temperature with 0.1M sodium phosphate buffer (pH 6.9) containing 0.1% (w/v) SDS at a constant flow of 0.7 mL min^{-1} and absorbance was recorded at 214 nm.
The total area under each chromatogram obtained from SDS extractable and un-extractable protein extracts was expressed as a percentage (EP% and UP%, respectively) of the sum of the total area of both chromatograms.
Grain nitrogen data were used to calculate the μg of nitrogen in each protein fraction. The nitrogen contents of EP and UP were calculated using their percentages (calculated by the SE-HPLC) multiplied by grain nitrogen

(GNμg). The nitrogen contents of albumin-globulins, gliadins and glutenins were calculated using their percentages calculated by RP-HPLC multiplied by the nitrogen content of EP, whereas those of gliadin groups and glutenin subunits (alpha/beta, omega and gamma and HMW and LMW-GS) were calculated using their percentages calculated by RP-HPLC multiplied by the nitrogen content of gluten. From here on, the nitrogen content of a fraction or subunit will simply be referred to as 'content'; for example, the μg of nitrogen of gliadin will be referred to as 'gliadin content'.

Electrophoresis

Gliadins were analyzed by A-PAGE and the gliadin extraction was carried out on30 mg durum wheat flour as described by Clement (1988). HMW-GS were separated by SDS-PAGE and protein extraction was performed on 20 mg durum wheat flour as described by Singh et al. (1991).
The identification of high-molecular-weight glutenin subunit (HMW-GS) alleles was based on the classification proposed by Payne and Lawrence (1983).

Statistical analysis

Agronomic data were analysed by a combined ANOVA, using R software (R core team), according to a split-split plot design where years were assigned to the main plot, cultivars to the sub plots and nitrogen treatment to the sub-sub plots. Cultivar means were separated by means of a multiple t-test once the significance of the cultivar effect had been tested. The Pearson correlation coefficient was used to evaluate the existence of causal relationships between couples of traits.
Principal component analysis (PCA) based on the cultivar means across seasons was performed.

RESULTS

Weather conditions

In 2017, the rainfall recorded between October and May was only 60% of the long-term mean (a 40-year mean for the period spanning 1970 to 2010). In 2016, the rainfall was higher than that of 2017, but still lower than the long-term mean (Table 2). When rainfall was combined with ETo to quantify the rainfall deficit, the 2017 season was confirmed as having been extremely dry (the rainfall deficit was more than 2-fold the long-term reference value), whereas 2016 was better than the long term mean.
Rainfall was particularly scarce in the spring of both seasons, and 2017 was even worse than 2016 (a total of only 8 mm of rain fell in the months of April and May in 2017). 2017 was also characterised by higher maximum temperatures compared with 2016 and the long term mean, as well as a greater number of days in which the maximum temperature exceeded 25°C in the month of May – the period in which most of the grain filling occurs.

Table 2. Main meteorological data for the two years and as long-term means

		1970-2010	2016	2017
1rst October-31rst May				
	Rainfall (mm)	475±115	363	286
	ETo (mm)	614±75	454	583
	Rainfall deficit (mm)	-140±134	-91	-297
1rst April - 31rst May				
	Rainfall (mm)	89±47	25	8
	Rainfall deficit (mm)	-146±40	-180	-248
May				
	Minimum temperature (°C)	11.8±1.7	11.3	11.8
	Maximum temperature (°C)	22.5±1.8	21.2	24.1
	N° of days with Tmax>25 °C	6.5±4.7	4	17

Height and phenology

The severe meteorological stress of 2017 resulted a reduction in average plant height by 20 cm and a shortening of the grain filling period by an average of four days. In both years, the distinct plant heights of the three cultivar groups were evident – a result of the specific breeding strategies directed at modifying this trait. The group comprising the 12 oldest cultivars displayed an average plant height of 154 cm in 2016 and 131 cm in 2017; the intermediate group of cultivars released in the 70s reached 117-120 cm in 2016, but only 103-105 cm in 2017; and the semi-dwarf modern cultivars Svevo and Aureo reached a height of 83 and 88 cm, respectively, in 2016, 73 and 77 cm in 2017 (Table 3).
Clear differences in phenology were observed between the groups: the oldest cultivars flowered on average at the beginning of May, and no later than the 7th May, whereas the intermediate and modern cultivars flowered between the 17th and the 20th April. The earlier anthesis of the intermediate and modern cultivars resulted in a longer grain filling period compared with the older varieties, which, in some cases, had less than one month to fill their grains in 2017.
These differences in phenology brought about different thermal conditions during grain filling: the oldest cultivars were exposed to higher maximum temperatures compared with the intermediate and modern cultivars (24.5°C vs. 22.3°C in 2017 and 21.7°C vs. 20.4°C in 2016) and had more days in which the maximum temperature exceeded 25°C, especially in 2017 (14 days vs. 10 in 2016).

Table 3. Plant height, anthesis date and grain filling duration of the 16 cultivars studied

	Height (cm)		Anthesis (doy*)		Grain filling duration (d^)	
2016	141	*a*	120	a	34	a
2017	120	*b*	120	a	30	b
46	130	*a*	120	a	32	a
86	131	*a*	120	a	32	a
Calabria	141	*ce*	123	e	31	d
Cappelli	142	*ce*	123	de	30	dg
Dauno	150	*ab*	126	b	29	gh
Russello	136	*e*	124	de	30	df
Saragolla	152	*a*	126	b	29	eg
Scorzonera	128	*f*	127	a	27	h
Taganrog	142	*ce*	125	bc	29	fh
Timilia	140	*de*	115	f	33	c
TriguBiancu	141	*de*	125	bd	29	eg
TriguMurru	143	*be*	123	de	30	dg
Triminia	148	*ac*	124	ce	30	dg
Trinakria	144	*ad*	123	de	30	de
Ichnusa	113	*g*	109	g	39	a
Maristella	110	*g*	110	g	39	ab
Aureo	83	*h*	108	g	39	a
Svevo	78	*h*	109	g	37	b

Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$
*DOY: day of the year
^d: day

Grain yield and grain yield components
Mean GY (grain yield) was less than 3 t ha^{-1} (Table 4). The bad weather conditions of 2017 caused a 28% reduction in GY compared with 2016 deriving from a corresponding decrease in GNO (number of grains per m^2) and total dry matter, whereas the HI (harvest index) and GW (grain weight) were unaffected in spite of the more severe terminal water stress observed in 2017.

The N treatments did not affect GY because the slight increase in total dry matter observed at N86 was compensated by a reduced HI. GW was negatively affected by the additional N treatment applied to N86.
On average, the intermediate cultivars Maristella and Ichnusa were the most productive, followed by the modern cultivars Svevo and Aureo, for which GY was comparable to that obtained with the old cultivars Timilia, Trinakria and Calabria.
The modern cultivars Aureo and Svevo were more negatively affected by the stressful conditions in 2017 (their GYs were only 61% and 50% of those achieved in 2016) compared with both intermediate cultivars, Maristella and Ichnusa (70 and 99%, respectively), and some old cultivars (Saragolla, Timilia and Cappelli) whose GYs in 2017 exceeded those of 2016. Several old cultivars produced a GY comparable to the two semi-dwarf cultivars in 2017.
Aureo, Trigu murru and Ichnusa (data not shown) were the cultivars showing the greatest difference in GY between the two N treatments (+28%, +28% and +21%, respectively, under N86 compared with N46), accompanied with an increase in total dry matter and GNO. Two old cultivars (Calabria and Trigu biancu), on the contrary, had a lower GY under the higher nitrogen rate.
The genotypic variation in GY was strongly associated with the corresponding variation in GNO (r = 0.94***), but not with GW. GNO was, in turn, strongly associated with the variation in fruiting efficiency (r = 0.93***), which was the highest in the intermediate and modern cultivars. The intermediate cultivars Ichnusa and Maristella exhibited a fruiting efficiency even higher than the modern cultivar Aureo, exceeding 70 grains per g of ear at anthesis.

Table 4. Grain yield and its components; total dry matter, harvest index (HI), grain weight, number of grains per m^2 (GNO), fruiting efficiency and dry matter in ears as $g.m^2$ and percentage of total dry matter.

	Grain yield		Total Dry matter		HI		Grain weight		GNO		Fruiting efficiency		Ear DM anthesis			
	($g\ m^{-2}$)		($g\ m^{-2}$)				(mg)		(n grains m^{-2})		(n grains g ear^{-1})		($g\ m^{-2}$)		(% total DM)	
Year																
2016	330	*a*	1298	*a*	0.26	*a*	43.6	*a*	7731	*a*	53	*a*	151	*b*	0.14	*b*
2017	238	*b*	859	*b*	0.28	*a*	44.6	*a*	5421	*b*	35	*b*	169	*a*	0.20	*a*
Nitrogen																
N46	286	*a*	1045	*b*	0.28	*a*	45.2	*a*	6432	*b*	45	*a*	154	*b*	0.17	*a*
N86	283	*a*	1111	*a*	0.26	*b*	43.0	*b*	6720	*a*	43	*a*	166	*a*	0.17	*a*
Cultivar																
CALABRIA	324	*b*	1293	*b*	0.26	*e*	47.7	*ac*	6861	*cd*	41	*de*	172	*ad*	0.14	*fg*
CAPPELLI	233	*cd*	1045	*cf*	0.23	*f*	47.4	*bd*	4952	*e*	34	*ef*	148	*cf*	0.15	*df*
DAUNO	204	*df*	1053	*ce*	0.19	*h*	42.2	*ef*	4842	*ef*	35	*ef*	165	*bd*	0.17	*bd*
RUSSELLO	261	*c*	1020	*dg*	0.27	*e*	43.9	*cf*	6085	*d*	34	*ef*	195	*ab*	0.18	*b*
SARAGOLLA	217	*de*	1045	*cf*	0.21	*fh*	43.7	*df*	4973	*e*	29	*fg*	173	*ad*	0.14	*fg*
SCORZONERA	166	*f*	841	*h*	0.21	*fg*	37.7	*gh*	4463	*ef*	35	*ef*	126	*f*	0.15	*ef*
TAGANROG	175	*ef*	921	*fh*	0.19	*gh*	45.5	*be*	3895	*f*	24	*g*	168	*bd*	0.17	*bd*
TBIANCU	239	*cd*	1050	*ce*	0.23	*f*	51.3	*a*	4708	*ef*	27	*fg*	177	*ac*	0.17	*be*
TIMILIA	337	*b*	1132	*cd*	0.30	*d*	47.4	*ad*	7160	*c*	45	*d*	162	*ce*	0.17	*be*
TMURRU	221	*cd*	1165	*bc*	0.20	*gh*	48.5	*ab*	4632	*ef*	34	*eg*	141	*df*	0.14	*fg*
TRIMINIA	241	*cd*	1067	*ce*	0.23	*f*	40.1	*fg*	6145	*d*	36	*df*	172	*ad*	0.16	*ce*
TRINAKRIA	337	*b*	1465	*a*	0.23	*f*	48.8	*ab*	6889	*cd*	36	*df*	202	*a*	0.13	*f*
ICHNUSA	421	*a*	1158	*c*	0.36	*c*	47.1	*bd*	8947	*b*	73	*b*	124	*f*	0.16	*ce*
MARISTELLA	447	*a*	1144	*cd*	0.39	*ab*	41.8	*ef*	10682	*a*	86	*a*	131	*ef*	0.17	*bc*
SVEVO	364	*b*	907	*gh*	0.41	*a*	36.1	*h*	9996	*a*	80	*ab*	128	*f*	0.24	*a*
AUREO	362	*b*	948	*eh*	0.38	*bc*	36.3	*gh*	9986	*a*	59	*c*	171	*ad*	0.24	*a*

Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$

Nitrogen uptake and allocation

Anthesis

The decrease in biomass accumulated by anthesis in 2017 compared with 2016 was responsible for the lower uptake of N in this year, whereas the greater N percentage deriving from the additional N fertilization was the main reason behind the greater N uptake under N86 (Table 5). No 'cultivar x N' interaction was observed for the total N accumulated by anthesis, whereas year did interact with cultivar because most cultivars, Aureo and Svevo included, accumulated less N by anthesis in the less favourable year of 2017 compared with 2016, whereas six cultivars, including some old and the two intermediates Ichnusa and Maristella, accumulated more N in 2017.

On average, old cultivars had accumulated more nitrogen in their biomass by anthesis compared with both intermediate and modern cultivars, due to the higher N present in their vegetative tissues (leaves plus stems) that derived from similar N percentages, but higher dry matter content compared with modern cultivars. Differences between cultivars in the total N present at anthesis were, therefore, strongly associated with differences in total dry matter (r = 0.86***), but not with N percentage. As expected, modern cultivars allocated more N to the ears and less to culms and leaves compared with old and intermediate cultivars. Genotypic differences in phenology between cultivars explained 48% of the variation in total N uptake by anthesis (Figure 1), but the relationship was spurious; i.e., it was only due to the difference between the group of earlier modern and intermediate cultivars and the group of later old cultivars. No effect of anthesis date was detected within each group.

The NNI (Nitrogen Nutrition Index) was used in this experiment to evaluate the crop N stress incurred by the cultivars. As expected, NNI was higher at the higher N rate. A large genotypic variation was observed in the mean NNI with intermediate cultivars and Svevo exhibiting the lowest NNIs, ranging between 0.70 and 0.76, while the old cultivars had an NNI ranging from 0.73 to 1.05. Aureo's NNI was much higher than that of Svevo. There was no evidence for a relationship between the NNI and N partitioning index at anthesis across year x N treatments. It should be noted, however, that the NNI was much lower under the lower N rate (N46), with values below 0.7 for seven cultivars, indicating that many cultivars endured N stress under the lower N rate (data not shown).

Table 5. Total dry matter and nitrogen content in gm^{-2} and percentage, their contents in the various plant parts (culms and leaves and ears) at anthesis, critical nitrogen percentage, nitrogen nutrition index (NNI) and nitrogen partitioning index in ears and vegetative tissues (VEG).

	Total		Culms and leaves (VEG)		Ears						N Partitioning Index	
	Dry matter	N	Dry matter	N	Dry matter	Nitrogen		Critical N	NNI		EAR	VEG
	($g\ m^{-2}$)	($g\ m^{-2}$)	($g\ m^{-2}$)	(%)	($g\ m^{-2}$)	($g\ m^{-2}$)	(%)	($g\ m^{-2}$)	(%)			
2016	1141 *a*	17.1 *a*	991 *a*	1.44 *a*	14.0 *a*	151 *a*	2.05 *a*	3.1 *a*	1.86 *a*	0.83	*a*	0.19 *a*	0.81 *a*
2017	890 *b*	15.5 *b*	721 *b*	1.65 *a*	11.7 *b*	169 *a*	2.23 *a*	3.8 *a*	2.10 *a*	0.85	*a*	0.26 *a*	0.74 *a*
46	989 *b*	14.3 *b*	835 *b*	1.39 *b*	11.1 *b*	154 *b*	2.03 *b*	3.1 *b*	2.02 *a*	0.75	*b*	0.23 *a*	0.77 *b*
86	1042 *a*	18.3 *a*	876 *a*	1.70 *a*	14.6 *a*	166 *a*	2.26 *a*	3.8 *a*	1.94 *b*	0.93	*a*	0.21 *b*	0.79 *a*
CALABRIA	1274 *ad*	17.4 *cd*	1102 *b*	1.23 *g*	13.7 *de*	172 *ad*	2.18 *bd*	3.7 *bd*	1.76 *e*	0.78	*df*	0.23 *ce*	0.77 *eg*
CAPPELLI	983 *cf*	14.0 *eh*	835 *eg*	1.31 *g*	11.3 *ef*	148 *cf*	1.82 *h*	2.7 *ef*	1.96 *cd*	0.73	*f*	0.21 *eg*	0.79 *cf*
DAUNO	994 *bd*	17.4 *cd*	828 *fg*	1.60 *cd*	13.1 *de*	165 *bd*	2.59 *a*	4.3 *ab*	1.94 *cd*	0.91	*bc*	0.25 *cd*	0.76 *g*
RUSSELLO	1093 *ab*	21.6 *a*	898 *dg*	1.80 *b*	16.5 *bc*	195 *ab*	2.57 *a*	5.1 *a*	1.88 *de*	1.05	*a*	0.25 *bc*	0.75 *gh*
SARAGOLLA	1237 *ac*	20.6 *ab*	1064 *bc*	1.60 *ce*	16.9 *ab*	173 *ac*	2.13 *cf*	3.7 *bd*	1.76 *e*	0.95	*bc*	0.19 *fi*	0.82 *ad*
SCORZONERA	909 *f*	17.2 *cd*	783 *g*	2.01 *a*	14.5 *bd*	126 *f*	2.20 *bd*	2.7 *ef*	2.09 *bc*	0.97	*ab*	0.16 *i*	0.84 *a*
TAGANROG	1053 *bd*	14.7 *dg*	885 *dg*	1.28 *g*	11.2 *ef*	168 *bd*	2.03 *dg*	3.4 *ce*	1.93 *cd*	0.74	*f*	0.25 *cd*	0.75 *g*
TBIANCU	1133 *ac*	18.6 *bc*	956 *cd*	1.59 *cf*	14.5 *cd*	177 *ac*	2.31 *b*	4.1 *bc*	1.87 *de*	0.92	*bc*	0.23 *ce*	0.77 *eg*
TIMILIA	995 *ce*	16.7 *ce*	833 *eg*	1.65 *bc*	13.5 *de*	162 *ce*	1.97 *fh*	3.2 *df*	1.95 *cd*	0.88	*bd*	0.20 *eh*	0.80 *be*
TMURRU	1059 *df*	15.7 *cf*	918 *df*	1.42 *dg*	13.0 *de*	141 *df*	1.92 *gh*	2.7 *ef*	1.90 *de*	0.79	*df*	0.18 *gi*	0.82 *ac*
TRIMINIA	1120 *ad*	16.3 *cf*	947 *ce*	1.36 *g*	12.9 *de*	172 *ad*	1.99 *eg*	3.4 *ce*	1.86 *de*	0.79	*df*	0.22 *cf*	0.78 *dg*
TRINAKRIA	1602 *a*	23.1 *a*	1401 *a*	1.40 *fg*	19.2 *a*	202 *a*	1.93 *gh*	3.9 *bd*	1.57 *f*	0.93	*bc*	0.17 *hi*	0.83 *ab*
ICHNUSA	769 *f*	12.6 *gh*	645 *h*	1.60 *ce*	10.0 *f*	124 *f*	2.11 *cf*	2.6 *f*	2.19 *b*	0.76	*ef*	0.21 *dg*	0.79 *cf*
MARISTELLA	764 *ef*	11.8 *hi*	633 *h*	1.41 *eg*	9.0 *fg*	131 *ef*	2.13 *cf*	2.8 *ef*	2.20 *b*	0.71	*f*	0.25 *cd*	0.76 *fg*
SVEVO	551 *f*	9.7 *i*	423 *i*	1.65 *b*	6.9 *g*	128 *f*	2.14 *be*	2.8 *ef*	2.58 *a*	0.70	*f*	0.29 *a*	0.71 *i*
AUREO	712 *ad*	13.7 *fh*	541 *h*	1.82 *bc*	9.8 *f*	171 *ad*	2.26 *bc*	3.9 *bd*	2.25 *b*	0.86	*ce*	0.29 *ab*	0.71 *hi*

Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$

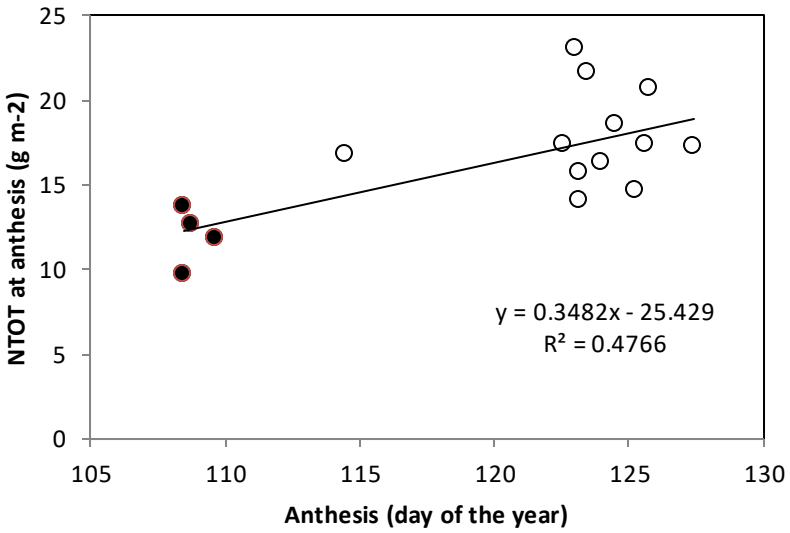

Figure 1. Relationship between total nitrogen (NTOT) in gm^{-2} in the crops per unit surface at anthesis and anthesis date. Black symbols represent the groups of modern and intermediate cultivars and the empty symbols represent the old cultivars.

Maturity

The N source available to the growing grains (Tab. 6) was higher in 2016 than in the following season, partly as a consequence of the greater uptake after anthesis, permitted by the higher spring rainfall in this year. The N source was limited to the N present at anthesis for almost all the old cultivars in the drier 2017 season, when only Aureo, Svevo, Maristella and Cappelli were able to take some N up after anthesis (data not shown). By contrast, in 2016 N uptake continued after anthesis in the modern and intermediate cultivars, contributing in the end to 14-26% of the N source. This was also the case for several old cultivars (Calabria, Dauno, Scorzonera, Trigu murru and Triminia). The earlier anthesis of the most recent releases surely created more favourable conditions for post-anthesis N uptake under the terminal water stress characterizing the two seasons, but, at the same time, resulted in less N uptake before anthesis, as shown by the negative association between the N uptake before and after anthesis (Figure 2. r = -0.78***). Despite this additional uptake, the N source available to modern and intermediate cultivars (13-16 g m^{-2}) was lower than that available to almost all old cultivars (15-23 g m^{-2}) (Tab. 6).

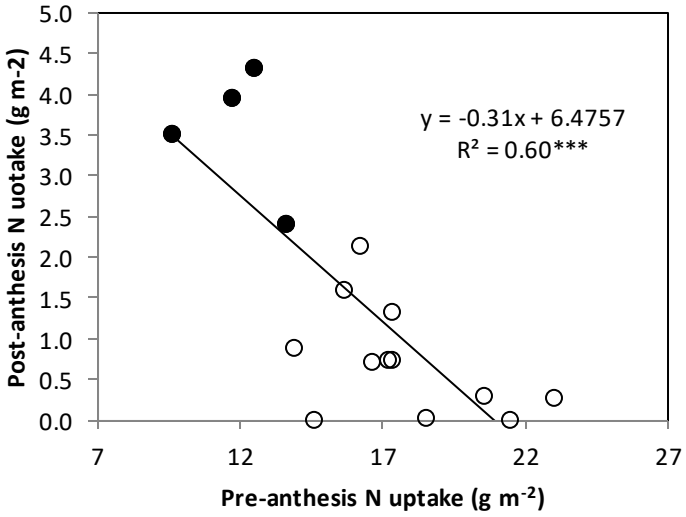

Figure 2. Relationship between pre- and post-anthesis N uptake. Points are cultivars means across years and N treatments. Black symbols represent the groups of modern and intermediate cultivars and the empty symbols represent the old cultivars.

Table 6. Nitrogen content in the various plant parts (straw, chaff and grain) at maturity, nitrogen harvest index (NHI), nitrogen remobilization efficiency in ears (EARNRE) and vegetatitive tissues (VEGNRE) and post-anthesis nitrogen (NPOSTANT).

Treatment	Nsource ($g\ m^{-2}$)		Straw N (%)		Chaff N (%)		Grain N ($g\ m^{-2}$)		NHI		NRE: N remobilization efficiency EARNRE		VEGNRE		NPOSTANT ($g\ m^{-2}$)	
2016	18.9	*a*	0.94	*a*	0.91	*a*	8.07	*a*	0.47	*b*	0.52	*a*	0.45	*b*	2.15	*a*
2017	16.0	*b*	0.87	*b*	1.13	*a*	6.04	*b*	0.52	*a*	0.67	*a*	0.60	*a*	0.57	*a*
N46	15.8	*b*	0.81	*b*	0.98	*b*	6.82	*b*	0.52	*a*	0.59	*a*	0.53	*a*	1.56	*a*
N86	19.2	*a*	1.00	*a*	1.06	*a*	7.30	*a*	0.47	*b*	0.59	*a*	0.51	*a*	1.17	*b*
CALABRIA	18.0	*bd*	0.91	*c*	1.13	*bd*	8.13	*c*	0.48	*ef*	0.55	*cf*	0.44	*gh*	1.32	*ef*
CAPPELLI	14.6	*ef*	0.87	*cd*	1.05	*ce*	5.86	*de*	0.44	*fh*	0.35	*h*	0.45	*eh*	0.88	*fg*
DAUNO	17.9	*bd*	0.89	*cd*	1.21	*ac*	5.45	*ef*	0.40	*hi*	0.65	*ad*	0.49	*dg*	0.74	*fg*
RUSSELLO	21.6	*a*	0.71	*ef*	0.84	*gh*	6.79	*d*	0.55	*d*	0.74	*a*	0.72	*a*	0.00	*g*
SARAGOLLA	20.6	*ab*	0.88	*cd*	1.17	*bd*	6.08	*de*	0.45	*fg*	0.59	*bf*	0.62	*bc*	0.27	*g*
SCORZONERA	17.6	*cd*	1.48	*a*	1.37	*a*	4.13	*g*	0.32	*j*	0.59	*eg*	0.46	*eh*	0.72	*fg*
TAGANROG	14.7	*ef*	0.80	*de*	0.88	*fh*	4.42	*fg*	0.43	*gi*	0.63	*ad*	0.55	*ce*	0.00	*g*
T.BIANCU	18.6	*bc*	0.90	*cd*	1.02	*df*	6.10	*de*	0.46	*fg*	0.69	*ab*	0.59	*bd*	0.01	*g*
TIMILIA	16.7	*ce*	0.72	*ef*	0.66	*i*	7.97	*c*	0.60	*bc*	0.63	*ad*	0.65	*ab*	0.69	*fg*
T.MURRU	17.3	*ce*	0.90	*cd*	1.27	*ab*	6.38	*de*	0.42	*gi*	0.41	*gh*	0.45	*fh*	1.58	*df*
TRIMINIA	18.1	*bd*	1.17	*b*	0.91	*eh*	6.05	*de*	0.40	*i*	0.64	*ad*	0.38	*h*	2.12	*de*
TRINAKRIA	23.1	*a*	0.69	*f*	0.77	*hi*	8.54	*c*	0.51	*e*	0.54	*df*	0.67	*ab*	0.26	*g*
ICHNUSA	16.1	*ce*	0.75	*ef*	0.90	*eh*	10.27	*a*	0.64	*a*	0.49	*fg*	0.54	*cf*	4.32	*a*
MARISTELLA	15.6	*df*	0.81	*de*	0.96	*eg*	9.83	*ab*	0.63	*ab*	0.62	*ae*	0.50	*dg*	3.21	*bc*
SVEVO	13.0	*f*	0.88	*cd*	1.02	*df*	8.09	*c*	0.64	*a*	0.66	*ac*	0.42	*gh*	3.48	*ab*
AUREO	15.9	*ce*	1.13	*b*	1.13	*bd*	8.84	*bc*	0.57	*cd*	0.72	*a*	0.43	*gh*	2.38	*cd*

Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$

In spite of this larger source, most old cultivars delivered a lower amount of N per square meter to their mature grains compared with the intermediate and modern cultivars, leaving more N in the straw (4.6 - 8.7 g m^{-2}) compared with modern and intermediate cultivars (3.9-5.5 g m^{-2}) (Figure 3). The greater proportion of structural N could be one of the reasons behind the limited capacity of old cultivars, like Scorzonera, Calabria, Trigu murru and Triminia, to remobilize their large N source; i.e., the cultivars that leave more N in their straw at maturity were also the ones with the highest amounts of structural N at anthesis (from 11.4 to 5.8 g N m^{-2}, data not shown). As a consequence, their NHI was lower compared with the values of 0.57-0.64 calculated for the more recent releases. Modern cultivars were amongst the most efficient cultivars in remobilizing N from the ears, and amongst the least efficient in remobilizing N from the vegetative tissues

(leaves and stems). Another advantage of modern vs. old cultivars for N translocation is the higher proportion of biomass made up by their ears in relation to the total biomass at anthesis (24% vs. 20%). The ear is the organ with the highest N percentage at anthesis (2.14% in modern and 1.54% in old cultivars), and also the organ that contributes the most to N translocation, as shown by their greater decrease in N percentage between anthesis and maturity (-1.12%) compared with that exhibited by leaves and stems (-0.64%).

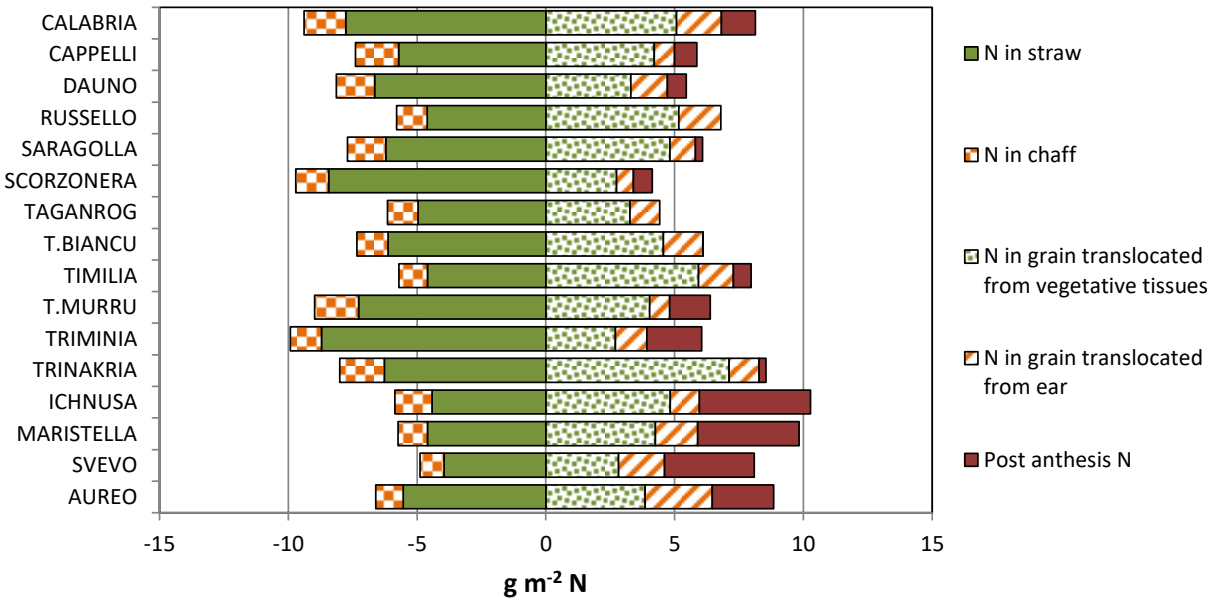

Figure 3. Average amounts of N found in straw and chaff at maturity (negative values), and total N present in the grains per m^{-2} (positive values), divided in the quote deriving from remobilization of the N present at anthesis in the vegetative tissues (stems + leaves) and in the ears, and the quote pertinent to post-anthesis N uptake.

Grain nitrogen

No effect of year or N input was detected on the nitrogen content of the grain, whereas the grain protein percentage increased by 7% at the higher N rate (Table 7). The effect of the fertilization treatment was only marked in 2017, thus generating a significant N x year interaction. The average protein percentage in this year was equal to 15.8% in the N86 treatment against only 13.7% in N46, and the corresponding µg of N per grain were 1200 vs. 1080 µg.

The ranking of the cultivars in relation to the percentage of N in the grains is reliable because it is not influenced by the year or by the nitrogen treatment as demonstrated by the lack of any significant interaction. The lowest N contents were observed in the grains of the intermediate Maristella and modern Svevo, both in percentage and in GNµg (µg of nitrogen in the grain), whereas the highest was observed in the old Trigu murru (17.3 %, 1453 µg). Old cultivars displayed a higher GNµg (1190 µg $grain^{-1}$) and protein percentage (15.2%) compared with intermediate and modern cultivars (940 µg $grain^{-1}$ and 13.3%).

Differences between cultivars in grain protein percentage were not a consequence of the corresponding differences in grain weight, since old cultivars combined the highest grain weights with the highest grain protein percentages. Instead, they derived from the corresponding differences in the GNµg, being the two traits (grain protein percentage and GNµg) associated with r = 0.81***. GNµg depends on both N source and GNO: traits that exhibit large genotypic variability in the set analyzed. The source over sink ratio (source:sink) was therefore calculated as the ratio between N source and GNO. Modern and intermediate cultivars had only 1300-1800 µg of N available for each grain compared with 2300-4100 µg available for the grains of old cultivars. The source:sink was associated with the variation in GNµg (r = 0.64**, Figure 4), and GNO was the main driver of the variation in source:sink (r = - 0.94***) with source variation showing a weaker association (r = 0.56*).

No effect of the N rate was observed on the pattern or strength of these relationships, which, therefore, also existed under the moderate N stress signalled by the crop NNIs grown at N46.

Table 7. Grain protein percentage and grain nitrogen content in µg.

	Grain Protein (%)		N in grains (µg grain^{-1})	
Year				
2016	14.6	*a*	1110	*a*
2017	14.8	*a*	1146	*a*
Nitrogen				
N46	14.3	*b*	1114	*a*
N86	15.2	*a*	1142	*a*
Cultivar				
CALABRIA	14.3	*df*	1158	*bc*
CAPPELLI	14.6	*df*	1190	*bc*
DAUNO	16.8	*ab*	1235	*b*
RUSSELLO	15	*ce*	1090	*ce*
SARAGOLLA	15.8	*bc*	1160	*bc*
SCORZONERA	14.5	*df*	1000	*df*
TAGANROG	15.2	*cd*	1203	*bc*
TBIANCU	13.8	*cd*	1125	*a*
TIMILIA	15.2	*f*	1380	*bd*
TMURRU	17.3	*a*	1453	*a*
TRIMINIA	15.1	*ce*	1030	*de*
TRINAKRIA	14.8	*cf*	1245	*b*
ICHNUSA	14.4	*df*	1158	*bc*
MARISTELLA	12.5	*g*	893	*f*
SVEVO	12.4	*g*	735	*g*
AUREO	14	*ef*	993	*ef*

Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$

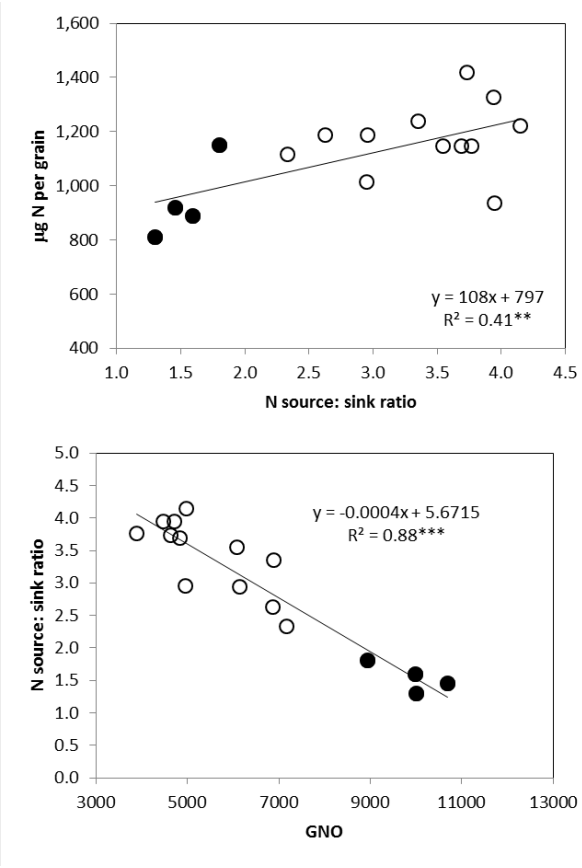

Figure 4. Dependence of the µg of N per grain on the source:sink ratio (upper panel), and of the source:sink ratio on the grain number per unit surface (lower panel). Points are cultivars means across years and N treatments. Black symbols represent the groups of modern and intermediate cultivars and the empty symbols represent the old cultivars. *** $P<0.001$, ** $P<0.01$.

Grain nitrogen partitioning into protein groups and subunits

At the higher N rate N86, a lower gliadin and a higher glutenin content were obtained (Table 8). As a consequence, their ratio GLI/GLU was higher for N46 (1.04) than for N86 (0.84)

The gliadins constituted the only protein fraction to be affected by year, with a higher gliadin content being observed in 2017 – the year characterized by severe water and temperature stress (437 µg grain^{-1} against 383 µg grain^{-1} in 2016). The content of gliadin groups were increased accordingly, with the exception of omega gliadins. Of consequence, the gliadin to glutenin ratio (GLI/GLU) was higher in 2017 (Table 8).

To evaluate whether the genotypic variation in GNµg also implied a variation in the various protein fractions, cultivar means were used to calculate the relationships between albumin-globulin, gliadin and glutenin with GNµg (Figure 5). These relationships provided information about the strength of the relationship between genotypic variation in protein fractions and the GNµg (coefficient of correlation) and the extent of the variation in protein fractions for each unit of variation of GNµg (slope of the regression).

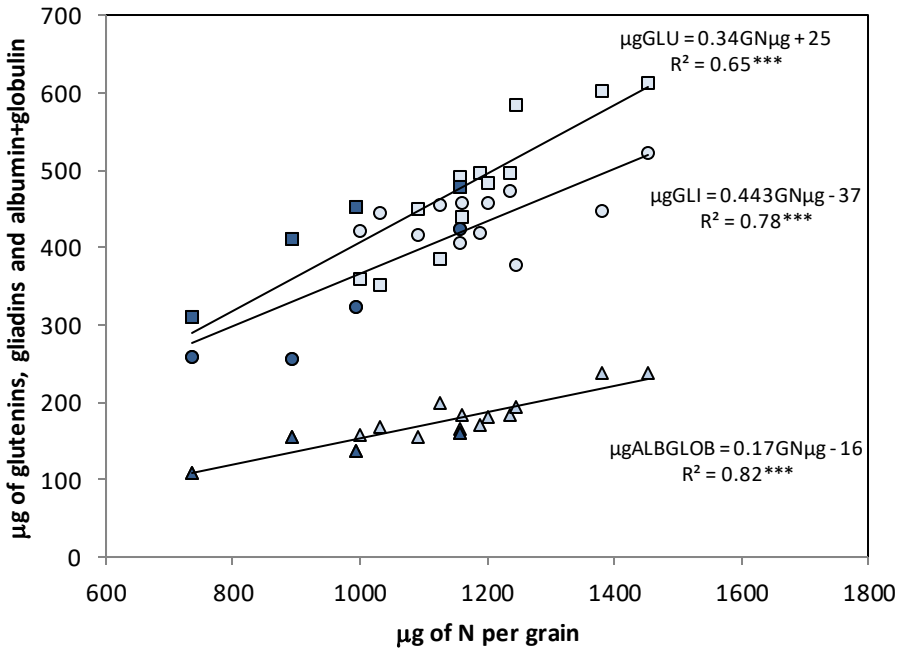

Figure 5. Relationships between the variation in the µg of total nitrogen per grain and the content of the three protein fractions, glutenins (GLU) (squares), gliadins (GLI) (circles) and albumins-globulins (ALBGLOB) (triangles). Points are cultivars means across years and N treatments. Darker symbols represent the groups of modern and intermediate cultivars and the lighter symbols represent the old varieties. *** $P<0.001$.

Variation in the protein fraction albumins-globulins was more tightly linked with the genotypic variation in GNµg (µg of nitrogen in the grain). On the other hand, this protein fraction was also the one with the lowest slope of regression (0.17± 0.56) compared with the storage proteins, gliadins (0.34±0.37) and glutenins (0.44±0.24); i.e., it was the fraction that varied the least in response to the genotypic variation in GNµg. The relationship between GNµg and gliadin was stronger than the one between GNµg and glutenins and no significant difference was detected in the slope of these two relationships. One interpretation of this result could be that no variation in the GLI/GLU ratio was associated with the genotypic variation in GNµg, however a significant effect of cultivars on the GLI/GLU ratio was detected by ANOVA due to the variation in GLI/GLU from 0.63 (Maristella) to 1.35 (Triminia) (Table 8), with the more recent cultivar released showing the lowest values. When directly regressed against the genotypic variation in GNµg, the variation in GLI/GLU ratio did not exhibit any relationship with the variation in GNµg (data not shown).
Within the gliadin groups, the sulphur(S)-rich alpha/beta and gamma were generally present in larger amounts than the S-poor omega gliadins (Figure 6). Alpha/beta content ranged from 101µg found in Maristella to 261µg found in Taganrog. Gamma content did not vary between genotypes. The relationship between total gliadin content and alpha/beta gliadins was stronger than the relationship with gamma gliadins, whereas omega gliadin levels varied independently from the total gliadin content in spite of the large genotypic variation in this component (the lowest content was observed in Taganrog at 31µg, and the highest was in Saragolla at 108 µg) and the significant genotypic effect as shown by ANOVA. The slope of regression of alpha/beta (0.56±0.14) was two times higher than that of gamma (0.27±0.45). This suggests that the cultivars with a higher gliadin content had a different proportion of S-rich gliadin and S-poor gliadin groups compared with the cultivars with a lower gliadin content.

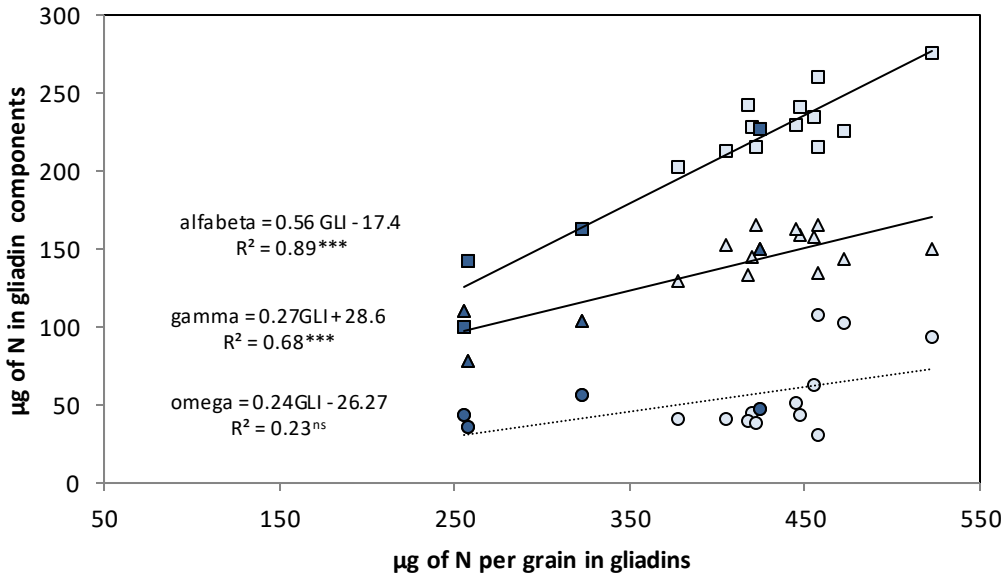

Figure 6. Relationships between the variation in the µg of total gliadins (GLI) per grain and the content of the three gliadin components alpha/beta (squares), gamma (triangles) and omega (circles). Points are cultivars means across years and N treatments. Darker symbols represent the groups of modern and intermediate cultivars and the lighter symbols represent the old cultivars. *** $P<0.001$, ns not significant

The S-rich LMW-GS were present in larger amounts than the S-poor HMW-GS (Figure 7) and showed greater genotypic variation, from 0.27 to 0.51 µg grain^{-1} (coefficient of variation (CV) = 36% vs.19% for HMW-GS). The genotypic variation in grain N allocated to glutenins was more tightly associated with the variation in LMW-GS (r = 0.96***) than in HMW-GS (r = 0.68**).
The slope of regression of LMW-GS (vs. µg of total glutenins per grain) (0.80±0.08) was more than four times greater than that of HMW-GS (0.20±0.66), suggesting that cultivars with a higher total glutenin content could have a different balance between HMW- and LMW-GS than cultivars with a low glutenin content. In fact, the HMW-GS to LMW-GS ratio (HMW/LMW) was significantly different between genotypes, ranging from 0.13 (in Aureo) to 0.33 (in Trigu murru) (Table 8), but no relationship was found between that ratio and the total grain glutenin content (data not shown).

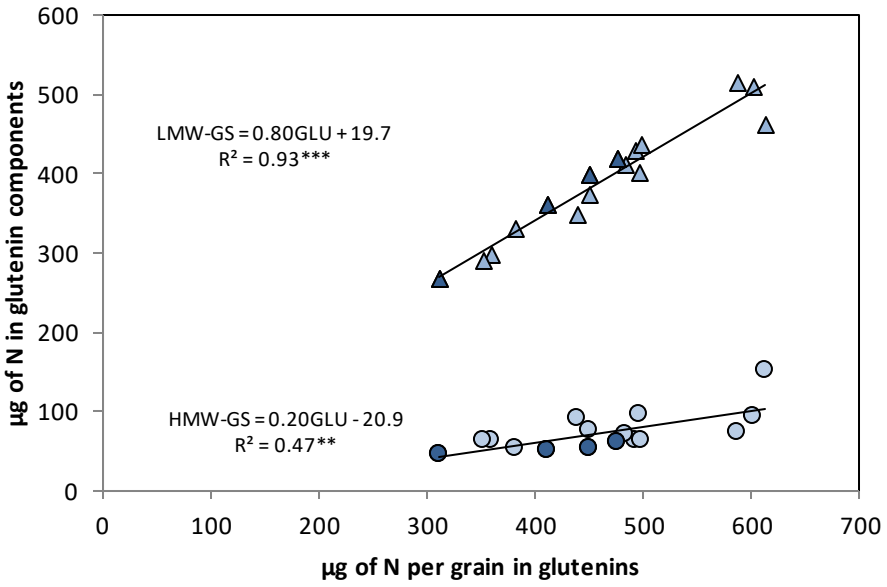

Figure 7. Relationships between the variation in the µg of total glutenins (GLU) per grain and the amount of the two glutenin components LMW-GS (triangles) and HMW-GS (circles). Points are cultivars means across years and N treatments. Darker symbols represent the groups of modern and intermediate cultivars and the lighter symbols represent the old cultivars. *** $P<0.001$, ** $P<0.01$.

Proteins subunits can be categorized as S–poor (HMW-GS and omega gliadins) or S–rich (LMW-GS and alpha/beta and gamma gliadins). The ratio S-rich over S-poor did not vary with GNµg, but it was significantly different among genotypes, with Trigu murru having the lowest ratio (3.07) and Aureo the highest one (7.63) (Table 8).

Table 8. Ratios between various protein components

	HMW/LMW		GLI/GLU		Srich/Spoor	
Year						
2016	0.19	*a*	0.85	*b*	6.2	*b*
2017	0.18	*a*	1.03	*a*	6.4	*a*
Nitrogen						
N46	0.18	*a*	1.04	*a*	6.6	*a*
N86	0.19	*a*	0.84	*b*	6.0	*b*
Cultivar						
CALABRIA	0.15	*gi*	0.83	*cf*	7.7	*ab*
CAPPELLI	0.15	*hi*	0.85	*cf*	7.5	*bc*
DAUNO	0.24	*c*	0.98	*bd*	4.0	*h*
RUSSELLO	0.20	*d*	0.93	*ce*	6.5	*eg*
SARAGOLLA	0.26	*b*	1.09	*ac*	3.6	*h*
SCORZONERA	0.21	*d*	1.26	*ab*	6.9	*ce*
TAGANROG	0.17	*ef*	0.96	*bd*	8.3	*a*
TBIANCU	0.16	*fh*	1.26	*ab*	6.3	*eg*
TIMILIA	0.18	*e*	0.80	*cf*	6.8	*cf*
TMURRU	0.33	*a*	0.95	*ce*	3.7	*h*
TRIMINIA	0.22	*d*	1.35	*a*	6.1	*fg*
TRINAKRIA	0.15	*hi*	0.65	*ef*	7.4	*bd*

ICHNUSA	0.14	*ij*	0.91	*cf*	7.4	*bc*
MARISTELLA	0.14	*ij*	0.63	*f*	6.2	*eg*
SVEVO	0.16	*fg*	0.85	*cf*	6.0	*g*
AUREO	0.13	*j*	0.70	*df*	6.6	*dg*

Means with the same letter are not statistically different at the Tuckey Test for $P<0.05$

PCA was performed on the correlation matrix of the grain N content and composition traits plus N source and GNO (number of grains per m^2) (Figure 8). The first two components explained 73.3% of the total observed variability. The first component captured 58.6% of the total variance and was mainly correlated with N in the grain (r = 0.35), grain protein percentage (r= 0.32) and GNO (r = -0.26). The PCA results confirmed some of the points discussed above: the leading role of GNO vs. N source in explaining the genotypic differences observed; the strong relationship between GNµg and the quantity of the albumin-globulin fraction; and the stronger dependence of total GLU content per grain on the variation of LMW-GS than on HMW-GS. On the other hand, PCA also highlighted the existence of a strong relationship between grain protein percentage and the gliadin components and between GNµg and HMW-GS, and this allowed us to group the studied cultivars. The extreme cultivars according to the first axes were: Svevo, Aureo and Maristella on the negative side due to their high GNO; and Trigu murru on the positive side, which was very close to the GNµg due to it having the highest value for this trait. Scorzonera and Triminia represented a group opposite to Trigu biancu and Trinakria for the second axis, which was positively related to gliadins and negatively related to glutenins.

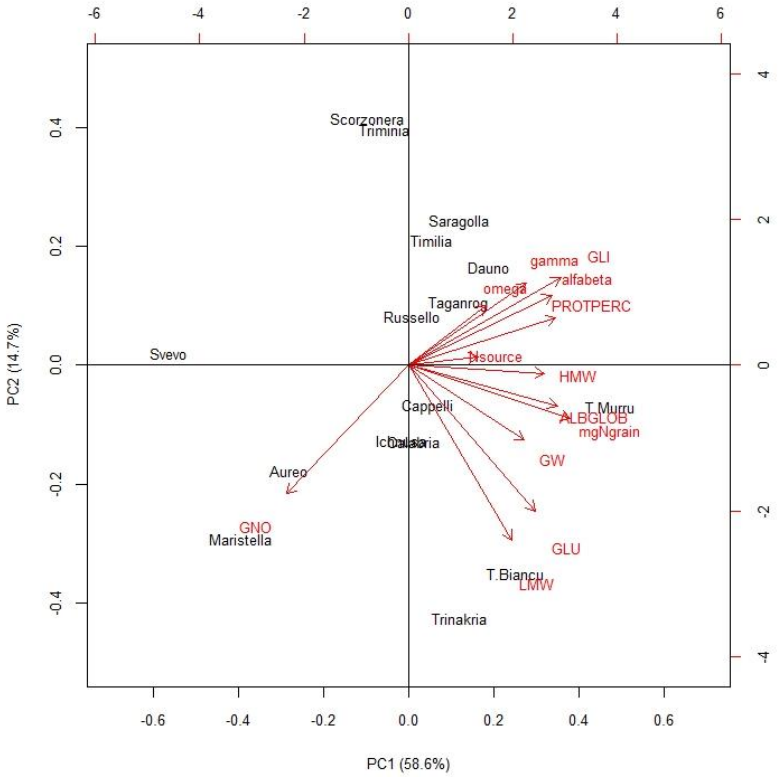

Figure 8. PCA of grain components obtained from the sixteen cultivars means across year and nitrogen treatments: grain number (GNO), grain weight (GW), grain nitrogen content (mgNgrain), protein percentage (PROTPERC), N source, the contents of albumins-globulins (ALBGLOB), gliadins (GLI), alpha/beta, omega, gamma, glutenins (GLU), high molecular weight (HMW) and low molecular weight (LMW) glutenins subunits (GS).

DISCUSSION

Growing interest in the recovery of abandoned marginal land areas and the diversification of cropping systems in response to the spread of new social values (Lynch, 2007 and Desclaux et al., 2008) are what fuelled our

interest in analysing wheat grain nitrogen content and composition in a crop system suited to old durum wheat cultivars. The study takes into consideration low soil fertility, terminal water stress typical of the Mediterranean environment and two relatively low N rates, for which the higher rate is split between sowing and the stem elongation phase to maximize the effect of nitrogen on grain nitrogen content (Sander et al., 1987).

Nitrogen effect

The additional application of N at the stem elongation phase, i.e., during the period of maximum crop growth, by improving the nutritional status of the crops as evaluated by the NNI (and increasing the NNI (nitrogen nutrition index)) increased the crop growth rate and hence the total biomass at anthesis and its N concentration. This was the likely reason behind the lower GNO observed at N46 compared with that seen at N86. Our results are in accordance with those of Desmotes-Maynard et al. (1999) and Martre et al. (2003) who found that the limiting crop growth rate induced by N deficiency around flowering can decrease the GNO and GY in wheat. On the other hand, we did not find N deficiency to change biomass partitioning into ears and stems or fruiting efficiency. Indeed, Desmotes-Maynard and Jeuffroy (2004) suggested that the N concentration in the ear at anthesis has no effect on fruiting efficiency.

The greater biomass and presumably greater leaf area developed due to the greater amount of N available could have induced more severe water stress following anthesis as a consequence of a higher transpiration rate, considering that water availability rapidly decreased to very low levels after this phase. The greater sensitivity of dry matter over protein deposition to drought and high temperatures (Jenner et al., 1991; Triboi and Triboi-Blondel, 2002) thus explains why grain weight was lower at the higher fertilisation rate, whereas the GNμg did not change, resulting in a higher grain protein percentage.

In the end, the higher GNO obtained with the higher N rate was associated with a higher grain protein percentage, but unchanged GNμg (μg of N in the grain), a frequent observation in wheat grown in Mediterranean environments (Giunta et al., 2007 and Giunta et al., 2019). The effect of the N treatment on grain protein fraction was not, therefore, mediated by an effect on GNμg and was limited to an increase in glutenin and a decrease in the gliadin fraction, in contrast with some previous studies on bread wheat (Jia et al., 1996; Doekes and Wennekes, 1982 and Gupta et al., 1992) but in agreement with other authors who have suggested that the N effect depends on the genotype considered (Pechanek et al., 1997; Wieser and Seilmeier, 1998).

Year effect

The greater grain yield decrease that affected the modern cultivars Aureo and Svevo compared with the old cultivars under the more stressful meteorological conditions of 2017 was due to the combination of a reduction in both GNO and grain weight. Old Italian cultivars are characterized by a higher grain filling rate compared with modern ones (Motzo et al., 2010), and this allowed them to maintain high grain weights even under the severe water stress that characterised 2017 and in spite of the higher temperatures and shorter time available for grain filling due to their later anthesis. High temperature and water deficit during the grain filling period were expected to increase grain protein concentration consequent to a decrease in carbohydrates due to a shortened duration of accumulation, whereas the quantity of protein did not change because the shorter duration of accumulation is compensated for by the higher rate (DuPont et al., 2006). What we observed in our experiment was a similar GNμg and grain protein percentage for the two years, in spite of a shorter duration of grain filling under the more severe stress of 2017. It is worthwhile noting that both years suffered from temperature and water stress and that the grain filling duration was simply calculated as the difference between anthesis and maturity. The only protein fraction affected was the gliadins, as previously noted by Daniel and Triboi (2001).

Genotypic effect

The set of cultivars compared in this experiment comprised two modern cultivars selected for their good grain quality associated with low to medium GYs, and a larger set of old cultivars with different medium to high grain protein contents even under low N inputs (Mefleh et al., 2019b). Under the crop system described, and in the two seasons characterized by a terminal drought of distinct severities, some old cultivars exhibited a GY that was not different to that for the modern ones, in agreement with a previous study by Mefleh et al. (2019b), and this was associated with a greater N uptake. This result lies in apparent contradiction with those reported in Giunta et al. (2007), who concluded that modern durum wheat cultivars were able to accumulate more N compared with old durum wheat cultivars as a consequence of a greater GY at similar biomass levels. The higher N rate (60 and 100 kg of N ha^{-1}), given entirely at sowing, the lack of any water limitation, and the set of modern cultivars used by those authors were the likely reasons for this discrepancy, because the modern

cultivars used by Giunta et al. (2007) were able to produce a significantly higher GY compared with the older ones.
GNO was crucial for the variation in GY in this experiment, as generally noted for wheat, and clearly discriminated the old (GNO <7200) from the intermediate and modern cultivars (GNO>9000). At the same time, some old cultivars had GY levels comparable to those of modern ones thanks to their particularly large grains. The genotypic variation in GNO and in fruiting efficiency were strongly dependent upon one another, as previously demonstrated for wheat (Stapper and Fischer 1990; Abbate et al. 1998) and triticale (Motzo et al., 2011). Hence, thanks to the high proportion of total biomass attributed to the ear at anthesis and a high number of grains per g of ear biomass at anthesis, modern cultivars were able to develop a large sink: source ratio for biomass, and also for N, given the strong relationship between biomass accumulation and N uptake (Lemaire et al., 2008).

NNI was particularly useful to describe the nutritional status of our crops given the large genotypic variability in biomass at anthesis. On average, the genotypic variation in NNI at anthesis was within the 0.7 - 1.1 range observed by Gaju et al. (2014) for wheat crops fertilized with 210 kg N ha^{-1} and did not discriminate between old and modern cultivars. On the other hand, several cultivars suffered N stress under the lower N rate (N46). A large genotypic variation was observed in the processes of nitrogen accumulation and redistribution, not only between cultivars from different eras of breeding, but also within the group of old cultivars. The longer duration of nitrogen accumulation deriving from the later anthesis of old cultivars and their greater biomass (Lemaire et al., 2008), presumably associated to a larger root system (Gaju et al., 2014; Siddique et al., 1990), allowed them to take up more N by anthesis compared with modern cultivars. Root depth and extension were particularly important in this experiment given the low N fertilization rates adopted (Lynch, 2007).
On the other hand, the later anthesis had a drawback effect on the capacity of old cultivars to prolong their N uptake after anthesis; nevertheless, the N source (pre + post anthesis N uptake) available to the grains of old cultivars was greater than that for modern ones. Post-anthesis N uptake in Mediterranean environments, although important, is extremely aleatory due to the uneven amount and distribution of spring rainfall. N translocation is therefore extremely important in order to feed the growing grains, although the NRE values for the vegetative tissues calculated in these experiments were lower in comparison with the genotypic variation reported by other authors that ranges between 0.52 and 0.92 (Gaju et al, 2014, and papers cited therein).
Given the similar NRE values for old and modern cultivars, we might expect that the greater N source of the old cultivars would have permitted more N remobilization per unit surface compared with modern cultivars. However, this was not observed since we found much more N per unit surface in the grains of modern cultivars than in those of old cultivars. One possible explanation is that the high N concentration and nitrogen remobilization efficiency of ears favoured modern cultivars because ears constituted a greater proportion of their total biomass at anthesis. Secondly, the higher amount of N left in the straw of old cultivars at maturity suggests that the proportion of structural N in these cultivars was higher (Pask et al., 2012). In the end, we cannot exclude that the low level of GNO - coherent with the agricultural system adopted – generated a sink limitation, particularly in old cultivars, whose grains achieved a very high level of protein content. According to Pask et al. (2012), N remobilization, generally driven by N source, can become sink limited when the N content of the grains exceeds 1.1-1.2 mg N $grain^{-1}$. In fact, many of the old cultivars in the present work had higher N contents.
Genotypic variation in grain protein percentage was mediated by the variation in GNμg. Considering that the partitioning of the different protein fractions is regulated at the individual grain level (Martre et al., 2006), it is thus reasonable to presume that we can quantify whether, and to what extent, the genotypic variation in GNμg at maturity is also responsible for a variation in the amount and proportion of the different protein fractions and subunits analyzed. To the best of our knowledge, this approach has only been used to assess the effect of N nutrition and environmental factors on the quantitative ratios between the various grain protein fractions within a limited set of bread wheat genotypes (Martre et al., 2006; Triboi et al., 2003). By expressing nitrogen on a per grain basis in μg instead of as a percentage, it was also possible to exclude the confounding effect of the variation in carbohydrate accumulation, the two processes of N and carbohydrate accumulation being almost independent (Jenner, 1991).
With the genotypic variation in GNμg, the variation in the content of the storage proteins was greater than that of the metabolic or structural proteins; i.e., each μg of total grain N difference between cultivar was associated with an increase in the μg of gliadins and glutenins that was more than twice the increase in μg of albumins-

globulins. Metabolic protein accumulation is sink limited (Martre et al., 2003), in contrast with the source-limited storage proteins. Therefore, the variation of metabolic proteins and GNμg are both dependent on GNO. This explains the strength of the relationship between GNμg and metabolic protein content and also the limited variation in metabolic proteins. The genotypic variation in GNμg was more tightly associated with the variation in gliadins than in glutenins, at least in the set of cultivars and environmental conditions analyzed here. In turn, the variation in gliadins was able to explain most of the variation in the alpha/beta gliadin group, and the genotypic variation in glutenins was better able to explain the variation in LMW-GS than the variation in HMW-GS.

The S concentration of the grain is an important indicator of the size distribution of the gluten macropolymers that govern the rheological properties of dough (Randall and Wrigley, 1986; Zhao et al., 1999; and Zörb et al.). The cultivars with the highest gliadin content also had a higher proportion of S-rich gliadin subunits (alpha/beta and gamma), while the S-poor omega subunit did not vary with the variation in the total μg of gliadins per grain (Shewry et al., 1986), in accordance with Uthayakumaran et al. (2001).

Moreover, the genotypic variation in the S-rich LMW-GS was strongly dependent on the variation in total glutenin content; i.e., the cultivars with high glutenin content also had a high S-rich LMW-GS content. This means that genotypes with different grain gliadin and glutenin contents should have a different proportion of S-rich and S-poor gliadins and glutenins subunits. The stronger dependence of LMW on the total quantity of glutenins in the grains compared with that of S-poor HMW-GS can be explained by the lack of polymorphisms in the LMW glutenin subunits characterizing the set of cultivars analyzed, in contrast with the six different HMW-B1 types found among our 16 cultivars, which also implied differential levels of expression of specific storage proteins (De Santis et al., 2017).

The significant genotypic differences in the ratios GLI/GLU, Srich/Spoor and HMW/LMW were not influenced by the corresponding variation in grain N content even when the slope of the regressions of the two terms of the ratios against the total, as in the case of HMW and LWM, were different. This is because correlations only explained part of the genotypic variation in the content of the different protein components, leaving an unexplained and/or unexamined part. The cause behind the difference in genetic behaviour remains to be fully understood (Triboi et al, 2000), but another factor that could have affected these ratios is the already mentioned difference in the allelic composition and expression of our cultivars.

GLI/GLU is one of the most meaningful traits of grain quality because an appropriate balance between gliadin and glutenin is one of the determinants of gluten rheology (Melnyk et al., 2012). According to Martre et al. (2006) and Triboi et al. (2003), the protein fraction composition of wheat grains from widely different cultivars could be deduced directly from the total quantity of N per grain, and differences observed at maturity in protein fraction composition are mainly because of differences in the total quantity of N accumulated during grain-filling. We were not able to confirm the consistency of the N allocation in the grain when the change in N was induced by cultivar because, in spite of the similar slopes for the relationships between gliadins and glutenins vs. the GNμg, the quantitative ratio between gliadins and glutenins changed with the cultivar and was not affected by the GNμg. The reasons for this discrepancy lie in the difference in the species used for analysis, bread wheat was addressed in Martre et al (2006) and Triboi et al (2003), whereas durum wheat was the subject of this experiment, and in the set of cultivars analyzed, which in the present study included cultivars from different eras of breeding, expressing a large polymorphism for protein fractions.

Finally, our results on the protein fractions suggest that: a) the quantitative variation in gliadin and/or glutenin found between cultivars can be accompanied by a change in the relative content of their constitutive subunits; b) grain nitrogen content was able to explain in part the variation in protein fractions and subunits, at least for the varieties studied; and c) another factors such as allelic composition should be always considered.

CONCLUSION

The value of recovering marginal areas unsuited to high-input agricultural systems in the Mediterranean environment makes selecting the cultivars that can be adapted to this type of agricultural system an important challenge. One potential solution could be old durum wheat cultivars, which in these agricultural systems can guarantee yield levels similar to those obtained by modern cultivars, but with a higher protein percentage. In the specific set of cultivars examined here, and in the context of two particularly dry seasons and a management approach suited to low-input systems, the higher grain protein percentage of the old cultivars was mainly due to their lower GNO, and not to their larger N source since a large proportion of their N source was not translocated to the growing grains. At the same time, the low-fertility conditions resulted in

GNO differences between old and modern cultivars that were small enough such that they could be compensated by the larger grains of old cultivars.
GNO variation was the main determinant underlying the variation in µg of N per grain, and also influenced the partitioning between the different protein fractions and subunits, giving new insights into the regulation of protein accumulation, at least for the cultivars studied. On the other hand, the relationships between the total quantity of N accumulated per grain and the quantity of the different protein fractions and subunits could only explain part of the genotypic variation in grain protein composition, leaving room for genotypic differences independent of the grain N content. The results of our studies confirm that the large genotypic variation present in durum wheat for grain protein content also impacts grain protein composition.

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SUMMARY AND CONCLUSIONS

Using old durum wheats in low input agriculture system is a perfect scenario due to the great sustainability of their cultivation, their good grain productivity combined with a good grain protein content and their rich genetic variability.

In a low input environment, old cultivars presented a grain yield not different from that of the modern cultivars consequently to their greater N uptake and larger grains.

Grain number per unit area showed to be strongly associated with grain yield among genotypes and to play a critical role in determining grain nitrogen content. Old varieties showed to have higher protein percentage and content than modern varieties due to their lower grain number.

The variation in grain N among genotypes was more associated with a variation in the storage proteins than in the metabolic ones. However, the total N in the grain only explained a part of the genotypic variation in the content of proteins fractions and subunits. Other factors such as difference in the allelic composition and expression could have affected these contents. This highlights the following statement, mentioned in the review (chapter one): 'quality is not defined by a single allele or trait but, instead, by the combination of allele composition and the quantitative ratios of their products determined by their level of expression'. The results of our studies confirm the high importance of varietal behavior in assessing wheat grain and end-product quality.

The increase in N fertilization did not increase the content of N per grain but, instead, resulted in a higher grain protein percentage as an indirect effect of the decrease in grain weight.

A large genotypic variation was observed among cultivars from different eras of breeding and within the group of old cultivars in the processes of N accumulation and redistribution, grain quality traits and bread quality.

The results of the bread-making trial highlighted the importance of protein percentage, gliadin content and gluten index in predicting bread quality among old durum wheat varieties. While the improved gluten strength of modern durum varieties had an opposite effect on dough extensibility and bread volume.

The world of agronomy and agriculture economics is witnessing a dilemma of improving the quantity as well as the quality of wheat production in a low input agriculture system. New genotypic variability can be of help and old durum wheat cultivars can be considered a worthy option because they proved to be able to combine good productivity levels with good grain quality suitable for many end-products including the staple food, bread.