Agronomic strategies for Sustainable Managment of Durum Wheat Cultivation in Mediterranean Area

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Preface

This Ph.D. dissertation is the result of the experimental work carried out at the Department of Sciences of Agriculture, Food, Natural resources, and Engineering (DAFNE) of the University of Foggia (Italy), at the Research Center for Cereal and Industrial Crops (CREA-CI) of Foggia (Italy) and at the Department of Analytical Chemistry of Complutense University of Madrid (Spain).

The Ph.D. project was carried out under the supervision of Prof. Marcella Michela Giuliani. The field trials at the Research Center for Cereal and Industrial Crops (CREA-CI) and the research period at the Complutense University of Madrid have been possible thanks to the collaboration with Dr. Pasquale De Vita and Prof. Yolanda Madrid Albarrán, respectively.

The Ph.D. dissertation deals with different agronomic strategies to improve the sustainability of durum wheat cultivation and its quality in the Mediterranean environment, and it is organized in seven chapters as described below.

Chapter I is an introduction section in which the key topics about the sustainable production of durum wheat in the Mediterranean environment are reviewed, focusing on nitrogen fertilizers management, a significant constraint in wheat production. The chapter is divided into the following sections:

- **WHEAT: PAST, PRESENT, AND FUTURE** – This section reviews the past, the present, and the future of the relationship between human and wheat cultivation.
- **DURUM WHEAT PRODUCTION AND END USES** – This section reviews the durum wheat production and consumption, overviewing the data and the primary area of production and its final utilization.

- **DURUM WHEAT IN MEDITTRANEAN REGION** - In this section, the characterization of the Mediterranean region is reported, and in particular, the durum wheat growing condition.

- **SUSTAINABLE AGRICULTURE** – This section introduces sustainable agriculture, focusing on low-input and organic agricultural systems.

- **RULE OF NITROGEN (N) IN DURUM WHEAT CULTIVATION** – This section reviews the most crucial N-related parameters as N uptake, N utilization efficiency, and N remobilization, the essential N sources used in durum wheat cultivation, and the relationship between N and durum wheat yield and quality parameters.

- **NUE: FROM THE GENERAL FOOD SYSTEM TO THE CEREAL CROPS PRODUCTION** – This section introduces the nitrogen use efficiency concept, going from the general food system to the specific cereal crops system. It reviews agronomic, environmental, and breeding progresses to improve the low NUE values.

Chapter II describes the aims of the experimental activities performed during the three-year Ph.D. period.

Chapters from III to IV deal with research experiments conducted during the Ph.D. project; in particular, the **chapters III** and **IV** correspond to original papers published in International Peer-Reviewed Journals:

- **Chapter III** – "Strobilurin Effects on Nitrogen Use Efficiency for the Yield and Protein in Durum Wheat Grown Under Rainfed Mediterranean Conditions": original paper published in 2020 in Agronomy. DOI: https://doi.org/10.3390/agronomy10101508.

- Chapter IV – "Effects of Genotype, Growing Season and Nitrogen Level on Gluten Protein Assembly of Durum Wheat Grown under Mediterranean Conditions": original paper published in 2020 in Agronomy. DOI: https://doi.org/10.3390/agronomy10050755.

- **Chapter V** – "Foliar application strategies to improve grain yield and N-related traits in old and modern durum wheat varieties grown under organic management in Mediterranean area."

- **Chapter VI** – "Selenium agronomic biofortification of organic fertilized wheat: Se grain content and selenium speciation".

The final part of the dissertation, chapter **VII**, defines the most relevant conclusions deduced from the experimental activities and introduces future investigations about improving the sustainability and quality of durum wheat cultivation.

I. Introduction

WHEAT: PAST, PRESENT, AND FUTURE

Wheat (*Triticum* spp.) is a cereal with very ancient origins since humans consumed it before the beginning of agriculture. The first "green revolution" for wheat started during the "Neolithic revolution" along the Fertile Crescent about 10,000 years ago (Hillman and Davies, 1990), with the intuitive idea of picking the biggest grains for sowing. From that moment on, progressive domestication of some species of wheat started, remarkably the emmer wheat (*Triticum turgidum* L. ssp. *dicoccum*) (Mac Key, 2005), from which modern durum wheat derivates (Salamini et al., 2002). From the Fertile Crescent zone, emmer wheat later spread to Asia, Africa, and Europe (Italy in particular), becoming the most widespread wheat species during the Neolithic and Bronze Age periods. It was a staple food for the Babylonians, Assyrians, and Egyptians, who were the first to make oven-baked bread. Its cultivation started to vanish at the end of the Bronze Age and, by the beginning of the 20th century, the derived species, durum and bread wheat, almost wholly substituted the ancient species (Mefleh et al., 2019).

Nowadays, wheat is still one of the most important cereals feeding humanity (Awika, 2011). It is a staple source of nutrients for around 40% of the world's population; it is the leading source of vegetal protein in human food, having a protein content of about 13%, which is relatively high compared to other major cereals (Giraldo et al., 2019). In general, wheat is more adaptable to a wide range of growing conditions than other major cereal crops. Thus, it is the most widely cultivated food plant globally, with 214 million hectares cultivated in 2018, producing 734 million tons of grain (FAOSTAT, 2018).

What does the future hold? With the latest estimates, concerns are growing about whether food production will not meet demand in the near future. By 2050 the world's population will reach 9.1 billion, 34% higher than today. Urbanization will continue at an accelerated rate, and about 70% of the world's population will be urban (compared to 49% today). To feed this larger population, it will be necessary to increase food production by 70% (FAO, 2009). Annual cereal production will need to rise to about 3 billion tons, from 2.1 billion today.

Additionally, the global mean surface temperature is expected to rise from 1.8 °C to 4.0 °C by 2100 (IPCC, 2000 SRES). Such changes will severely impact food production and availability: yield losses between 2.5 to 16% for every 1°C of increase in seasonal temperature are expected (Battisti and Naylor, 2009). The impacts of climate change on crop production are geographically very unevenly distributed. The Mediterranean basin has been identified as one of the two most responsive regions to climate change globally (IPCC, 2014). The most critical impacts of climatic changes in the Mediterranean region are likely associated with water availability since the whole region is already vulnerable to water scarcity and drought. At the current state of knowledge, we can say that climate change is more than a risk. It is a challenge to take effective action to mitigate its effects and adapt to its unavoidable consequences.

In response to this, it is crucial to promote technical improvements to increase future wheat production under the new ongoing environmental conditions. Advancements associated with wheat production may include the development and selection of well-adapted genotypes, the use of modern agricultural techniques, which retain soil health and improve nutrient and water efficiencies, and germplasm development, which improves resistance to biotic and abiotic stresses.

DURUM WHEAT PRODUCTION AND END USES

Although durum wheat constitutes only 6% of world wheat production, it is an economically important crop because of its unique characteristics and its use in making essential food products such as pasta (International Grain Council, 2010). Pasta is a key component of the diet of many countries. According to the International Pasta Organisation (IPO,2020), the pasta per capita per year consumption is, now, 23.1 kg in Italy, 9 kg in the USA, 8 kg in French and Germany, and 3.5 kg in the United Kingdom. These countries consume a third of the global pasta production and represent the most important Italian pasta markets (60% of production is destined for export). In addition to its use in pasta, the popularity of new durum products such as bulgur, couscous, freekeh, puffed cereals, hot cereal, desserts, filler for pastries, and, in some areas of the world, various types of bread (flatbread and double-sided kebab bread) is increasing (Elias and Manthey, 2005; Sissons, 2008).

Durum wheat is grown mainly in temperate world areas, particularly in North America and the Mediterranean basin. The Mediterranean basin accounts for more than half of the worldwide durum wheat-growing area (IGC, 2010). Europe is the largest world durum wheat producer: in 2018, 2.5 million hectares are dedicated to this crop, with an estimated output of about 9 million tons. The cultivation area of durum wheat in Europe is mainly concentrated in Italy, Spain, and France, making up 80% of total European production. Italy is the top European producer country being the traditional durum wheat-growing region; it accounts for about 50% of the entire European production, with about 4 million tons of grain produced in 2018 and medium (2016-2018) yield 3.5 t ha^{-1} (European Commission, 2020).

Remarkable is also the increase of the organic wheat superficies cultivated worldwide, which has risen by 250% since 2005 (FiBL, 2020). Market demands strongly stimulated this trend (Kubota et al., 2019). Thereby, a further increase in organically managed land, which now accounts for 1.4% of the current total world arable land, is predicted in the coming decades (Willer and Lernoud, 2019). In Europe in 2019 have been cultivated 13.8 million hectares under organic management (EUROSTAT, 2021). Italy is the third top European country, after Spain and France, with 1.9 million hectares of organically managed farmland and 310 thousand hectares of organic durum wheat (SINAB, 2020).

DURUM WHEAT IN MEDITTRANEAN REGION

Köppen (1931) was the first to introduce the idea of "Mediterranean climate" formally. His classification based upon temperature and precipitation, and "Mediterranean climate" is one of the five world types. Afterward, Rudloff in 'World Climates' (1981), utilizing Köppen's system, defined the areas which possess a Mediterranean climate, that are: the Mediterranean coast of France and the French hinterland, Corsica, Sicily, Sardinia, the greater part of Spain except for northern Spain and parts of eastern Spain, the Balearic Islands, Gibraltar, southern Portugal, the Madeira Islands, southern Italy, Malta, Greece, most parts of Turkey, Cyprus, northern and western Syria, The Lebanon, northern Israel, and northern Algeria (Fig. 1).

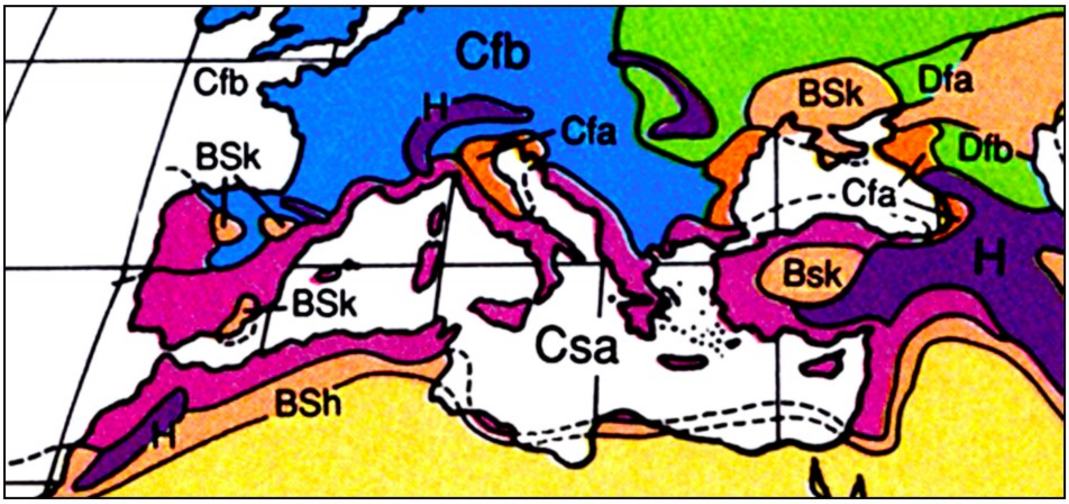

Figure 1. Mediterranean Köppen Climate Classification (from Ahrens, 1991). (Bs=Steppe; Bw=Desert; Cs=Mediterranean; Cf=Humid sub-tropical. Subdivisions: a=Hot summer; b=Cool summer; k=Cool, dry climate; H=Highland)

In general, the Mediterranean area is characterized by a dry season that can last from less than 2 to approximately 5 months. The mean annual precipitation ranges from 300 to more than 1500 mm, which occur erratically from winter to spring. The mean annual temperature ranges from about 11 °C to approximately 19 °C, with absolute maxima above 45 °C and minima below −20 °C (Hillel & Hatfield, 2005). However, there are some differences across the basin: i) temperatures decrease to the north, with some mitigation by the Mediterranean Sea occurring along coastlines in the winter; ii) rainfall decreases southwards in summer, while in winter region-to-region contrasts are less dependent on latitude; iii) in the east, minimum temperatures are generally lower than in the west; iv) periods of dryness tend to increase west to east as conditions become more continental and less exposed to the Atlantic; v) the orientation of coasts has a significant effect on rainfall due to prevailing winds (Trewartha, 1962).

In the Mediterranean basin, durum wheat is a winter crop, usually sown in autumn-winter and harvested from June to July. In the Mediterranean area, the principal stresses affecting cereal yield are drought and high-temperature events, which influence spike and kernel formation and development (Rharrabti et al., 2003; Guzman et al., 2016; Flagella et al., 2010; Giuliani et al., 2011b). On the other hand, the Mediterranean area shows suitable conditions to obtain durum wheat with high grain quality. During the crop cycle, usually, rainfall and temperature are favorable until anthesis, while during the grain filling period, limited rainfall and high temperatures frequently occur (Ercoli et al., 2008). However, in the Mediterranean area, precipitation and temperatures are highly variable within and across growing seasons, causing yield and quality to fluctuate intensively from season to season (Symeonidis et al., 2013). The low and erratic rainfall distribution can explain as much as 75% of the wheat yield variation (Del Moral et al., 2003).

SUSTAINABLE AGRICULTURE

The idea of "sustainable agriculture" appeared for the first time in 1987 in the World Commission on Environment and Development Report, titled "Our Common Future", produced by several countries for the United Nation. Also known as Brundtland Report, it defined the term "sustainable development" as meeting "the needs of the present without compromising the ability of future generations to meet their own needs" (Tait and Morris, 2020). For the first time, it was understood that the world is not as limitless and that there is a need to study and identify the impact that human activity has on the environment.

Sustainable agriculture is ambiguous in its meaning; the scientific literature has been prolific on this subject, and consequently, it is not a unique definition of sustainable agriculture. Collections of definitions are found in Goldman (1996) and in Hansen (1996), from which the following three definitions have been selected:

Sustainable Agriculture comprises **"management procedures that work with natural processes to conserve all resources, minimize waste and environmental impact, prevent problems and promote agroecosystem resilience, self-regulation, evolution and sustained production for the nourishment and fulfillment of all".**

MacRae et al., 1989

"For a farm to be sustainable, it must produce adequate amounts of high-quality food, protect its resources and be both environmentally safe and profitable. Instead of depending on purchased materials such as fertilizers, a sustainable farm relies as much as possible on beneficial natural processes and renewable resources drawn from the farm itself".

Reganold et al., 1990

Sustainable agriculture is an **"integrated system of plant and animal production practices having a site specific application that will, over the long term: (a) satisfy human food and fiber needs; (b) enhance environmental quality; (c) make efficient use of non-renewable resources and on-farm resources and integrate appropriate natural biological cycles and controls; (d) sustain the economic viability of farm operations; and (e) enhance the quality of life for farmers and society as a whole".**

U.S. Farm Bill, 1990

Sustainable agriculture is often described in contrast with conventional agriculture. The term of conventional agriculture itself was developed to clarify and justify alternative approaches to agriculture. Sustainable agriculture includes several ideological approaches to agriculture: i.e., organic, low-input, permaculture, agroecology. In this work, we will take into consideration low-input and organic systems.

Low-input agriculture

There is no official definition of low-input farming systems. One of the main definitions that are addressing the concept is:

Low-input farming systems "**seek to optimize the management and use of internal production inputs (i.e. on-farm resources)[...] and to minimize the use of production inputs (i.e. off-farm resources), such as purchased fertilizers and pesticides, wherever and whenever feasible and practicable, to lower production costs, to avoid pollution of**

surface and groundwater, to reduce pesticide residues in food, to reduce a farmer's overall risk, and to increase both short- and long-term farm profitability".

Parr et al. 1990

In general, low-input sustainable farming could be defined as a reduction, but not necessarily elimination, of extra-farm chemical input (chemical fertilizers, insecticides, and herbicides). Farmers adopt these practices primarily to reduce costs but also because they want to minimize the impact on the environment. Low-input agriculture is regulated mainly by Directive 2009/128/EC of the European Parliament and of the Council "establishing a framework for Community action to achieve the sustainable use of pesticides" (Directive 2009/128/EC) and by Council directive 91/676/EEC of 12 December 1991 "concerning the protection of waters against pollution caused by nitrates from agricultural sources" (Council directive 91/676/EEC). However, there is still no precise framework to respect. Low-input farming methods include rotations, crop and livestock diversification, soil and water-conserving practices, mechanical cultivation, and biological pest controls.

Organic agriculture

Organic agriculture is "**a production system that sustains the health of soils, ecosystems, and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic Agriculture combines tradition, innovation, and science to benefit the shared environment and promote fair relationships and good quality of life for all involved".**

IFOAM, 2008

Organic agriculture movements emerged in the 1930s and 1940s in the major industrial countries - in Britain by Lady Eve Balfour and Sir Albert Howard in Switzerland by Hans Mueller, in the United States by J. I. Rodale, and in Japan by Masanobu Fukuoka (Lockeretz, 2007) - as an alternative to the increasing intensification of agriculture, particularly the use of synthetic nitrogen (N) fertilizers (Lotten, 2003).

In the 1960-1970s, there has been a proliferation of organizations and associations promoting organic farming also in the Mediterranean countries (e.g. Soil Association in

United Kingdom and FNAB - FŽdŽration Nationale d'Agriculteurs Biologiques - in French), which, in 1972 in France, joined to found the IFOAM (International Federation of Organic Agriculture Movements), a body that even today sets standard regulations and guarantees to safeguard organically produced foodstuffs and protect consumers.

One of the aspects of organic production that separates it from the other alternative agricultural movements is its history of regulations (Rigby and Caceres, 2001). On 24 June 1991, the European Economic Community (EEC) Council approved the first regulation" on organic production of agricultural products and indications referring to it on agricultural products and foodstuffs" (Regulation ECC 2092/91). Following this, Regulation EEC 2078/92 granted subsidies to holdings converting to organic, favoring the huge and rapid development of organic farming in Europe. A further and important step was the Community approval of the regulation to recognize a logo for organic production (Regulation EEC 331/2000) and the approval of the regulation on organic livestock farming (Regulation EEC 1804/99). At the moment, in the European Union (EU), organic farming is regulated according to the European Council Regulation No 834/2007 (Regulation EC 834/2007), which sets the basis for national standards in the EU. Regulation is also complemented by several implementing acts of Commission on the production, the distribution, and the marketing of organic goods. Organic farming practices are unique, since they are the only ones codified as a law. Certified organic products are produced, stored, processed, handled, and marketed following precise technical specifications and certified as "organic" by a certification body, which may be private or managed by the government.

Currently, the organic sector is the fastest-growing agricultural sector in much of the developed world; worldwide, organic farmland has increased by 400% (377%) since 2000 (IFOAM, 2020). This trend is predicted to increase further due to concerns with pesticides and interests in the environment and food safety. (Rigby and Caceres, 2001). Despite the considerable growth of the organic sector, the rate of articles including organic management has grown little, and most of them proposed a holistic approach to study the entire cropping system management. Certainly, this approach represents the most informative one (Mäder et al., 2002), but detailed field studies on specific aspects of crop management remain essential for fine-tuning concrete guidelines for farmers and policymakers (Bengtsson et al., 2005). Indeed, the application of the organic production method is not the result of improvisation or the return to traditional techniques; instead, it requires huge investments in knowledge-oriented production and conservation techniques in compliance with the regulations in force.

Furthermore, the benefits of organic agriculture are widely debated: if for someone organic is the best solution to achieve sustainability, for others, it is an ideologically driven and inefficient approach (Trewavas, 2001; Emsley, 2001). Indeed, skeptics argued that organic agriculture needs more land to produce the same amount of food as conventional agriculture (Reganold and Wachter, 2016). Currently, the studies are focusing on the benefits of organic management, asking whether organic agriculture is good or bad. Seufert and Ramankutty (2017), in a review on the costs and benefits of organic agriculture, concluded that, on the positive side, organic agriculture delivers higher biodiversity and improved soil and water quality per unit area, enhanced profitability, and higher food nutritional value. On the negative side, there are many costs, including lower yields and higher consumer prices. Maybe we are looking at it in the wrong way. Indeed, no organic agriculture alone will safely feed the planet; rather, a blend of organic and other innovative farming systems, including agroforestry, integrated farming, conservation agriculture, mixed crop, and livestock. For example, integrated farming systems that blend organic practices with some conventional ones are more sustainable than conventional farming systems.

RULE OF NITROGEN IN DURUM WHEAT CULTIVATION

Nitrogen (N) fertilization is a key agricultural factor for durum wheat production. However, it is expected to be less used in agricultural systems because of its rising production costs, related to the rises in petrol prices (Abas et al., 2015). Moreover, governmental policies will restrict N use (Winiwarter and Hettelingh, 2011) as a consequence of its significant role in global warming and water pollutions (Vitousek, 1994; Pinder et al., 2012).

The use of N by plants involves several steps: uptake, assimilation, and remobilization. Field crops N uptake is highly variable within a single year, between years, between sites, and between crops. N uptake occurs at the root level. In general, plants absorb from the soil nitrate (NO_3^-) and ammonium (NH_4^+), as inorganic nitrogen sources, and amino acids under particular soil composition conditions. Most N uptake is in the form of NO_3^-. The nitrogen sources taken up by the plant are then assimilated. During the N assimilation, the NO_3^- is reduced to NH_4^+ both in roots and shoots. In the cytosol, NO_3^- is reduced into nitrite, catalyzed by the enzyme nitrate reductase (NiR) (Meyer and Stitt, 2001). Then, nitrite is translocated to the chloroplast and reduced to NH_4^+ by the NiR. NH_4^+ is assimilated to produce more complex compounds containing N (Lam et al., 1996) such as chlorophyll, which captures light used during photosynthesis, nucleic acids, amino acids, vitamins (e.g., biotin, thiamine, niacin, and riboflavin), all proteins, and other several organic molecules. Accumulation and redistribution of N are critical processes determining grain yield and grain quality (Simpson et al., 1983, Hirel et al., 2007, Gaju et al., 2011).

In literature, it is widely reported that N accumulated before anthesis provides the primary source of grain N (Palta and Fillery, 1995; Kichey et al., 2007; Gaju et al., 2013). Nitrogen remobilization has been studied in several plant species through the “apparent remobilization” method, which is the determination of the amount of total nitrogen present in the different plant organs at different times of development and through 15N long-term labeling, which allows the determination of fluxes (Gallais et al., 2006).

The use of ^{15}N tracing in cereals showed that the beginning of grain filling is a critical phase because N uptake declines during plant maturation and seed filling (Salon et al., 2001). N uptake and assimilation during the grain filling period is generally insufficient for the kernels’ high demand, and remobilization is necessary to address N to the kernels. Leaves are the most important sources of N for the grain; in wheat, their contribution varies from 50% to 90%, depending on the genotypes (Masclaux et al., 2001). Roots and chaff contribute about 10 and 15%, respectively (Dalling, 1985). Also, the environment plays a role in N remobilization, which is favored by limited N and water availability (Ercoli et al., 2008, Giuliani et al., 2011a).

Moreover, it is well known that the grain N content is correlated with flag leaf senescence. On the one hand, leaf senescence is essential for N mobilization; indeed, the photosynthesis-related proteins (particularly Rubisco) are degraded during senescence, providing the nitrogen's main source that plants can tap to supplement the nutrition of growing organs, such as seeds. On the other hand, delaying leaf senescence results in a prolongation of photosynthetic activity, resulting in increased grain yield and carbon filling into seeds. So, delay leaf senescence leads to higher yields but decreasing N remobilization and grain protein content (Masclaux-Daubresse et al., 2010). Thus, N is for sure the most critical constraint in durum wheat plant growth and productivity. However, its chemical nature makes it a dynamic and highly mobile element in agricultural soils, causing environmental problems through increased N pollution that acts locally and globally (Gruber and Galloway, 2008).

In the past half-centuries, synthetic nitrogenous fertilizers have highly contributed to the remarkable increase in food production; but their extensive use in agriculture has created pollution problems worldwide.

Nitrogen sources in durum wheat cultivation

The commercial mineral fertilizers commonly applied to soils during durum wheat cultivation are urea, ammonium sulfate, and ammonium nitrate. Urea is the most used N fertilizer in agricultural system worldwide. More than half of all fertilizer used globally is in the form of urea (Gilbert et al., 2006) because it is the most concentrated solid nitrogen fertilizer (46% N); moreover, it is highly soluble and low expensive to manufacture, store, and transport (Prasad et al., 1998). In the soil, urea changes to ammonium, and it may be lost from the surface of the soil, particularly in chalk and sandy soils. It is most efficiently utilized on soils with good moisture content so that the gaseous ammonia can go quickly into the solution. In dry conditions, during the summer, for instance, it is probably better to use ammonium nitrate, also to avoid gas ammonia volatilization. In ammonium nitrate, the N concentration is from 33.5 to 34.5% N and half of the nitrogen is in the form of NO_3^-, then it is very readily available. It is mostly used as top-dressing fertilizer. Finally, ammonium sulfate has lower N concentration than the other fertilizers (21 % N, 60 % SO_3). By now, it is seldom used, but at one time, it was the source of nitrogen; it has a high acidifying action on the soil. In general, all nitrogen fertilizers are particularly soluble for easy assimilation by crops and are subject to various loss mechanisms by volatilization and runoff after heavy rainfall and leaching into groundwater (Vitosh et al., 1995; Jarvis et al., 2011). For this reason, in the last time, it has been worked to produce slow-acting nitrogen fertilizers. Good results are achieved by such products as resin-coated granules of ammonium nitrate (26% N), sulfur-coated urea prills (36% N) (soil bacteria slowly break down the yellow sulfur in the soil), urea condensates and urea-formaldehyde (30–40% N). However, at present, these fertilizer types are not widely used because they are considered too expensive. (Finch et al., 2002).

Livestock manure is the second most used nutrient inputs in agricultural land (Hooda et al., 2000). The manure application has frequently been shown to stimulate soil microbial activity (Watson et al., 2002) and increase soil fertility (Glaser et al., 2002). There are two forms of N in manure: inorganic N and organic N. The inorganic form is initially present as urea and may account for about 50% of the total N. Urea in manure is no different from urea in commercial fertilizer, and it converts rapidly to ammonium, available for plant growth in a short time. The organic N fraction in manures is more stable and slowly released. Part of the organic N breaks down in the first year, and some in the following years, resulting in a slow-release N source.

In the organic cultivation system, N management is usually based on i) increasing the presence of legumes in the crop rotation (Thomsen et al., 2013), ii) broadcasting organic compounds before sowing (Mazzoncini et al., 2015; Peterson et al., 2013), and iii) adopting cereal-legume intercropping (Pelzer et al., 2012).

Besides livestock manures, other founts of organic N are the extra-farm source of N. The two more popular families of fertilizers that can be used in the EU as fertilizers and soil conditioners in organic farming (Regulation EC 889/2008) are:

- **Plant-based fertilizer:** it has a low content of N, and other macronutrients (phosphorus and potassium) and, for this reason, is not a stand-alone fertilizer. At this family of fertilizer belong: soybean meal (6-1-4.2 N-P-K), cottonseed meal (6-2-1 N-P-K), alfalfa meal (4-3-1 N-P-K), and kelp meal (1-0-2 N-P-K). These fertilizers can be used on organic crops, regardless of whether they came from certified organic plants. However, none of these meals can be used on certified organic crops if they come from genetically modified crops.
- **Animal-based fertilizer:** Every year, million tons of animal by-products are produced worldwide; for instance, the rendering industry processes nearly 15 million tons per year in the European Union (Swisher,2006). In this family are included blood meal, bone meal, feather meal, and fish meal. Some of this are protein-rich materials and offer a high potential as N-fertilizers (Mondini et al., 2008). For instance, feather meal and blood meal are highest in nitrogen (from 12 to 14%). Some of these residuals are commercially available as certified organic fertilizers and their application as soil amendments is promoted as a beneficial agricultural practice.

Nitrogen and its relationship with grain yield and quality

N is for sure the most important constrain influencing wheat grain yield (Ehdaie and Waines, 2001) and is a decisive factor in improving grain quality, particularly by its effect on grain protein concentration and composition (Gooding and Davies, 1997). The protein composition and the gluten strength are important quality characters in durum wheat because they are related to the pasta-making quality (See box 1).

Grain yield and grain protein content are strictly related to N fertilizer application and its effective uptake from the soil solution. Increasing N uptake seems to be the most promising strategy to enhance the N amount within the plant to increase both yield and grain protein. For wheat, maximum N uptake occurs after tillering and before flowering, and affecting mainly yield. In this phase, the good nitrogen availability stimulates the leaves' activity of the leaves and the chlorophyll functionality and quantity; N causes an increase in the rate of CO_2 assimilation and in the accumulation of the dry matter (Mosca et al., 1985; Bonciarelli and Santilocchi, 1997). Furthermore, a good supply of nitrogen delays the leaf senescence, and under favorable conditions, allows to extend the grain filling stage (Spiertz, 1977). While N accumulated after flowering has little effect on yield, it can increase grain protein content in absence of water deficiency and high temperatures (Flowers et al., 2007).

When fertilizer rates satisfy both yield and protein synthesis requirements, N significantly contributes also to the durum wheat quality parameters and, in particular, to the protein content (Giuliani et al., 2011b).

Several studies showed that high N rates could induce a further increase in protein content but not in protein quality (Triboï et al., 2000; Saint Pierre et al., 2008). Indeed, the extra N accumulated in the grain is mainly represented by gliadins (See box 1), which depress durum wheat dough strength (Sissons, 2007). Protein content and composition are the primary determinants of pasta cooking quality, and gluten strength plays an essential role in durum dough extrusion properties and cooked pasta quality (Feillet and Dexter, 1996). Most studies suggest that the increase in protein content also caused increased gluten strength (Abad et al., 2000; Johansson et al. 2001). However, Dexter et al. (1982) concluded that N fertilizer increased protein content of durum wheat characterized by weak to moderate gluten strength, without affecting gluten strength.

So, dough and gluten strength parameters continue to be used to assess quality of durum wheat, and the increasing demand for extra-strong durum types suggests that the protein strength also plays a significant role in end-product quality (Ames et al., 2003).

In conclusion, to meet farmer, industrial and environmental requirements, complementary processes have to be developed to manage crop N nutrition better.

BOX 1. PROTEIN COMPOSITION

Wheat proteins were among the first to be studied, and different classifications of them were proposed during the years:

Classification of wheat proteins on solubility - the Osborne groups

According to their extractability and solubility in different solvents, wheat proteins are traditionally separated into albumins, soluble in water, globulins, soluble in saline solution, gliadins, soluble in alcoholic solution, and glutenins, soluble in diluted acids or alkalis (Osborne, 1924). The first two fractions (albumins and globulins, with molecular weights ranging between 20-30 kDa) are characterized by proteins not involved in forming the gluten network but mainly having a metabolic and structural function. The other two fractions, gliadins and glutenins (with molecular weights of 30-70 kDa up to 100 million kDa), constitute about 80% of total semolina protein content and are exclusively storage proteins that form gluten and confer viscosity and extensibility for flour quality (Shewry and Halford, 2002) (Figure B1).

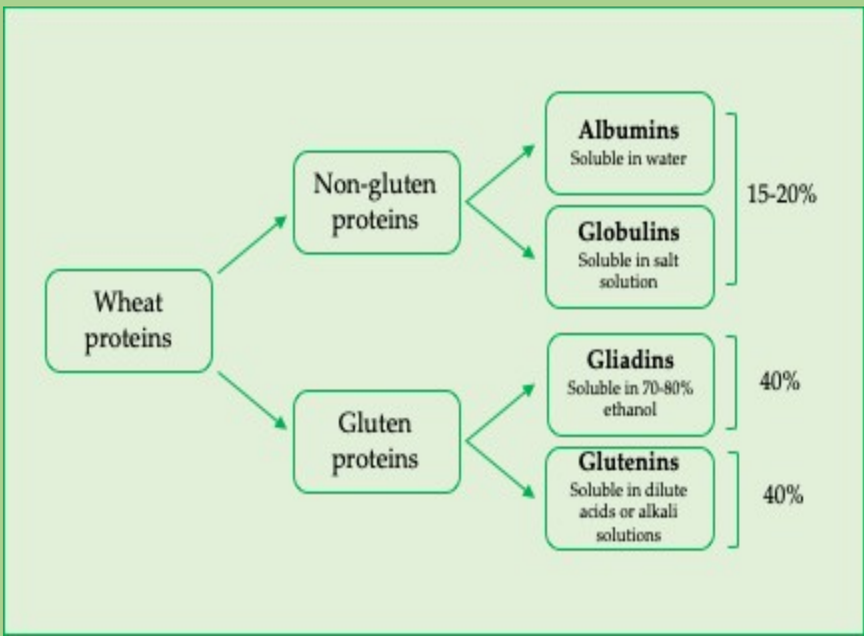

Figure B1. Protein composition of durum wheat endosperm (modified from Feillet, 1980).

Classification of gliadins and glutenins

Gluten proteins are classified, based on their electrophoretic mobility, into monomeric gliadins (α-, γ- and ω-) and polymeric glutenins (comprising high and low molecular weight glutenin subunits, HMW-GS and LMW-GS) (D'Ovidio and Masci, 2004) (Figure B2).

Also, Shewry et al. (1986) classified gliadins and glutenins proteins in three groups by their sulfur (S) content: ω-gliadins, poor in S; HMW-GS, intermediate content of S; α- and γ-gliadins and LMW-GS, rich in S (Figure B3).

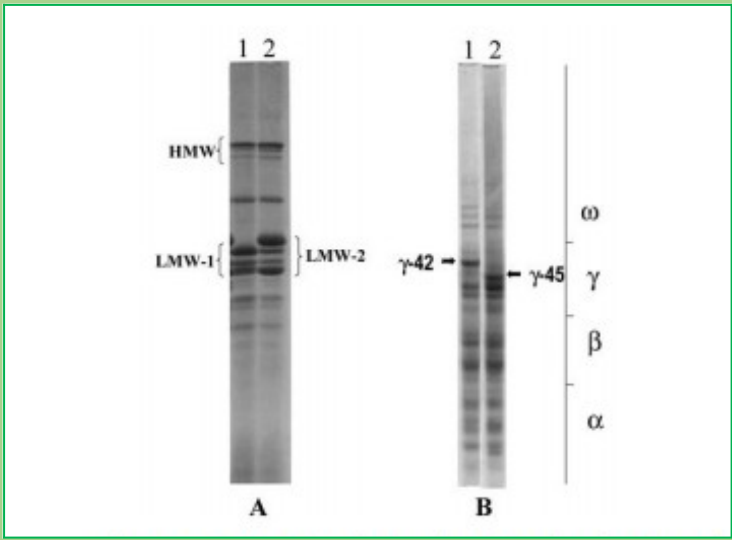

Figure B2. SDS-PAGE of glutenins (A) and acid polyacrylamide gel electrophoresis (A-PAGE) of gliadins (B) in durum wheat biotypes (from d'Ovidio and Masci, 2004).

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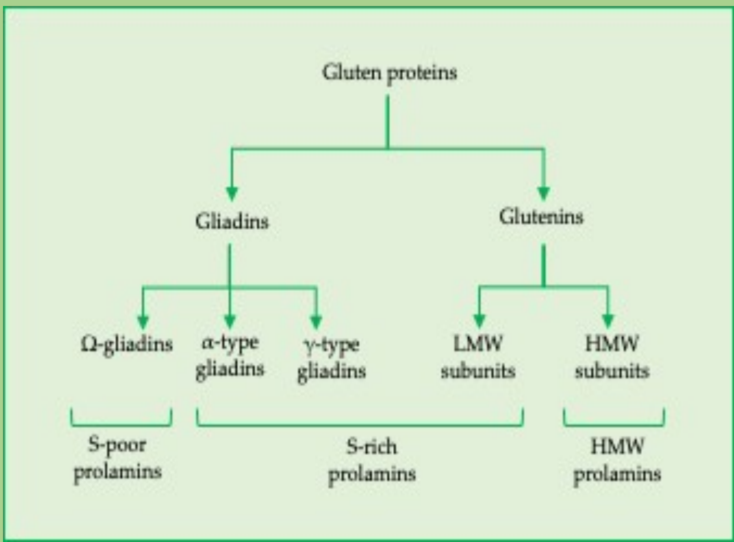

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Figure B3. Classification of gluten proteins in relation to sulfur content (modified from Shewry et al., 1986)

Qualitative parameters of gluten composition

Quantity and type of gluten proteins, as well as their aggregation/polymerization level, are responsible for the pasta-making quality of semolina. The semolina technological quality is usually described as a combination of elasticity and viscosity, with the balance between them being referred to as dough strength. In mature grains, gliadins and glutenins are aggregated in polymers characterized by different size and solubility. The distribution of the monomeric and polymeric proteins as well as their solubility is very important for the technological properties of the semolina. There is a certain amount of polymers which remains insoluble and therefore, not extracted, even using different media (such as phosphate buffer in sodium dodecyl sulfato, SDS). This fraction, named UPP (SDS- insoluble polymers), is very important for wheat technological characteristics and especially the proportion of UPP in total polymeric proteins (%UPP) since is highly correlated with dough strength.

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NUE: FROM THE GENERAL FOOD SYSTEM TO THE CEREAL CROPS PRODUCTION

In the past half centuries, the development and adoptions of the Haber–Bosch process (see Box 2) for the synthetic nitrogenous fertilizer production have highly contributed to the remarkable increase in food production. However, when N fertilizers are used at the wrong time and rate, negative environmental impacts can occur: reduction of water and air quality, eutrophication of freshwater and coastal ecosystems, climate change and stratospheric ozone depletion (Erisman et al., 2018). So, N resources management is becoming more critical, especially in food production, as agriculture is the biggest user of anthropogenic N globally.

The negative environmental impact of N used on food production is due to a general nitrogen use efficiency (NUE) low values (Yadav et al., 2017). NUE is defined by the Organization for Economic Co-operation and Development (OECD) as "**the ratio between the amount of fertilizer N removed from the field by the crop and the amount of fertilizer N applied**".

Nitrogen use efficiency is a critical indicator in agricultural systems, and several studies have estimated NUE in whole food systems and crop production.

At general level, four approaches are proposed to assess the NUE of food production:

- **Life Cycle Analysis (LCA):** a technique to assess the potential environmental and human health impacts associated with a product, process, or service by (1) compiling an inventory of relevant energy and material inputs and environmental releases and (2) evaluating the potential environmental impacts associated with the identified inputs and releases.
- **Nitrogen Footprint:** Total N losses into the environment resulting from the production of a defined unit of food.
- **Nitrogen Budget:** The inputs and outputs of nitrogen across the boundaries of a system; it can contain information about internal nitrogen fluxes within the system. The farm-gate nitrogen budget is a standard indicator for assessing the inputs and outputs across a farm's boundaries. The nitrogen balance indicator measures the difference between the nitrogen available to an agricultural system and the nitrogen

harvested and exported from the system in agricultural products. Also, the food system waste/loss indicator has received particular attention by studies that estimate nitrogen's loss through waste (Grizzetti et al., 2012; Gustavsson et al., 2011; Parfitt et al., 2010).

- **Environmental Impact Assessment:** A process to evaluate the likely environmental impacts of a proposed project or development, considering inter-related socio-economic, cultural, and human health impacts both beneficial and adverse (Erisman et al., 2018).

By now, the global NUE has remained stably low since 1980 (Conant et al., 2013; Lassaletta et al., 2014; Ladha et al., 2016), and only 47% of the reactive nitrogen added globally onto cropland is converted into harvested products (Lassaletta et al., 2014); this means that more than half the nitrogen used for crop fertilization is currently lost into the environment. Only European countries have shown substantial increases in NUE over time (Conant et al., 2013; Lassaletta et al., 2014). Indeed, in most European countries, a regular increase in fertilization and yield during the 1960–1975 period occurred, followed by a shift towards improved yields without further increasing fertilization (Lassaletta et al., 2014). This trend is related to reducing N inputs prescribed by European environmental policies and regulations (Van Grinsven et al., 2012), and interestingly, it did not prevent significant yield increases thanks to better N management. Despite the NUE increase, the nitrogen emitted into the environment in many cases remains high (Lassaletta et al., 2014).

A focus on cereal NUE is essential since they account for nearly 60% of global N fertilizer use (IFA, 2002). Cereal world NUE was estimated at 33%, implying that 67% of all the applied N can be lost within the soil system through leaching and/or gaseous forms, contributing to air and water pollution (Omara et al., 2019).

In cereal crop production, NUE is evaluated through agronomic indices. The agronomic NUE is most helpful in understanding the factors governing N uptake and fertilizer efficiency, comparing short-term NUE in different environments, and evaluating different N management strategies or technologies. Many authors defined NUE in the context of crop production, and the literature contains many different definitions depending on whether authors are dealing with agronomic, genetic, or physiological studies (Good et al., 2004; Fageria et al., 2008).

Moll et al. (1982) first defined NUE as “the ratio between grain weight and N supply (Gw/Ns) and N supply as the amount of N in the soil available for the plant”.

There are also alternative definitions of NUE in the literature (Cassman et al., 1998; Tilman et al., 2002; Raun et al., 2002; Semenov et al., 2007). Also, the measure of N available for the plant is complex; for this reason, many authors proposed to substitute it with the applied fertilizer N when calculating NUE (Van Sanford et al., 1986; Salvagiotti et al., 2009; Giuliani et al., 2011a; Yu et al., 2018; Omara et al., 2019).

NUE measures the plant’s ability to extract N from the soil and the plant’s ability to convert the absorbed N into harvested grain yield. Indeed, NUE can be partitioned into UPE (nitrogen uptake efficiency) and NUtE (nitrogen utilization efficiency) (Moll et al., 1982; OrtizMonasterio et al., 1997).

Moreover, another measure of NUE in cereal crops was proposed: the nitrogen use efficiency for protein (NUEp), defined as grain protein per unit of available N (Le Gouis et al., 2000). Similar to what was proposed by Moll et al. (1982), also NUEp can be partitioned into UPE and NHI (N harvest index) (Giuliani et al., 2011a).

The low NUE values relative to the cereal crops led the studies to focus on agronomic, environmental, and breeding progresses to improve this parameter (Raun et al., 2002; Presterl et al., 2003; Fageria and Baligar, 2005; Hirel et al., 2007; Garnett et al., 2009). The biggest challenge to improve NUE is the ability to combine genetic, agronomic, and environmental variables. Over time, the improvement in crop genetics has led to increases in cereal crop grain yields (Cassman, 1999; Hoisington et al., 1999). The advanced crop selection methods use faster techniques, such as marker-assisted selection and genetic engineering, which promise increased grain yield despite increasing environmental stresses. (Tester and Langridge, 2010).

However, the farmers still need to apply high fertilizers to satisfy the high-yielding cultivars’ potential (Dalrymple, 1986; Cassman, 1999). It is then clear that, besides crop genetic improvements, agronomy advances are fundamental to improve the nutrient use efficiency. It was demonstrated that improvements in agronomic techniques, including nutrient, herbicide, and pesticide applications (Omara et al., 2019) have resulted in a linear increase in global food production trends (Tester and Langridge, 2010). It is also noted that the environment has a central role in the nitrogen utilization by the plant. A significant portion of the N loss or inefficiencies in uptake is due to spatial variability in the landscape (Khosla et al., 2002). Thus, the farming approach has a central rule to optimize use of agricultural inputs for maximum economic output.

Regarding sustainable agricultural, low-input and organic systems present unique environmental conditions. These systems use an integrated approach to maximize nutrient cycling and maintain soil fertility and crop productivity in order to improve NUE. In particular, in organic farming, most studies show that less N is used due to organic system regulations (no mineral N), but this does not always mean high NUE because the yields are lower (Chmelíková et al., 2021). The big criticism is the release rate of inorganic N compounds. Plant capacity to take up organic molecules containing N, such as amino acids, has been demonstrated but is not yet well understood (Masclaux-Daubresse et al., 2010). Genotypic differences exist to take up amino acids in wheat cultivars, which may affect their performance in organic systems (Dawson et al., 2008). Despite this capacity, most plant N uptake is likely to be inorganic N (Ukalska-Jaruga et al., 2020), so mineralization rates and timing of N availability from organic fertilizers are crucial to understanding the NUE of cereals grown under organic management practices. Another cause of the yield reduction may be that the cultivars used in organic systems have been bred for conventional systems and thus are not adapted to organic conditions. There is more attention towards breeding crops specifically for organic (and low-input systems) as we are beginning to realize that beneficial traits for these systems may be very different from those that produce high yields in conventional systems (Murphy et al., 2007).

In conclusion, to maximize NUE, mineral N fertilizers are crucial in intensive conventional farming; in contrast, in organic farming, it is highly dependent on closed nutrient cycles, biological N fixation, and crop rotations (Chmelíková et al., 2021).

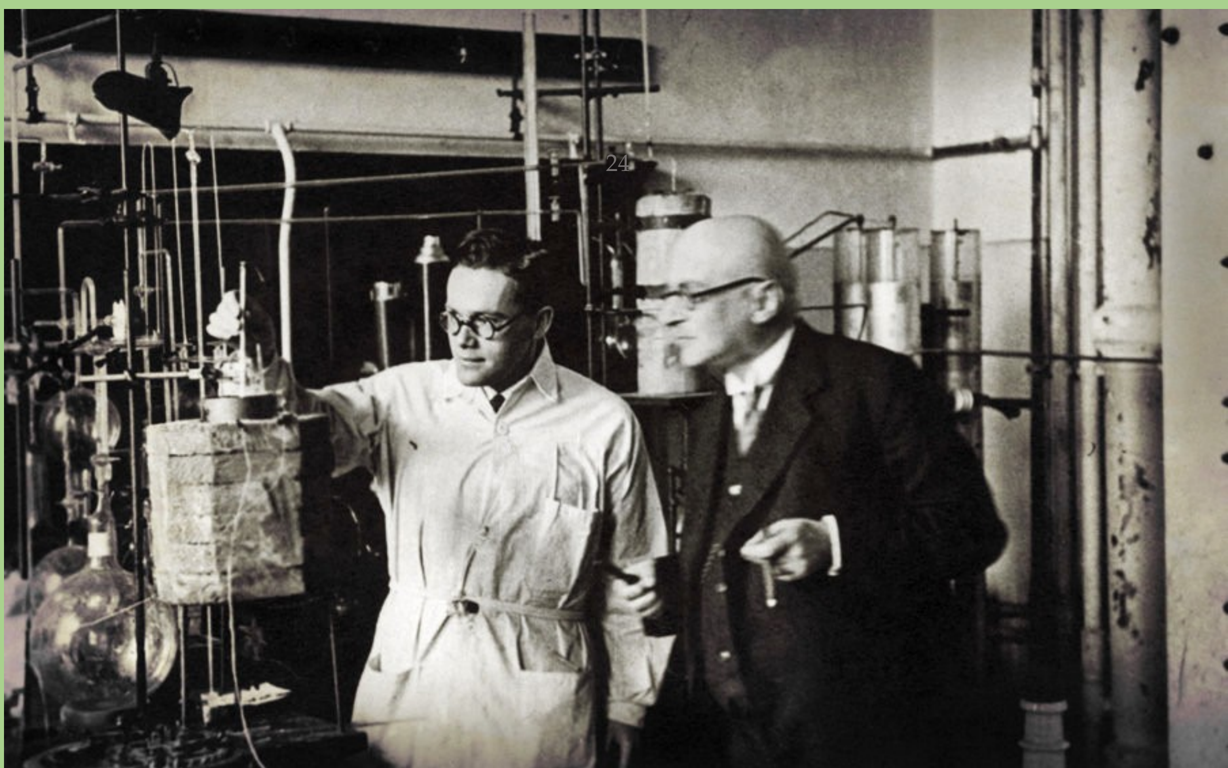
BOX 2. HABER-BOSCH PROCESS

The Haber-Bosch process is a method to synthesize ammonia (NH_3) directly from hydrogen (natural gas) and nitrogen (from the air). The method, developed by the German physical chemist Fritz Haber, was translated into a large-scale process using catalyst and high-pressure methods by Carl Bosch. Both chemists received the Nobel Prize for Chemistry: Fritz Haber in 1918 and Carl Bosch in 1931, jointly with Friedrich Bergius for high-pressure studies.

Haber-Bosch was the first industrial chemical process to use high pressure for a chemical reaction: it combines nitrogen from the air with hydrogen under extremely high pressures and moderately high temperatures, using iron as catalyst. The reaction is carried out at pressures ranging from 200 to 400 atmospheres and at temperatures ranging from 400° to 650° C for commercial production (Vojvodic et al., 2014).

Today the Haber-Bosch process is considered the most important invention in modern history. It permitted the manufacturing of fertilizers by using the abundant nitrogen reserves in the atmosphere. Currently, ammonia is one of the most highly produced inorganic chemicals. There are numerous large-scale ammonia production plants worldwide; a third of the total production occurs in China (32.1%), followed by India, Russia, and United States (US Geological Survey).

More of the ammonia produced is used for fertilizing crops; the remaining part for plastics, fibers, explosives, nitric acid, and intermediates for the pharmaceutic sector.



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II. Aim

The general objective of this Ph.D. was to investigate the sustainability of nitrogen fertilization in durum wheat. Nitrogen is the most requested element in cereal systems and has the most significant impact on the environment. The research work addressed the sustainability of nitrogen fertilization by considering low-input agricultural systems and organic farming systems in the Mediterranean area. This area is extremely interesting to investigate, as it is particularly subject to climatic changes, making the prudence of the agronomic use of nitrogen even more critical. The main objective was pursued through the four original works reported in the doctoral thesis, evaluating the response of different Italian durum wheat genotypes to the management of nitrogen fertilizers combined with different agronomic strategies. This work will likely bring essential indications on strategies to improve the efficiency of nitrogen use by the plant in both low input and organic systems, accompanied by improvements in both grain yield and grain quality.

In particular, chapter **III** – "Strobilurin Effects on Nitrogen Use Efficiency for the Yield and Protein in Durum Wheat Grown Under Rainfed Mediterranean Conditions" – reported the results relative to the investigation of the effects of strobilurin treatment and N fertilization on NUEy, NUEp (nitrogen use efficiency for yield and for protein), and their components, on two durum wheat cultivars. We aim to provide ready-to-use indications on the possible benefits connected to strobilurin application on durum wheat, considering the standard N management strategies used in the semi-arid area as Southern Italy.

Chapter **IV** – "Effects of Genotype, Growing Season and Nitrogen Level on Gluten Protein Assembly of Durum Wheat Grown under Mediterranean Conditions" –explored the effect of different nitrogen levels, including a low input rate on the variation in gluten proteins quality, in terms of their capacity to assembly in a visco-elastic structure, of four durum wheat genotypes, in relation to the growing season.

Chapter **V** – "Foliar application strategies to improve grain yield and N-related traits in old and modern durum wheat varieties grown under organic management in Mediterranean area " - focused on the evaluation of the effect of different organic fertilization strategies based on foliar application of N, S, and Se on yield, grain protein, and N-related traits in 4 different durum wheat varieties grown under the organic system in the Mediterranean area. Indeed, in the scientific literature, there is a lack of key information on the potential synergistic effects of these fertilizers in organic systems. Moreover, the study of the effect of Se in the durum wheat organic system, never done before, gave essential information for its use and integration in the European Organic Production Regulation.

Finally, the aim of chapter **VI** – "Selenium agronomic biofortification of organic fertilized wheat: Se grain content and selenium speciation" - investigated whether the application of selenate and organic N and S sources *via* foliar, individually and in combination, alters the content of total Se in grains of old and modern Italian durum wheat varieties under Mediterranean conditions, and a possible alteration of produced Se species in the grains.

III. Strobilurin effects on Nitrogen Use Efficiency for the Yield and Protein in Durum Wheat Grown Under Mediterranean Condition

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Strobilurin Effects on Nitrogen Use Efficiency for the Yield and Protein in Durum Wheat Grown Under Rainfed Mediterranean Conditions

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Box 3. STROBILURIN

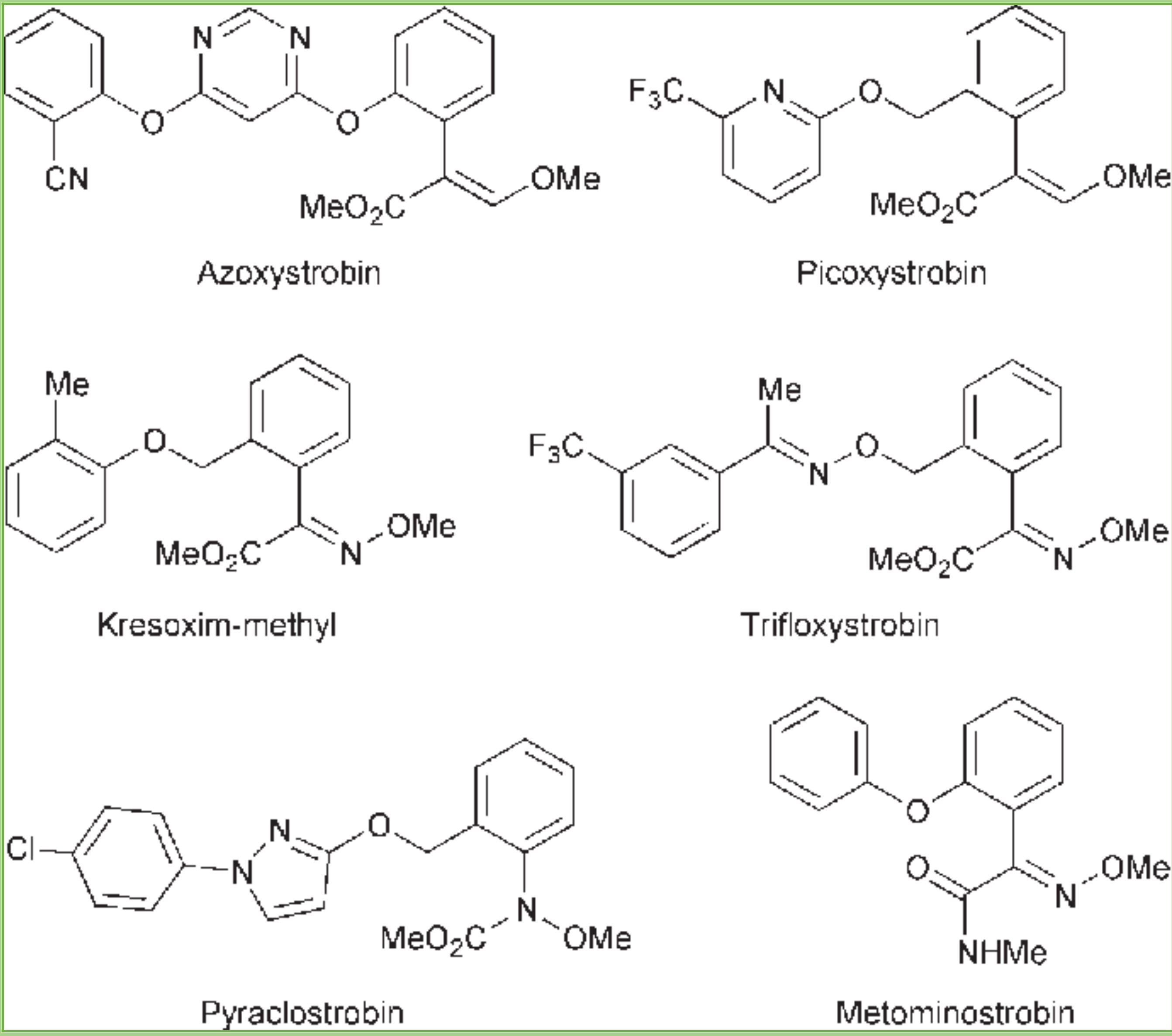


Figure B4. The strobilurin fungicides (from Bartett et al., 2001)

Today the strobilurins are one of the most utilized fungicides; in particular, azoxystrobin and the pyraclostrobin are the most applied forms (Giuliani et al., 2018; Cantore et al., 2016) (Figure B4). Strobilurins come from the discovery of *Stroblilurus tenacellus*, the mushroom fungus that causes wood-rotting. The discovery of strobilurins led scientists to isolate and produce synthetic strobilurins by chemically altering the compound to tolerate sunlight. The strobilurins have a unique mode of action, which targets the mitochondrial respiration of fungi through the interruption of electron transfer, and, therefore, the ATP production (Bartlett et al., 2002). Specifically, they bind at the Q_0 site of the mitochondrial cytochrome bc_1 complex (Hnátová et al., 2003).

Besides the traditional utilization in plant protection, foliar applications of strobilurins

demonstrated to positively affect plant physiological processes (Swoboda & Pedersen, 2009; Giuliani et al., 2018). In cereals, the first studies on this class of fungicide have concentrated mainly on phytohormone-mediated effects on the plant senescence process's physiology.
Therefore, a prolonged green leaf area duration is suggested to be the main factor for yield increases obtained with strobilurin fungicides because the more extended period of photosynthetic activity would increase the quantity of the assimilate available for grain filling (Bertelsen et al., 2001).
Another effect of strobilurins on plant physiology concerns the increase of the activity of NADH-nitrate reductase, which catalyses the first stage of plant nitrate assimilation (Ishikawa et al., 2012), with a positive effect on N grain filling. As a consequence, the soil N uptake increases after strobilurins application, as well as N harvest index (NHI) and the net N remobilization from vegetative tissues during ripening (Ruske et al., 2003). Strobilurin fungicides demonstrated to improve the NUE (Gooding et al. 2005) and to enhance the recovery of late-season N fertilizers in grain (Gooding et al., 2007).

ABSTRACT

In wheat the increase of the nitrogen use efficiency (NUE) and optimization of the nitrogen doses to be used, are both very important aspects for improving sustainable and productive agriculture. The aim of this study was to investigate, under rainfed Mediterranean conditions, the influence of strobilurin treatment and N fertilization on durum wheat N use efficiency for yield (NUEy) and protein (NUEp) and on the contribution of their components nitrogen uptake efficiency (UPE) and nitrogen utilization efficiency (NUtE). Two durum wheat cultivars (Saragolla and Sfinge) were grown for two years in field condition under five nitrogen treatments (60 kg ha^{-1} N60; 90 and 120 kg ha^{-1} given in two and three times; N90, N90T3, N120 and N120T3) comparing a control without strobilurin treatment (ST0) and one application of strobilurin (STaz). In Sfinge, STaz caused a decrease of UPE and NUEp and an increase of NUtE and NUEy. In Saragolla, an opposite behaviour was observed. Moreover, strobilurin positively affected the contribution of UPE and negatively that of NUtE to NUEy only in Saragolla. Also, strobilurin determined higher NUEy and NUEp values under most of the N treatments adopted in the drier year. With this study we supported the hypothesis that in Mediterranean conditions, the possibility of reducing N rate application from 120 to 90 kg ha^{-1} with a strobilurin-based treatment, even in the absence of fungal diseases, could represent a useful agronomic strategy for durum wheat grown under drought condition as those predicted under the ongoing climate change.

KEYWORDS

wheat, nitrogen fertilization, N uptake, N utilization, NUE, azoxystrobin

INTRODUCTION

Durum wheat (*Triticum turgidum* var. *durum* Desf.) is widely cultivated in the Mediterranean basin, for making pasta, couscous, semolina, and other products. Italy is one of the major producers in the world with almost 4.5 million tons [1]. Local wheat farmers operate in a harsh semi-arid environment, characterized by large inter-annual variability of precipitations [2]. These climatic conditions determine frequent drought and heat stress on rainfed durum wheat systems, with detrimental effects on spike and kernel formation leading to large inter-annual yield fluctuations [3].

Under these climatic conditions, the main challenge for durum wheat farmers is to achieve the best trade-off between grain yield and protein content by optimizing the agronomic practices, especially regarding the choice of the variety and the rationalization of nitrogen (N) fertilization.

Nitrogen is one of the most important yield-limiting nutrients in crop production in all agro-ecological regions of the world, but it is also the most expensive. However, since only 30-40% of the nitrogen applied is generally absorbed and used by cereal plants [4], it also represents one of the major causes of pollution attributed to agriculture. In fact, the N applied and not utilized by the plants (about 60%) is lost by leaching, denitrification, and volatilization [5]. Finally, the synthesis of N fertilizers is a very expensive industrial process and contributes to global warming through nitrous oxide emissions [6].

Nitrogen use efficiency (NUE) is the fraction of applied nitrogen that is absorbed and used by the plant, therefore increasing NUE by optimizing the doses and the N application time represents a very important strategy for improving sustainable and productive agriculture [7] On the contrary, limiting or excluding the application of N fertilizer in the cereal farming systems causes a reduction in the residual soil fertility with yield losses, insufficient inadequate quality standards and poor environmental sustainability [7].

Protein concentration is a critical factor determining grain quality and marketability in durum wheat since values equal or higher than 12% are requested from pasta making industry; thus, together with the evaluation of the NUE for grain yield (NUEy), the evaluation of the NUE for grain proteins (NUEp) appears to be equally important, for its role in the high-quality pasta-making. Both NUEy and NUEp may be subdivided into two components: nitrogen uptake efficiency and nitrogen utilization efficiency [8-10]. In the literature, the NUE has been widely studied in bread wheat [11] while little information is available in durum wheat [12, 13], especially about NUEp [9,10].

Recently, the use of substances as strobilurines, showing biostimulating actions on some metabolic and physiological processes, is providing a good strategy to improve the yield and grain quality. In fact, some strobilurines (e.g., azoxystrobin) are marketed as broad-spectrum fungicides with non-toxic effects for humans and the environment and with positive complementary effects on the yield and quality of different crops [14].

Strobilurins are natural antifungal antibiotics which were firstly isolated in 1977 from the mycelium of *Strobilurus tenacellus*, a saprobic Basidiomycete fungus causing wood-rotting on forest trees [15]. Strobilurin fungicides affect the electron transfer in the Krebs cycle of plant pathogenic fungi, and therefore halt the ATP production in mitochondrial respiration [16]. Therefore, it decreases ATPase activity, delaying proton (H+) pumping to the apoplast, causing the decrease in cytosol pH [17]. Besides the traditional utilization in plant protection, foliar application of strobilurins demonstrated to positively affect plant physiological processes such as the photosynthetic activity, the ethylene biosynthesis, and the stomatal opening [16, 18]. Another effect of strobilurins on plant physiology concerns the increase of the activity of NADH-nitrate reductase, which catalyses the first stage of plant nitrate assimilation [17]. Ruske et al. [19] in a study on bread wheat reported that the N uptake increased after strobilurin application, as well as N harvest index (NHI) and the net N remobilization from vegetative tissues to the grain. Moreover, Goodig et al. [20, 21] reported that strobilurin fungicides improved NUE of different wheat varieties compared to untreated plots enhancing the recovery of late-season N fertilizers, with also a significant increase of NHI. Finally, of special interest is the increase in biomass and yield achieved by application of strobilurin, even in plants not infected by fungi [22].

In order to fill the knowledge gap on NUE between bread and durum wheat the aim of this study was to investigate the effects of strobilurin treatment and N fertilization on NUEy, NUEp and their components, on two durum wheat cultivars. We aim at providing ready-to-use indications on the possible benefits connected to strobilurin application on durum wheat, considering the standard N management strategies used in semi-arid area as Southern Italy.

MATERIALS AND METHODS

Field trial

Field experiments were conducted in Foggia, Southern Italy, (41°46′ N, 16°54′ E). The study was carried out over 2 years, 2010-2011 and 2011-2012 (namely 2011 and 2012, respectively). Two cultivars, Saragolla (Syngenta Seeds AG, Basel, Switzerland) and Sfinge (CREA-CI, Foggia, Italy), both maturing early genotypes, were grown on a clay loam soil (United States Department of Agriculture classification) (Table 1).

The two cultivars were chosen based on their respective qualitative and quantitative traits. Saragolla is high-yielding variety with a medium grain protein content, while Sfinge is a variety that combines a high protein content with a medium yield potential. The experiment was sown on 18th and 17th November 2010 and 2011, respectively with a seeding rate of 350 germinable seeds per square meter; the preceding crop was durum wheat. Five nitrogen treatments were evaluated: 60, 90 and 120 kg ha^{-1} (N60, N90 and N120) given in two times according to the N fertilization strategy used in Southern Italy [23], and 90 and 120 kg ha^{-1} (N90T3 and N120T3), splitted in three times from pre-sowing to flag leaf appearance as reported in Table 2.

Table 1. Main soil physical and chemical properties relative to the two years under study.

Soil proprieties	Unit of measurement	2011	2012
Sand	%	35.2	31.7
Silt	%	31.1	36.2
Clay	%	33.7	32.1
Total N (Kjeldhal method)	‰	1.16	1.15
Available P (Olsen method)	mg kg^{-1}	80	81
Exchangeable K (Ammonium acetate method)	mg kg^{-1}	461	450
Organic matter (Walkley-Black method)	%	2.20	2.50

Table 2. Nitrogen fertilizer application management

Nitrogen application	Pre-Sowing	Tillering	Flag leaf appearance
	Biammonium phosphate (18% N)	Urea (46% N)	Ammonium nitrate (27% N)
		(kg ha^{-1})	
N60	36	24	-
N90	36	54	-
N90T3	36	27	27
N120	36	84	-
N120T3	36	54	30

In addition, the strobilurin fungicide effect was evaluated by comparing: i) ST0, control without strobilurin and ii) STaz, with one application of azoxystrobin, at a rate of 1 L ha^{-1} at flag leaf appearance stage (BBCH stage 41) [24], applied using a hand-held knapsack sprayer.

The experiment was arranged as a split-split plot design with three replications; the cultivar was the main plot, N fertilization was the plot and strobilurin application was the sub-plot (10.2 square meter).

All agricultural practices applied to durum wheat crop during the two years, were performed according to the agronomic techniques commonly adopted by local farmers. Weeds were controlled by means of specific herbicides: Tralcossidim (1.7 L ha^{-1}) + Clopiralid+MCPA+Fluroxypyr (2.0-2.5 L ha^{-1}).

Durum wheat grain was machine-harvested at full maturity (BBCH 91-92) [24], on 14th and 5th June in 2011 and 2012, respectively. During the experimental period, the daily climatic parameters of rainfall and temperature were recorded by a weather station near the experimental area.

SPAD measurements

The chlorophyll meter SPAD-502 (SPAD; Konica Minolta Holdings Inc. - Japan), was utilized to determine the relative amount of leaf chlorophyll at different growth stages. Measurements were recorded as mean value of 10 randomly selected main tiller per plot, taken approximately one third the length from the base of the youngest fully expanded leaf [25,26]. The measurements were done at about the same time (middle day).

For each cultivar, six SPAD measurements were collected during the two crop cycles: stem elongation phase (SPAD1; BBCH stage 31-39), flag leaf (SPAD2; BBCH stage 41); heading date (SPAD3; BBCH stage 53-59), flowering (SPAD 4; BBCH stage 63-67), development of grain to early-late milk (SPAD 5; BBCH stage 72-78) and ripening - soft dough (SPAD 6; BBCH stage 80-86) [24]. According to Noulas et al. [25], the SPAD values were used as a direct measure of leaf greenness because the plant material tested was restricted to a single crop species.

Yield, Protein content, Nitrogen plant content and N use efficiency indices

At harvest time the grain yield (GY) was assessed for each plot. Grain protein content (GPC) was also determined by near-infrared reflectance spectroscopy, using an Infratec 1229 grain analyzer (Foss Tecator, Hillerød, Denmark).

In each experimental year, at physiological maturity (BBCH stage 87), plant samples were taken from 0.5 linear meter of two adjacent rows, cutting-off the shoots at the crown level. Dry matter was determined by drying the sampled plants in an oven at 65 °C to constant weight. Subsequently, all samples were ground using a Cyclotec Sample Mill 1093 (Foss Tecator, Hillerød, Denmark). Nitrogen concentration of straw and grain was determined in triplicate by the Kjeldhal method using a Kjeltec Analyzer Unit 2300 (Foss Tecator, Hillerød, Denmark).

The straw and grain N content were calculated multiplying dry weight by N concentration of each fraction; finally, the plant N content was the sum of straw and grain N content. According to Lopez-Bellido et al. [27] and Giuliani et al. [10] the following N-efficiency parameters were calculated:

- N use efficiency for grain yield (NUEy; kg kg^{-1}) as the ratio of grain yield to N fertilizer applied;
- N use efficiency for protein (NUEp; kg kg^{-1}) as the ratio of grain N content to N fertilizer applied;
- N uptake efficiency (UPE; kg kg^{-1}) as the ratio of plant N content to N fertilizer applied;
- N utilization efficiency (NUtE; kg kg^{-1}) as the ratio of grain yield to plant N content;
- N harvest index (NHI; kg kg^{-1}) as the ratio of grain N content to plant N content.

NUEy was partitioned into the component UPE and NUtE, while NUEp into the components UPE and NHI [8, 10, 28].

Statistical analysis

The dataset was tested according to the basic assumptions of analysis of variance (ANOVA). The normal distribution of the experimental error and the common variance of the experimental error were verified through Shapiro-Wilk and Levene's tests, respectively. According to Levene's test significance ($P \leq 0.05$), the ANOVA procedure was performed separately for the two experimental years. When required, Box-Cox transformations [29] were applied prior to analysis. The ANOVA model was performed according to a split-split-plot design with three replicates considering cultivar, nitrogen fertilization, strobilurin treatment and their interactions (cultivar x nitrogen fertigation, cultivar x strobilurin treatment, nitrogen fertilization x strobilurin treatment and cultivar x nitrogen fertigation x strobilurin treatment). All the factors were considered as fixed, while the replicates as random. The statistical significance of the difference among the means was determined using Tukey's honest significance difference post hoc test at the 5% probability level.

To evaluate the strobilurin effect on the relation among NUEy and its component (UPE and NUtE) and among NUEp and its component (UPE and NHI), the analysis of covariance (ANCOVA) was performed on ln-transformed data. According to this procedure, the NUE components (ln UPE, ln NUtE and ln NHI), considered as the dependent variables (Y), were processed through a linear model, where ln NUEy and ln NUEp were used as the regressor (X, continuous numerical variable) and the strobilurin treatment was the covariate (categorical variable). The sum of the angular coefficients of the regressed components was equal to unity thus explaining the complementary contribution of each component to NUEy and NUEp, respectively [10,30].

ANOVA analysis was performed using "the lme4 package" of the 'R' statistical software, version 3.6.3 [31]; also, graphs were generated with 'ggplot2' [32]. Finally, ANCOVA analysis was performed using the JMP software package, version 14.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Weather conditions

The first year was characterized by higher rainfall compared to the second one, both during the vegetative and the grain filling period (Table 3 and Figure S1). During the first year, intense rainfall events were recorded in the third 10-days of January, in the second 10-days of February and in the first 10-days of March. As for the grain filling period, in the first year higher and well distributed rainfall occurred. On the contrary, in the second year only 21 mm of rainfall felt, the most of them concentrated in four days during the second 10-days of May. During the two growing seasons the trends of temperature (T) were quite mild compared to long term averages and similar in the two crop seasons. Due to the dry weather condition of the two years the incidence of fungal diseases was completely negligible or zero. However due to the low rainfall, the second growing season was drier than the first one.

Table 3. Climatic data related to the two experimental years.

		2011	2012
Crop cycle duration from sowing	days	209	201
Crop cycle rainfall	mm	362.2	242.1
From seeding to heading rainfall	mm	302.6	220.9
Grain filling rainfall	mm	59.6	21.2
Crop cycle Mean T	°C	11.4	10.7
Grain filling Mean T	°C	18.1	17.4
Grain filling Mean T max	°C	24.2	25.7

SPAD measurements

In the two years the time-course of SPAD readings have been done six times from stem elongation (control, before strobilurin and the third nitrogen application) to soft-ripening (Figure 1). In 2011, the SPAD values began to decrease from flowering stage (SPAD4) and gradually to the end (soft ripening; SPAD6). Instead, in 2012 the values began to decrease later, from development of fruit to early-late milk (SPAD5) and then they dropped drastically until SPAD6.

Clear differences were observed among the cultivars (Figure 1a); Sfinge, in general, showed higher SPAD values than Saragolla. In 2011, no significant difference was showed between the cultivars at stem elongation (SPAD1) but the differences increased after the strobilurin application until maturity (SPAD6). In 2012 the differences between Sfinge and Saragolla were less evident.

Respect to N fertilization, in 2011, as expected, from the first to the last readings, higher SPAD values were observed under higher N- fertilization level going from 45.4 to 11.8 for N60, from 49.4 to 15.9 for N90, from 48.3 to17.9 for N90T3, from 52.1 to 17.2 for N120, and from 49.4 to 19.0 for N120T3. At the last reading SPAD values decreased regularly, but N90T3 and N120T3 decreased less, showing higher SPAD values at the end of the cycle. In 2012, the differences among the N fertilization levels in the time-course of SPAD readings were less evident with respect to 2011. The SPAD values decreased regularly, excepted for third N split application plot (N90T3 and N120T3) where SPAD values slight increased during heading (SPAD3) and anthesis (SPAD4) stages. Also, in 2012, at last SPAD reading 90T3 and 120T3 showed higher values (Figure 1b). In both years, a positive effect of strobilurin application was evident. In 2011, significant higher values under STaz occurred starting from flowering stage (SPAD4), while in 2012 the positive effect was evident soon after the strobilurin application (flag leaf, SPAD2) (Figure 1c).

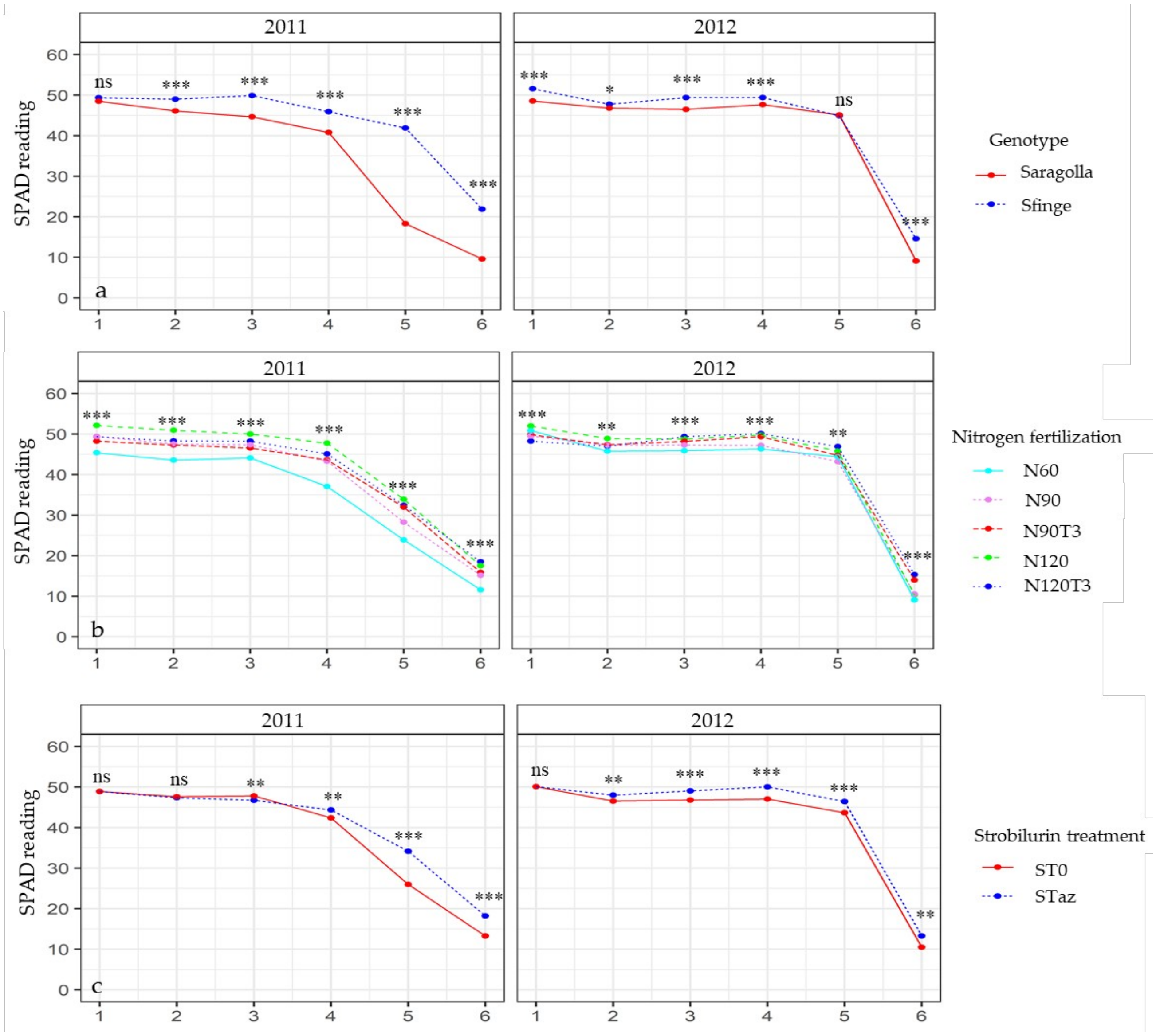


Figure 1. Effect of cultivar (a), nitrogen fertilization (b) and strobilurin treatment (c) on SPAD readings during the two growing seasons (2011 and 2012) from stem elongation to soft-ripening. 1=SPAD1 (BBCH 31-39). 2=SPAD2 (BBCH 41); 3=SPAD3 (BBCH 53-59); 4=SPAD4 (BBCH 63-67);
5=SPAD5 (BBCH 72-78); 6=SPAD6 (BBCH 80-86)[24].
N60, N90 and N120 correspond to 60, 90 and 120 kg ha^{-1} of nitrogen applied in two times.

N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.
ST0, no strobilurin treatment; STaz, strobilurin treatment.
, significant at P≤0.01, *, significant at P≤0.001; ns, not significant.

N content in plant, straw, and grain

The results of the ANOVA, performed on the two years, on plant, straw and grain N content are reported in Table S1 (Supplementary material). Sfinge showed higher values of plant, straw, and grain N content than Saragolla, in both years. The differences between the two genotypes were more marked in 2011 than in 2012 (Table 4). In the first year, N fertilization caused an increase in plant and straw N content with increasing N level. The same trend was observed for grain N content with higher values under N90T3 than under N90 in both cultivars and only for Saragolla also under N120T3 than under N120 (Table S2). On the contrary, in the second year the highest values of plant, straw and grain N content were observed under N60 and the three times fertilization (N90T3 and N120T3) always had a negative effect in both cultivars (Table S2). Finally, as for strobilurin treatment, in the first year it caused a decrease of plant and straw N content and an increase of grain N content, while in the second year both grain and plant N content increased under STaz.

Table 4. Effect of cultivar, nitrogen fertilization and strobilurin treatment in 2011 and 2012 for plant, straw and grain N content (kg kg $^{-1}$).

Experimental Factor	**Crop season**					
	2011			2012		
	Plant	Straw	Grain	Plant	Straw	Grain
Cultivar						
Saragolla	133.3±7.9^{b}	68.1±3.9^{b}	65.2±4.4^{b}	121.4±3.8^{b}	55.01±1.6^{b}	66.35±2.3^{b}
Sfinge	273.9±11.4^{a}	195.9±9.7^{a}	78.0±2.0^{a}	141.2±2.6^{a}	72.30±1.3^{a}	68.89±1.5^{a}
Nitrogen fertilization						
N60	146.2±16.5^{d}	92.4±12.6^{d}	53.8±4.0^{e}	147.4±6.5^{a}	71.1±4.5^{a}	76.31±2.9^{a}
N90	201.0±30.1^{c}	134.9±25.5^{c}	66.1±5.1^{d}	126.1±7.3^{d}	62.2±3.5^{c}	63.92±4.1^{d}
N90T3	213.8±30.4^{b}	142.0±25.6^{b}	71.8±5.7^{c}	119.9±3.2^{e}	58.8±2.7^{d}	61.12±1.7^{e}
N120	228.2±18.6^{a}	146.3±18.7^{a}	81.9±1.6^{b}	134.1±2.7^{b}	63.5±2.1^{b}	70.55±1.0^{b}
N120T3	228.8±25.6^{a}	144.5±24^{ab}	84.3±6.5^{a}	129.0±5.5^{c}	62.7±3.4^{bc}	66.22±2.7^{c}
Strobilurin treatment						
ST0	211.0±21.1^{a}	143±17.8^{a}	68.0±3.9^{b}	129.5±4.3^{b}	63.39±2.4 ^{a}	66.12±2.1^{b}
STaz	196.2±9.3^{b}	121±8.1^{b}	75.2±3.24^{a}	133.0±3.0^{a}	63.92±2.0^{a}	69.13±1.8^{a}

N60, N90 and N120 correspond to 60,90 and 120 kg ha^{-1} of nitrogen applied in two times.
N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.
ST0, no strobilurin treatment; STaz, strobilurin treatment.

For each experimental factor, values in column followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test.
Data are reported as means±standard errors.

However, the effect of the strobilurin treatment was different in relation to the two cultivars under study and to the different N fertilization applied. In Figure 2 the interactions cultivar x strobilurin treatment for the two years are reported. In 2011, for Saragolla the strobilurin treatment caused a significant increase of N content both in straw and in grain; as consequence also plant N content was higher under STaz. On the contrary, for Sfinge cultivar STaz had a negative effect determining a decrease in N content in straw content and grain (Figure 2-A). In 2012, the two cultivars confirmed the same behavior observed in 2011, with the strobilurin treatment showing a positive effect on Saragolla and a negative effect on Sfinge (Figure 2-B). The interactions N fertilization x strobilurin treatment, evaluated in the two years, are reported in Table 5. In 2011, the negative effect of the strobilurin treatment on the N content of plant and straw was significant under N90 and N120. On the contrary, the strobilurin caused a significant increase of the grain N content under N60 whereas under N90 and N120 the effect was significant only when splitted in three times (N90T3 and N120T3). In 2012, the STaz caused a significant increase in plant, straw, and grain N content under N60, N90 and N120T3.

Figure 2. Effect of the interactions cultivar x strobilurin treatment in 2011 (A) and 2012 (B) for plant, straw and grain N content. ST0, no strobilurin treatment; STaz, strobilurin treatment. Values with different letters are significantly different at $P \leq 0.05$ according to Tukey's test.Vertical bars indicate standard errors (n=15).

Table 5. Effect of the interactions nitrogen fertilization x strobilurin treatment in 2011 and 2012 for plant, straw and grain N content (kg kg^{-1})

Crop season	Strobilurin treatment									
	ST0					STaz				
	N60	N90	N90T3	N120	N120T3	N60	N90	N90T3	N120	N120T3
2011										
Plant	135.2±28.2^{f}	221.9±57.4^{b}	215.9±55.2^{bc}	243.0±37.4^{a}	239.7±53.1^{a}	157.3±18.6^{e}	180.3±23.0^{d}	211.7±32.0^{c}	213.4±5.87^{c}	217.9±4.4^{bc}
Straw	85.9±21.0^{f}	156.2±47.1^{ab}	152.6±46.1^{b}	159.5±37.2^{a}	161.5±46.6^{a}	99.0±15.6^{e}	113.7±21.6^{d}	131.4±26.6^{c}	133.1±9.1^{c}	127.5±15.3^{c}
Grain	49.3±7.2^{h}	65.7±10.3^{e}	63.3±9.2^{f}	83.5±0.3^{b}	78.2±6.5^{d}	58.3±3.1^{g}	66.6±1.5^{e}	80.3±5.3^{c}	80.3±3.3^{c}	90.4±11.2^{a}
2012										
Plant	136.4±11.7^{c}	118.2±14.4^{h}	129.0±2.7^{e}	142.5±0.8^{b}	121.5±10.5^{g}	158.3±1.4^{a}	134.1±1.6^{d}	110.8±2.0^{i}	125.6±1.8^{f}	136.4±0.9^{c}
Straw	65.2±7.5^{c}	58.7±6.8^{f}	63.2±2.8^{c}	69.1±0.4^{b}	60.8±6.4^{e}	76.9±4.3^{a}	65.7±1.4^{c}	54.3±4.0^{g}	58.0±2.5^{f}	64.7±2.7^{c}
Grain	71.24±4.1^{c}	59.48±7.6^{g}	65.75±0.3^{e}	73.42±0.4^{b}	60.7±4.1^{f}	81.4±3.0^{a}	68.4±3.0^{d}	56.5±2.0^{h}	67.7±0.7^{d}	71.7±1.9^{c}

N60, N90 and N120 correspond to 60,90 and 120 kg ha^{-1} of nitrogen applied in two times.

N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.

ST0, no strobilurin treatment; STaz, strobilurin treatment.

Values in each row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Data are reported as means±standard errors (n=6).

N use efficiency indices, yield, and grain protein content

The results of the ANOVA, performed on the two years, on NUEy, NUEp and their components and on grain yield (GY) and grain protein content (GPC) are reported in Table S3 (Supplementary material). Saragolla showed higher, NUtE, NHI, NUEy and GY values than Sfinge in both years. On the contrary UPE, NUEp and GPC were always higher for Sfinge than for Saragolla (Table 6). In general, GY and GPC values were higher in 2011 than in 2012. Moreover, in 2011 both GY and GPC increased with N level increasing, especially in Saragolla for which a positive effect of the three-timing fertilization on GPC was observed (Table S4). In 2012, N fertilization had not a significant effect on GPC; on the contrary, the GY increased going from N60 to N120, but the N90T3 and N120T3always showed lower values than N90 and N120, respectively. In both years, in general the UPE, NUEy and NUEp values decreased with N levels increasing (Table 6); however, in 2011 Sfinge showed the highest UPE value under N90T3, and Saragolla the highest NUEP values under N120T3 (Table S4). As for NUtE, the highest values were observed always under N60 for Saragolla and under N60 and N120 in the first and second year, respectively, for Sfinge (Table S4). As regards NHI, it showed highest value under N120T3 in 2011 for Sragolla and under N120 in 2012 for Sfinge. Finally, the strobilurin treatment (Table 6) always caused an increase of NHI, NUEy, NUEp, and GY. On the contrary the NUtE decreased under STaz in both years, while UPE only in 2011. No effect of STaz on GPC was detected. However, the effect of the strobilurin treatment on NUEy, NUEp and their components was different for the two cultivars under study (Table 7).

In Saragolla, strobilurin treatment caused an increase of UPE, NUEy, NUEp and GY and a decrease of NUtE, in both the years. Moreover, only in 2012 an increase of NHI was observed. On the contrary, in Sfinge strobilurin treatment caused a decrease of UPE and NUEp and an increment of NUEy, NUtE and GY. Finally, NHI increased under STaz in 2011 and decreased in 2012.

Table 6. Effect of cultivar, nitrogen and strobilurin treatment in 2011 and 2012 on uptake efficiency (UPE), nitrogen utilization efficiency (NUtE), nitrogen harvest index (NHI), nitrogen use efficiency for yield (NUEy), nitrogen use efficiency for protein (NUEp), grain yield (GY) and grain protein content (GPC).

Experimental factor	2011							2012						
	UPE	NUtE	NHI	NUEy (kg kg^{-1})	NUEp	GY	GPC	UPE	NUtE	NHI	NUEy (kg kg^{-1})	NUEp	GY	GPC
Cultivar														
Saragolla	1.39±0.1^{b}	43.8±2.0^{a}	0.48±0.071^{a}	58.3±1.5^{a}	0.67±0.03^{b}	5.43±0.12^{a}	10.1±0.2^{b}	1.36±0.1^{b}	35.5±1.0^{a}	0.55±0.010^{a}	46.5±2.3^{a}	0.74±0.05^{b}	4.20±0.0^{a}	8.7±0.1^{b}
Sfinge	2.95±0.1^{a}	14.4±0.6^{b}	0.29±0.061^{b}	41.5±2.1^{b}	0.85±0.03^{a}	3.74±0.14^{b}	16.0±0.2^{a}	1.59±0.1^{a}	19.7±0.6^{b}	0.49±0.010^{b}	30.4±1.5^{b}	0.78±0.05^{a}	2.75±0.0^{b}	10.4±0.2^{a}
Nitrogen fertilization														
N60	2.44±0.3^{a}	33.0±4.8^{a}	0.39±0.018^{b}	66.0±1.6^{a}	0.90±0.07^{a}	3.96±0.1^{d}	11.7±0.9^{d}	2.46±0.11^{a}	24.2±2.4^{d}	0.52±0.01^{a}	57.0±3.6^{a}	1.27±0.05^{a}	3.42±0.2^{bc}	9.3±0.4^{a}
N90	2.23±0.3^{c}	32.4±6.0^{a}	0.37±0.031^{c}	51.9±3.0^{b}	0.74±0.05^{c}	4.67±0.3b^{c}	12.5±1.0^{c}	1.40±0.08^{b}	29.9±3.5^{a}	0.50±0.008^{c}	39.0±2.6^{b}	0.71±0.05^{b}	3.51±0.2^{b}	9.3±0.4^{a}
N90T3	2.38±0.3^{b}	30.1±5.6^{b}	0.38±0.029^{bc}	51.2±2.6^{b}	0.80±0.06^{b}	4.61±0.2^{c}	13.7±1.0^{ab}	1.33±0.03^{c}	28.4±2.6^{b}	0.51±0.014^{b}	37.0±2.7^{c}	0.68±0.02^{c}	3.33±0.2^{c}	9.5±0.3^{a}
N120	1.90±0.1^{d}	23.6±3.3^{d}	0.39±0.031^{b}	39.6±3.2^{c}	0.68±0.01^{e}	4.76±0.4^{b}	13.1±1.0^{bc}	1.12±0.02^{d}	28.2±1.9b^{c}	0.53±0.007^{a}	31.1±1.7^{d}	0.59±0.01^{d}	3.74±0.2^{a}	9.7±0.3^{a}
N120T3	1.91±0.2^{d}	26.4±4.3^{c}	0.41±0.04^{a}	41.0±2.7^{c}	0.70±0.05^{d}	4.92±0.3^{a}	14.1±1.0^{a}	1.07±0.05^{e}	27.3±2.8^{c}	0.51±0.001^{b}	28.1±2.0^{e}	0.55±0.02^{e}	3.38±0.2^{c}	10.1±0.5^{a}
Strobilurin treatment														
ST0	2.23±0.2^{a}	31.8±3.9^{a}	0.38±0.021^{b}	48.6±2.4^{b}	0.72±0.04^{b}	4.47±0.2^{b}	12.9±0.6^{a}	1.44±0.09^{b}	27.8±1.3^{a}	0.51±0.004^{b}	36.7±2.4^{b}	0.74±0.05^{b}	3.32±0.1^{b}	9.4±0.2^{a}
STaz	2.11±0.1^{b}	26.4±1.9^{b}	0.40±0.017^{a}	51.3±2.4^{a}	0.81±0.03^{a}	4.70±0.2^{a}	13.1±0.6^{a}	1.51±0.11^{a}	27.4±2.0^{a}	0.52±0.008^{a}	40.2±2.5^{a}	0.78±0.06^{a}	3.63±0.1^{a}	9.8±0.3^{a}

N60, N90 and N120 correspond to 60, 90 and 120 kg ha^{-1} of nitrogen applied in two times.
N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.
ST0, no strobilurin treatment; STaz, strobilurin treatment.

For each experimental factor, values in column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors.

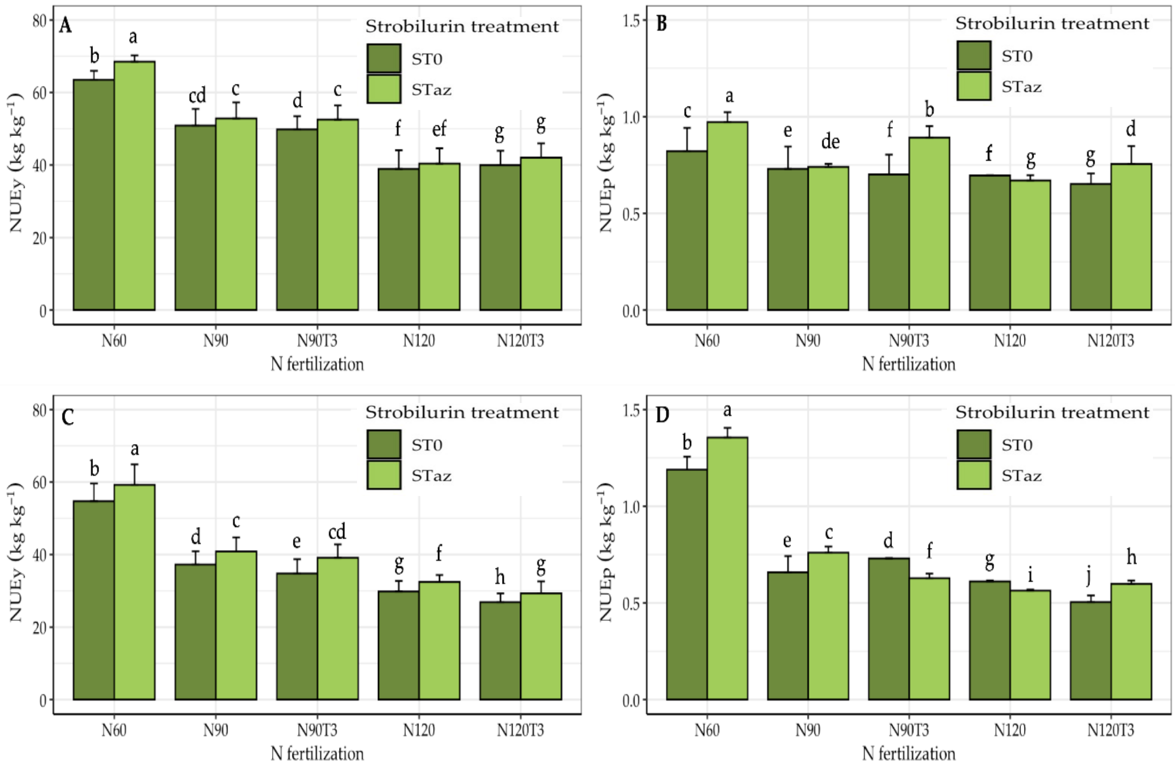
Table 7. Effect of the interactions cultivar x strobilurin treatment in 2011 and 2012 on uptake efficiency (UPE), nitrogen utilization efficiency (NUtE), nitrogen harvest index (NHI), nitrogen use efficiency for yield (NUEy), nitrogen use efficiency for protein (NUEp), grain yield (GY) and grain protein content (GPC).

Crop season	**Saragolla**		**Sfinge**	
	ST0	STaz	ST0	STaz
	(kg kg^{-1})		(kg kg^{-1})	
2011				
UPE	1.12±0.03^{d}	1.67±0.04^{c}	3.33±0.12^{a}	2.56±0.16^{b}
NUtE	51.7±2.1^{a}	35.8±1.5^{b}	11.9±0.8^{d}	16.9±0.5^{c}
NHI	0.49±0.08^{a}	0.48±0.01^{a}	0.27±0.08^{c}	0.31±0.04^{b}
NUEy	57.4±2.0^{b}	59.3±2.2^{a}	39.8±2.9^{d}	43.2±3.2^{c}
NUEp	0.55±0.02^{c}	0.80±0.02^{b}	0.90±0.04^{a}	0.81±0.06^{b}
GY	5.3±0.2^{b}	5.5±0.1^{a}	3.6±0.15^{d}	3.9±0.13^{c}
GPC	10.0±0.3^{b}	10.1±0.2^{b}	15.9±0.2^{a}	16.1±0.3^{a}
2012				
UPE	1.23±0.01^{d}	1.49±0.2^{c}	1.66±0.1^{a}	1.53±0.2^{b}
NUtE	37.1±1.6^{a}	33.8±1.1^{b}	17.7±0.3^{d}	21.7±0.9^{c}
NHI	0.53±0.06^{b}	0.56±0.03^{a}	0.49±0.03^{c}	0.48±0.05^{d}
NUEy	44.6±3.1^{b}	48.4±3.4^{a}	28.9±2.2^{d}	31.9±2.2^{c}
NUEp	0.65±0.1^{d}	0.84±0.1^{a}	0.82±0.1^{b}	0.72±0.1^{c}
GY	4.0±0.15^{b}	4.4±0.13^{a}	2.6±0.1^{d}	2.9±0.1^{c}
GPG	8.7±0.2^{b}	8.7±0.2^{b}	10.0±0.2^{a}	10.9±0.4^{a}

ST0, no strobilurin treatment; STaz, strobilurin treatment.
For each crop season, values in row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors (n=15).

The effects of the strobilurin treatment in relation to nitrogen fertilization are reported in Figures 3 and in Figures S2 and S3 (supplementary material). In 2011, STaz caused a general increase of NUEy (Figure 3-A) under all the N fertilization adopted with significant differences only under N60 and N90T3. Similar trend was observed for NUEp (Figure 3-B) for which the positive effect of STaz under N60 and N90T3 was more evident; moreover, also under N120T3 the STaz value was higher than the ST0. On the contrary, for the UPE (Figure S2A) and NUtE (Figure S2-B) the STaz had always a negative effect except for UPE under N60. Finally, STaz caused a marked increase of NHI (Figure S2-C) under N90 and N90T3, a slight increase under N120T3 and a decrease under N60. For GPC the positive effect of STaz was observed only under N90T3 (Figure S2-D).

In 2012 strobilurin treatment caused a significant increase of NUEy (Figure 3-C) under all the N fertilization adopted, while for NUEp (Figure 3-D) and UPE (Figure S3-A) this was true under N60, N90 and N120T3. As for NUtE (Figure S3-



B) the positive effect of STaz was evident only under N90T3 and N120. Finally, the higher NHI (Figure S3-C) values were observed under N120 and N120T3 when treated with strobilurin, and under N60 no treated.

Figure 3. Effect of the interactions nitrogen fertilization x strobilurin treatment for nitrogen use efficiency for yield (NUEy, A and C in 2011 and 2012, respectively) and nitrogen use efficiency for protein (NUEp, B and D in 2011 and 2012, respectively).
N60, N90 and N120 correspond to 60, 90 and 120 kg ha^{-1} of nitrogen applied in two times; N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.
ST0, no strobilurin treatment; STaz, strobilurin treatment.
Values followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.
Vertical bars indicate standard errors (n=6)

To evaluate the effect of the strobilurin treatment on the contribution of the two components UPE and NUtE to NUEy and UPE and NHI to NUEp, the ANCOVA analysis was performed on the ln-transformed data. The analysis was significant only for NUEy (Table S1) for cultivar Saragolla (Table S2).

For Saragolla (Table 8) there was a high association between NUEy and UPE (R2 = 0.63), while the association between NUEy and NUtE was lower (R2 = 0.43). The angular coefficients represent the percentage contribution of each individual component to the total NUEy. For Saragolla the contribution of UPE to NUEy was 69% and that of NUtE was 31%. Moreover, the STaz caused an increase in the contribution of UPE and a decrease in the contribution of NUtE to NUEy of 26%.

Table 8. Effect of strobilurin treatment on nitrogen uptake efficiency (UPE) and nitrogen utilization efficiency (NUtE) contribution to nitrogen use efficiency for yield (NUEy) in cultivar Saragolla.

Term	**ln UPE versus ln NUEy**			**ln NUtE versus ln NUEy**		
	Estimate	Std Error	Prob	Estimate	Std Error	Prob
Intercept *(contribution to ST)*	-2.47	0.37	***	2.47	0.37	***
STo	-0.12	0.02	***	0.12	0.02	***
STaz	0.12	0.02	***	-0.12	0.02	***
Angular coefficient *(contribution to NUEy)*						
ln NUEy	0.69	0.096	***	0.31	0.096	**
STo x ln NUEy	-0.26	0.096	**	0.26	0.096	**
STaz x ln NUEy	0.26	0.096	**	-0.26	0.096	**
	R^2 = 0.63*** RMSE = 0.17			R^2 = 0.43** RMSE = 0.17		

ST0, no strobilurin treatment; STaz, strobilurin treatment.
, significance at P≤0.01, *, significant at P≤0.001; ns, not significant.
R^2, coefficient of determination, RMSE, root-mean-squared error.

DISCUSSION

The two years differed for the rainfall during the crop cycle and mainly during the grain-filling period being the second year drier than the first one; this caused a lower N uptake in the second year and consequently a lower N content in plant and in grain. This was in agreement with the results reported previously by Giuliani et al. [10]. In general, in the second drier year higher SPAD values were observed in agreement with other studies which reported that SPAD values increased under drought stress and in warm environment [33,34].

Cultivars effects

The higher plant, straw and grain N content values showed by Sfinge in both years, were probably due to the higher capacity of this cultivar to uptake N from the soil, since UPE was always higher for Sfinge than for Saragolla. However, when stressed environment conditions occurred, as in the second year, Sfinge showed a marked UPE decrease associated to a marked decrease of plant, straw, and grain N content, while Saragolla showed a more constant behaviour. UPE is a component of both NUEy and NUEp and represents the plant's ability to extract nitrogen from the soil depending mainly on root structure and functioning [6,7].

The higher UPE values of Sfinge did not influence NUEy but caused higher NUEp and GPC values than Saragolla. On the contrary, in both years, Saragolla showed higher yield and NUEy values than Sfinge due to higher NUtE values. NUtE can be considered as the capability of the plant to use N stored in the canopy, through its remobilization to the sink [7]. In durum wheat, the contribution of N remobilization from vegetative tissue during grain filling approximately account for 53 to 82% of grain N content, with high variability due to differences in climate, soil type effect, cultivars, and crop management [10, 35].Thus, during grain filling, the vegetative tissues function as nutrient sources until the crop maturity, especially under dry condition when a limited N uptake during the grain filling period may force the plant to make greater use of its stored N [36, 37]. Under this point of view senescence of the canopy is essential for the remobilization of N [7] and lower SPAD values, as those observed for Saragolla respect to Sfinge, especially in the first year, could be linked to higher NUtE and grain yield of the cultivars. Indeed, also Tsialtas et al., [38] reported that under semiarid conditions, the cultivar with the lower SPAD values was more productive.

Therefore, the two cultivars were very different as the most productive, Saragolla, was characterized by higher NUEy linked to its capability to remobilize the N from the canopy (NUtE). On the other hand, Sfinge showed higher UPE but lower yield and capacity to remobilize the N from the canopy to the grain during the grain filling (NUtE), since also straw N content and SPAD values at maturity were higher. Together with higher UPE, Sfinge showed also higher GPC and NUEp values. These results are partially in agreement with those reported for bread wheat by Yu et al [39]; indeed, the authors, comparing six bread wheat cultivars concluded that the cultivar characterized by high grain yield showed also the highest UPE and NUEy values, while the cultivar that emphasized protein accumulation showed higher NUtE values than the other ones. On the contrary, in spring barley, comparing cultivars coming from 75 years of breeding, higher yield values were always associated with the increase of NUtE, while not necessarily matched by increased UPE [40].

Nitrogen fertilization effects

Respect to N management, the SPAD values increased with N fertilization level and with the split applications; this behavior may be explained with a higher N content in plant and then the increase in chlorophyll concentration [41].

In the first year the plant, straw and grain N content increased with increasing nitrogen fertilization level; on the contrary when conditions of water scarcity arise, as happened in the second year, the higher doses of N tended to limit the N accumulation both in straw and in grain.

As for NUEy, NUEp and their component UPE, similarly to other studies [10, 27, 42-44], N fertilizer application led to a general decrease in both years.

The N fertilizer given in three times (N90T3 and N120T3) had a positive effect in the first year only on N grain content, NUEp and GPC, while no significant effect was observed for NUEy even if in literature is reported that splitting the amount of N fertilizer increases the efficiency of nitrogen use efficiency [45]. However, giving N in three times always a negative effect in the second drier year was observed, indicating that under drought condition, late N fertilizations were not useful for the plant, worsening the water stress conditions. This was confirmed also by the lower values of GY, NUEy, NUEp and their components observed in the second year under NT3 conditions. Moreover, the lower rainfall registered in the second year canceled the beneficial effect of N given in three times on the GPC since no significant differences were observed. Several studies have shown that in wheat, applications of N later in the season (spring) and near anthesis are more effective than earlier applications in enhancing GPC [46-48] and sometimes also GY [49,50]; thus, also N fertilizer recovery and efficiency were reported to increase with late applications compared to fall application at sowing [51-53].

In our study this was true only for NUEp in the first year; moreover, we found that the effect of the N given in three times on nitrogen use efficiency was strongly linked with the weather conditions. Indeed, the limited moisture, due to dry weather and rainfed conditions, caused limited root exploitation and therefore a limited UPE, N plant content, and NHI [10]. According to Velasco et al. [54], splitting N rate would be a strategy to increase N efficiency and then reduce N losses mainly in no water stress conditions.

Strobilurin effect

Usually, fungicides are used to control and prevent diseases; in this paper strobilurin treatment was considered only to evaluate its physiological effects [55-57], since weather conditions recorded in the two years of study were not favorable to fungal diseases. The SPAD values recorded during the grain filling period confirmed that strobilurin had an effect on the duration of the wheat canopy [58] and therefore on stay green of the plant [59-61] probably due to the reduction of ethylene synthesis and the protective effect of the antioxidative system [62], which postpones the degradation of chlorophyll, and therefore the yellowing of the leaves [63].

The effect of the strobilurin treatment was different for the two cultivars under study, which indicates that there was genetic difference between Saragolla and Sfinge for response to STaz. In Sfinge, surprisingly, strobilurin treatment caused a decrease of the straw and grain N content. Indeed, for this cultivar, the higher N uptake ability discussed above decreased under strobilurin treatment. This result disagreed with the literature in which is reported that strobilurin fungicides can increase soil N uptake [57,59]. Also, Khole et al. [22] reported that strobilurin stimulated nitrate uptake in hydroponically grown wheat plants. In our experimental condition, the increase of NUtE under strobilurin treatment (about 42% in 2011 and 23% in 2012) seems to have compensated for the decrease in UPE (-23% in 2011 and -8% in 2012), as the NUEy increased, especially in the first year, as well as the grain yield. Nitrogen-utilization efficiency depends on both the nitrogen source-to-sink remobilization efficiency and nitrogen assimilation efficiency at the sink [64]. However, the mechanism of source-to-sink nitrogen remobilization in wheat remains unclear [39]. The greater amount of nitrogen adsorbed is transported in the form of nitrate and ammonium and then assimilated in the leaves [65]. It has been demonstrated that NADH-nitrate reductase, which catalyzes the first step in nitrate-assimilation, is the relevant target for the yield effect of strobilurin [66]. Khole et al. [22] reported that when wheat plants (*Triticum aestivum* L.) grown under hydroponic condition were treated by strobilurin, nitrite and ammonia were accumulated in the leaves during the night period, due to the fact that nitrate reductase was not dark inactivated as in the control plants. Moreover, it is reported that strobilurin could cause an additional activation of nitrate-reductase which is probably mediated via acidification of the cytoplasm [17].

However, the decrease in UPE observed in Sfinge under STaz, strongly influenced the values of NUEp which always decreased, despite the increase in the NHI component in the first year. In Saragolla, an opposite behavior of the strobilurin treatment respect to Sfinge was observed. The significant increase of straw and grain N content under strobilurin treatment was due to the positive effect of the treatment on UPE, especially in the first year when the environment conditions for a higher N uptake were favorable (49% in 2011 and 21% in 2012).

The UPE increase led to an increment of NUEy, NUEp and GY, more evident in the first year, despite strobilurin surprisingly had a negative effect on NUtE (-31% in 2011 and -9% in 2012). Therefore, for Saragolla the increase in plant N content and yield due to the strobilurin treatment is to be charged to the increase in UPE. The results presented in the current paper showed that the positive effect reported in literature for strobilurin on uptake and remobilization efficiency, really depend on the cultivars and thus the interaction strobilurin treatment x cultivar seems to be very important.

Furthermore, the results of ANCOVA analysis showed a significant effect of the strobilurin treatment on the contribution of the two components only to NUEy for the cultivar Saragolla. In agreement with several authors [28, 30, 67, 68] a higher contribution of UPE (69%) than NUtE (31%) to NUEy was observed. The present study provides new evidence to suggest that strobilurin significantly and positively affected the contribution of UPE (from 69% to 95%) and negatively that of NUtE (from 31% to 5%) to NUEy only in Saragolla, while no significant effect was detected for Sfinge.

Finally, in our experimental condition, it is important to note that, for both cultivars, no reduction in GPC was observed despite a yield increase was promoted by strobilurin application [19]. This was contrary to the best-known negative relationship between protein concentration and yields of wheat cultivars [10, 69, 70]. The improvement in the duration of grain N accumulation during grain filling following the strobilurin application, as showed by the increase of the SPAD values, probably helped to maintain grain protein concentration, as yield increased [71], confirming that the effect of strobilurin on protein concentration is small [57].

Regarding the effects of the strobilurin treatment in relation to N fertilization, no clear trend was observed for plant and straw N content, while the effect of the treatment on the grain N content was clearer. The strobilurin caused a significant increment of the grain N content under N60 in both years (18% and 14% in the first and second year, respectively) and under N90 in the second drier year (15%). Concurrently, STaz caused an increase of both NUEy and NUEp under N60 and N90T3, in the first year. This is a very interesting result with a view to sustainable agriculture, since strobilurin seems to emphasize the effect of low nitrogen level. Moreover, in the second drier year, strobilurin treatment determined higher NUEy values under all the nitrogen fertilization adopted and NUEp under N60, N90 and N120T3. This result was also very interesting, since in the Mediterranean area situations of drought are predicted, due to the ongoing climate change.

Moreover, in the first year the STaz determined a positive effect of the N given in three times for grain N content (27% and 16% for N90T3 and N120T3, respectively); the value observed under N90T3 was very similar to that reached under N120 without strobilurin treatment. Once again, this result is very important because the strobilurin application could reduce the N rate application from 120 to 90 kg ha-1 with excellent results.

Finally, the negative effect of the nitrogen distributed in three times observed in the second drier year on most of the parameters considered was overcame following strobilurin application.

CONCLUSIONS

The influence of strobilurin treatment on durum wheat N use efficiency for yield and grain protein was investigated in interaction with cultivar and different N fertilization treatments under Mediterranean conditions. We partially confirmed results of previous experiments investigating the relationships between strobilurin and nitrogen use efficiency in durum wheat. In addition, our findings showed that the positive effect reported in literature for strobilurin on uptake and remobilization efficiency, really depend on the cultivars and thus the interaction strobilurin treatment x cultivar seems to be very important. Indeed, strobilurin had a positive effect on UPE, NUEy and NUEp in Saragolla, that was a cultivar characterized by high yield and low grain protein content, and on NUtE and NUEy in Sfinge, that was the cultivar characterized by low yield and high grain protein content. Moreover, we provide new evidence to suggest that strobilurin positively affected the contribution of UPE and negatively that of NUtE to NUEy only in Saragolla.

As for the interaction with N fertilization, we observed that strobilurin could reduce the N rate application from 120 to 90 kg ha^{-1} splitted in three time with satisfactory results in terms of grain N content. The most interesting results were observed in the second drier year, when only 27 mm of rain felt during the grain filling period. In this condition, strobilurin determined higher NUEy and NUEp values under most of the N fertlization adopted. These results are very interesting since conditions of drought are predicted in the Mediterranean area, due to the ongoing climate change. Thus, our results improved the knowledge related to complexity of managing durum wheat nitrogen fertilization in sustainable agricultural systems under Mediterranean climate conditions.

SUPPLEMENTARY MATERIALS

Table

Table S1. Mean square relative to genotype (G), nitrogen fertilization (N), strobilurin treatment (ST) and their interactions resulting from analysis of variance (ANOVA) performed in two crop seasons on straw, grain and plant nitrogen content.

		2011			2012		
	DF	**N plant content**	**N straw content**	**N grain content**	**N plant content**	**N straw content**	**N grain content**
Genotype (G)	1	296480 ***	244939 ***	2458.4 ***	5899.8 ***	4484.7 ***	96.9 ***
Nitrogen fertilization (N)	4	1090.1 ***	6107.2 ***	1843.6 ***	1287.8 ***	264 ***	426.1 ***
Strobilurin treatment (ST)	1	264.8***	7402.6 ***	776.9 ***	187.5 ***	4.2 **	135.9 ***
G x N	4	462.1 ***	3531.5 ***	1201.2 ***	359.3***	163.3 ***	99.3 ***
G x ST	1	5152.1 ***	36326.2 ***	4355.4 ***	3824.8 ***	244.2 ***	2135.6 ***
N x ST	4	144.1 ***	1358.1 ***	203.1 ***	1129.9 ***	302.3 ***	282.9 ***
Gx N x ST	4	249.6 ***	1567.3 ***	555.1 ***	762.0 ***	164.4 ***	224.6 ***

DF, degree of freedom; *** P ≤ 0.001; ** P ≤ 0.01; * P ≤ 0.05.

Figure

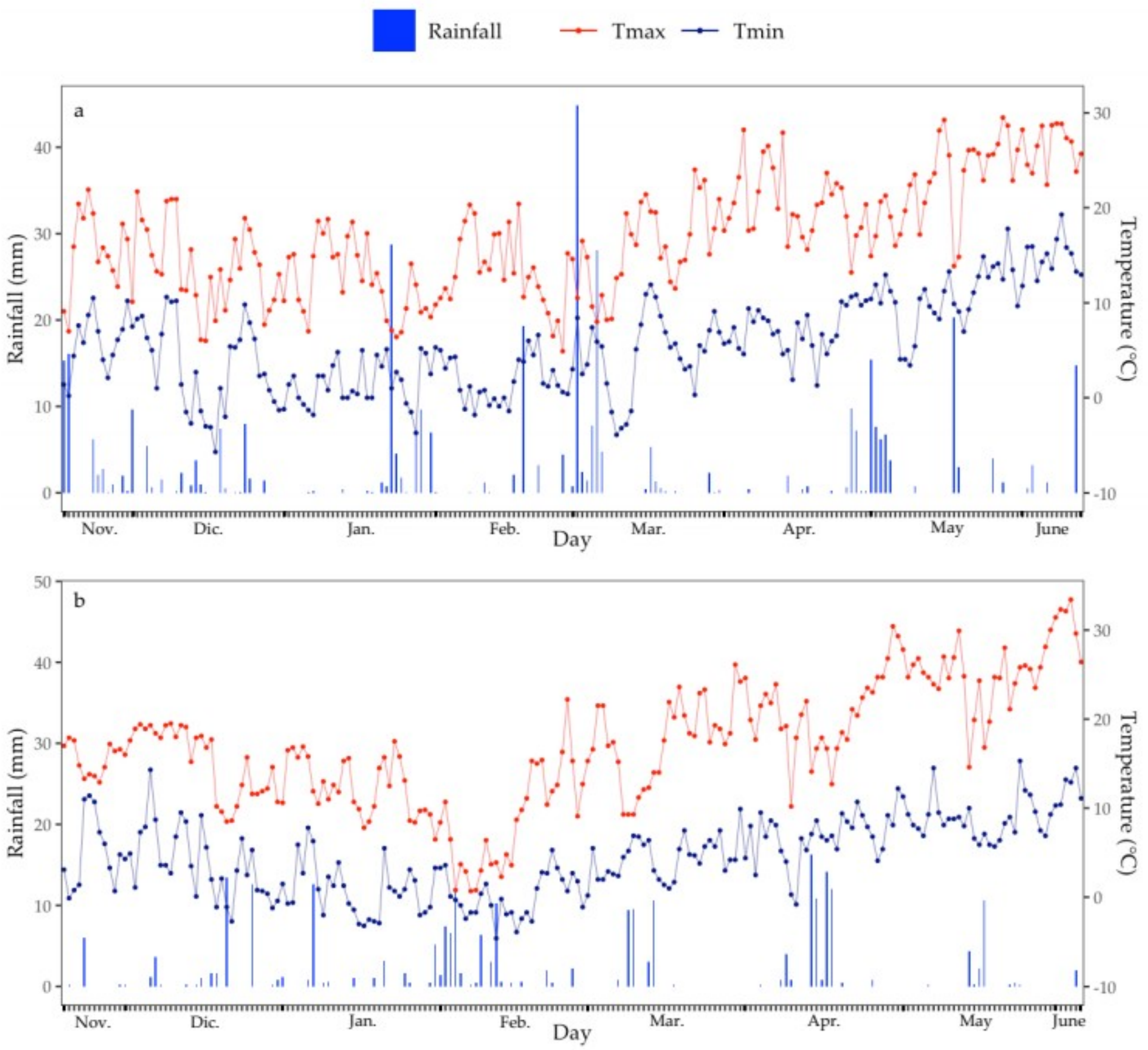


Figure S1. Daily rainfall and maximum and minimum temperatures for the two years under study 2011 (a) and 2012 (b).

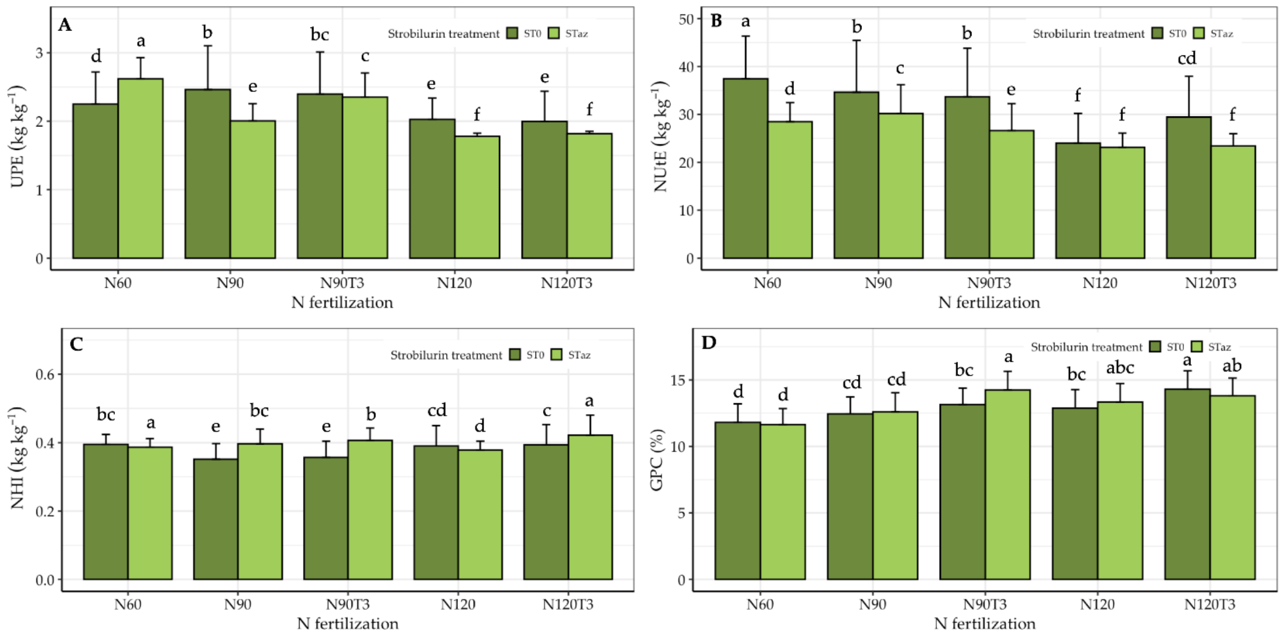


Figure S2. Effect of the interaction nitrogen fertilization x strobilurin treatment in 2011 for uptake efficiency (UPE, A), nitrogen utilization efficiency (NUtE, B), nitrogen harvest index (NHI, C), and grain protein content (GPC, D).
N60, N90 and N120 correspond to 60, 90 and 120 kg ha^{-1} of nitrogen applied in two times; N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.
ST0, no strobilurin treatment; STaz, strobilurin treatment.
Values followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.
Vertical bars indicate standard errors (n=6).

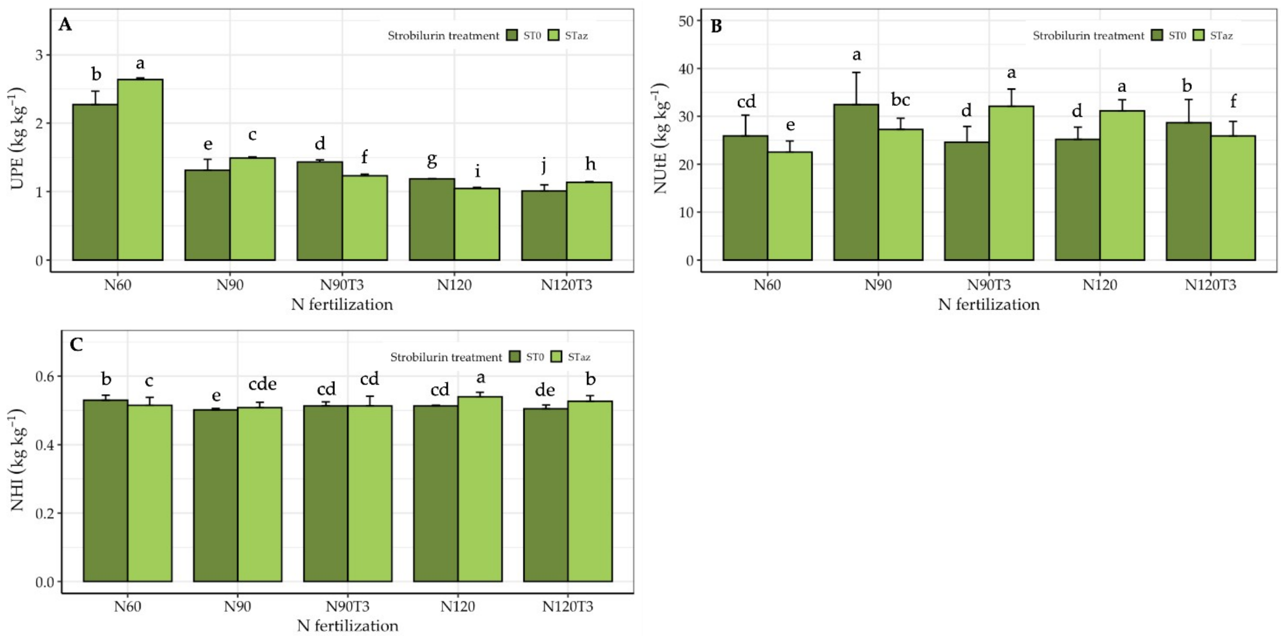


Figure S3. Effect of the interaction nitrogen fertilization x strobilurin treatment in 2012 for uptake efficiency (UPE, A), nitrogen utilization efficiency (NUtE, B), and nitrogen harvest index (NHI, C).
N60, N90 and N120 correspond to 60, 90 and 120 kg ha^{-1} of nitrogen applied in two times; N90T3, 90 kg ha^{-1} of nitrogen applied in three times; N120T3, 120 kg ha^{-1} of nitrogen applied in three times.
ST0, no strobilurin treatment; STaz, strobilurin treatment.

Values followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test. Vertical bars indicate standard errors (n=6)

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IV. Effects of Genotype, Growing Season and Nitrogen Level on Gluten Protein Assembly of Durum Wheat Grown under Mediterranean Conditions

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Effects of Genotype, Growing Season and Nitrogen Level on Gluten Protein Assembly of Durum Wheat Grown under Mediterranean Conditions

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ABSTRACT

Water deficit and high temperatures are the main environmental factors which affect both wheat yield and technological quality in the Mediterranean climate. The aim of the study was to evaluate the variation in the gluten protein assembly of four durum wheat genotypes in relation to growing seasons and different nitrogen levels. The genotypes, Marco Aurelio, Quadrato, Pietrafitta and Redidenari, were grown under three nitrogen levels (36, 90 and 120 kg ha^{-1}) during two growing seasons in Southern Italy. Significant lower yield and higher protein concentration were observed in the year characterized by higher temperature at the end of the crop cycle. The effect of the high temperatures on protein assembly was different for the genotypes in relation to their earliness. Based on PCA, in the warmer year only the medium-early genotype Quadrato showed positive values along the "protein polymerization degree" factor, while the medium and medium-late genotypes Marco Aurelio and Pietrafitta showed negative values along the "proteins assembly" factor. No clear separation along the two factors was observed for the early genotype Redidenari. The variation in gluten protein assembly observed in the four genotypes in relation to growing season might help breeding programs to select genotypes suitable for facing the ongoing climate changes in Mediterranean area.

KEYWORDS

durum wheat; glutenin polymers; gluten quality; high temperature; nitrogen fertilization.

INTRODUCTION

Durum wheat (*Triticum turgidum* L., subsp. *durum* Desf.) is the most widespread cereal crop in Mediterranean countries and is grown in various climatic conditions [1].

Water deficit and high temperatures are the main environmental factors, which affect both wheat yield and technological quality in the Mediterranean climate [2-3]. According to studies performed by the Intergovernmental Panel on Climate Change (IPCC), further increase in temperatures is predicted in Europe, especially in the Southern and Central parts [4-5]. In this context, the maintenance of adequate yield and quality standards is of particular interest, since the annual variability of product quality cannot be acceptable, especially for dry pasta production [6].

The wheat grain quality mainly depends on the quantity and type of gluten proteins, as well as on their aggregation/polymerization level [7-8]. In particular, gliadins, which are monomeric proteins, are mainly responsible for the viscous nature of the dough, and interact mostly via non-covalent links, while glutenin, which are polymeric proteins stabilized by disulphide bonds, determine its elasticity [9-12].

In the literature [13-22], conflicting results on the effect of high temperatures on the quality of the gluten proteins are reported. Studies made on bread wheat suggest that when high temperatures occur in the middle of grain filling, they positively affect dough strength [13], while very high temperatures near physiological maturity can have a negative effect [14]. Ciaffi et al. [15] reported that in bread wheat, high temperatures increased the accumulation of glutenins compared to gliadins. On the contrary, O'Leary et al. [16] reported that water or thermal stress conditions throughout the grain filling period determine a delay in the synthesis of glutenins while the synthesis of gliadins is not altered. Furthermore, for common wheat, it is reported that short periods of very high temperatures can significantly reduce the proportion of SDS-insoluble polymers (UPP) [15, 17], which in bread wheat (*Triticum aestivum* L.) have been positively correlated with dough viscoelasticity [7-8]. On the contrary, some authors have reported that short periods of very high temperatures can lead to an increase in the size of glutenin polymers in both soft and durum wheat [18-19]. While numerous are the studies available in the literature on the effect of high temperatures on gluten protein concentration, composition and on polymeric proteins size and distribution in common wheat [20-22], very few are the studies relative to durum wheat and to its pasta-making quality [8]. Moreover, pasta-making quality in durum wheat is mostly determined by low molecular weight glutenin subunits (LMW-GS), especially the B-type [23], whereas in bread wheat high molecular weight glutenin subunits (HMW-GS) play the major role in determining dough technological properties [24].

In the Mediterranean areas, after climate conditions, the nitrogen (N) availability represents the main constraint in obtaining adequate yield and quality in durum wheat [25]. Some studies on bread wheat have suggested that high doses of N tend to increase the amount of monomer proteins [26-27] and to reduce the percentage of UPP causing an increase in the extensibility of the dough [28-31]. Moreover, some authors have highlighted that the effect of nitrogen on gluten proteins composition and on polymers organization may vary according to the genotype [26, 30, 32]. Finally, for the same parameters, significant effect of the interaction between the high temperatures and N availability has been reported [29, 33]. Malik et al. [33] highlighted that the combinations of cultivars, nitrogen and temperature were needed to explain the variation in the quantity and size distribution of the polymer proteins and their effects on the quality of the end-product. To the best of our knowledge, for durum wheat, this type of information is still lacking.

Thus, the aim of the present study was to evaluate the variation in gluten proteins quality, in terms of their capacity to assembly in a visco-elastic structure, of four durum wheat genotypes in relation to the growing season and different nitrogen levels, including a low input rate.

MATERIALS AND METHODS

Field Trials

Four durum wheat cultivars, Marco Aurelio, Quadrato, Pietrafitta and Redidenari, that are used in an important Italian pasta supply chain, (Table 1), were grown in two rain-fed field experiments carried out at Foggia (latitude 41°46' N and longitude 15°54' E, 74 m a.s.l.) during two growing seasons (2016-2017 and 2017-2018, hereafter indicated as 2017 and 2018, respectively) in a clay loam soil.

Table 1. Main characteristics of the genotypes under study.

Genotype	Year of release	Pedigree	Earliness
Pietrafitta	1999	Grazia x Isa	medium-late
Quadrato	1999	Creso x Trinakria	medium early
Marco Aurelio	2010	Orobel//Arcobaleno/Svevo	medium
Redidenari	2015	Kofa x N185	early

The main chemical and physical soil characteristics in the two experimental year, 2017 and 2018, are reported in Table 2.

Table 2. Soil physical and chemical characteristics in the two experimental years.

Soil characteristics		2017	2018
Sand	%	21.5	25.2
Silt	%	39.8	36.2
Clay	%	38.7	38.6
pH		8.1	8.2
Organic Matter*	%	1.9	1.9

Total Nitrogen**	‰	1.3	1.3
Assimilable Phosphorus√	mg kg-1	80	64
Exchangeable Potassium◊	mg kg-1	461	422
Field Capacity (-0.03 MPa)	%	37.3	33.13
Wilting Point (-1.5 MPa)	%	19.7	18.5
Bulk Density	Mgm3	1.15	1.10

* Walkley-Black method; ** Kjeldhal method; √ Olsen method; ◊Ammonium acetate method

The four cultivars have been sown on November 17 in 2016 and November 25 in 2017, at a seeding rate of 240 kg ha^{-1}. In both years, the experiment was in a field where the previous crop was durum wheat.

Three different nitrogen levels were adopted corresponding to 36, 90 and 120 kg ha^{-1} (N36, N90 and N120, respectively). The fertilizers used were Yara Mila Supersemina (18% nitrogen) at pre-sowing fertilization and Yara Bela Sulfan (24% nitrogen) at tillering, stem elongation and inflorescence emergence fertilization.

Each year, the experiment was arranged in a split-plot design with two factors (genotype in plots and nitrogen levels in sub-plots) and three replications; each sub-plot was 20.4 m2.

The grain harvest was carried out at physiological maturity on 13 June 2017 and on 22 June 2018. During the experimental period, the daily climatic parameters of rainfall and temperature were recorded by a weather station near the experimental area.

Yield and technological quality parameters

At harvest, grain yield (t ha^{-1}) and thousand kernel weight (TKW) were determined. Moreover, grain protein content (GPC) was performed by NIR System Infratec 1241 Analyzer (Foss, Hillerod, Denmark).

Semolina flours have been obtained from kernels milled by Bona mill 4 cylinders (sieve 180 μm).

The gluten index (GI), an indicator of the gluten strength, was determined on semolina samples using the Glutomatic system according to ICC standard 155 [34].

2.3. Calculation of %UPP and analysis of gluten protein molecular size distribution

The percentage of Unextractable Polymeric Proteins (%UPP) was measured trough the SE-HPLC procedure according to the method reported in Tosi et al. [35] with minor modifications. The SDS-soluble fraction was obtained by adding to the semolina a solution consisting of 0.5% (w/v) SDS in 0.05 M sodium phosphate buffer, pH 6.9 to a final concentration of 10 mg/ml (0.3 g semolina on 30 ml buffer). The mixture was stirred for 30 min at room temperature and then centrifuged at 20.000 g for 20 min at 15 °C. The supernatant was filtered through 0.45 μm PVDF filters and 20 μl were injected into a Biobasic Thermo Scientific SEC-300 Columns (300 mm x 7.8 mm; flow rate: 0.7 ml/min) and run for 40 min, with an eluent consisting of 0.05 M sodium phosphate buffer pH 6.9, containing 0.08 M NaCl and 0.1 % (w/v) SDS, using the UHPLC Ultimate 3000 Thermo scientific. Detection was at 214 nm. The SDS soluble fraction profiles were divided into four areas, corresponding to HPLC fractions F1, F2, F3 and F4 (Figure S2a). The first two areas correspond to large and medium size polymers, with both being enriched in HMW-GS (mainly F1) and B-type LMW-GS (mainly F2) of glutenin. F3 corresponds to ω-gliadins and small oligomers enriched in C-type and D-type LMW-GS subunits [23], while F4 corresponds to monomeric gliadins (α-type and β-type) and non-gluten proteins [35].
The SDS-insoluble fraction was obtained from the residue of the centrifugation step. The pellet was resuspended in 30 ml of the same extraction buffer and sonicated in a probe type sonicator (SONICS Vibracell Model VCX 130 -max output power 130 W at a frequency 20 KHz) for 30 sec at 45% power setting. After centrifugation at 20.000 g for 20 min at 15 °C, the supernatant was filtered through 0.45 μm PVDF filters and 20 μl were injected into column in the same condition described above. The SDS-insoluble fraction profile (Figure S2b) showed only one peak (F1*) containing the largest glutenin polymers, insoluble in SDS solution alone, but rendered soluble by sonication.
Samples were extracted in duplicate and two replicate separations for each extraction were performed. The proportions of each peak (%F1* and%F1–%F4)

were calculated as percentages of the total areas of the two chromatograms (SDS-insoluble and SDS-soluble fractions). The amount of monomeric over polymeric proteins (mon/pol) was calculated as the ratio between the sum of F3 and F4 areas and the sum of F1*, F1 and F2 areas. %UPP was determined as the ratio between F1* area and the sum of F1 and F1* areas (*100).

Statistical analysis

The dataset was tested according to the basic assumptions of analysis of variance (ANOVA). The normal distribution of the experimental error and the common variance of the experimental error were verified through Shapiro-Wilk and Bartlett's tests, respectively. When required, Box-Cox transformations [36] were applied prior to analysis. The ANOVA procedure was performed according to a split-plot design with three replicates. Three-way ANOVA procedure was performed considering the factors (growing season, genotype and nitrogen level) as fixed factors. The statistical significance of the difference among the means was determined using Tukey's honest significance difference post hoc test at the 5% probability level. Principal component analysis (PCA) was performed on the correlation matrix of technological and SE-HPLC parameters. We obtained Principal Components (PCs) on centered and scaled variables, through diagonalization of the correlation matrix and extraction of the associated eigenvectors and eigenvalues. Grain protein content, gluten index, and SE-HPLC parameters were set as quantitative variables and used to define PCs, while genotype, N level and growing season were used as categorical variables, not considered in the computation of PCs. The coordinates of the categorical variables were calculated in order to enhance the interpretation of data and were represented as barycenter in the Principal Component biplot. The number of factors needed to adequately describe the data was determined on the basis of the eigenvalues and of the percentage of the total variance accounted by the different factors. The results of PCA were graphically represented in two-dimensional plot, using the SigmaPlot software (Systat Software, Chicago, IL, USA). ANOVA and PCA analyses were performed using the JMP software package, version 14.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Weather condition

The climatic data related to the two growing seasons are reported in Table 3, while the rainfall distribution and maximum and minimum daily mean temperatures of the 2017 (a) and 2018 (b) crop seasons are reported in Figure S1 (supplementary file).

The first growing season was characterized by lower rainfall compared to the second year (about 340 mm vs 401 mm). Moreover, in the first experimental year the rain distribution was not regular, with the most intense rainfall occurred in the second decade of January, the third decade of February, the second decade of April and the first decade of May. As for the second growing season, rainfall was observed throughout the crop cycle, especially during the grain filling period, in the first ten days of May and June. In addition to rainfall, the two years differed also for the maximum temperatures during the grain filling period showing the second year the highest values. Moreover, during 2018, more days with temperatures between 30 and 35 °C and three days with temperatures higher than 35 °C, compared to 2017, occurred.

Table 3. Climatic data related to the two growing seasons.

		2017	**2018**
Crop cycle duration	d	209	210
Crop cycle rainfall	mm	339.9	401.4
From seeding to heading rainfall	mm	204.2	198.6
Grain filling rainfall	mm	135.7	202.5
Crop cycle Mean T	°C	12.3	13.1
Grain filling Mean T	°C	18.3	21.7
Grain filling Mean T max	°C	25.5	29.1
30° C< T <35° C	d	15	23
T >35°C	d	-	3

Yield and technological parameters

The analysis of variance (ANOVA) generally showed a significant effect of year (Y), genotype (G) and nitrogen (N) on the parameters considered (Table S1). The two growing seasons differently influenced the yield and the technological parameters considered. In the second growing season (Table 4) significant lower yield, thousand kernel weight and gluten index were observed with respect to the first one. On the contrary, grain protein content was higher in 2018 than in 2017. Relative to the nitrogen level (Table 4), a significant positive effect on grain yield was evident only under N90, while for protein content the highest value was observed under N120. Finally, the gluten index values decreased with N level increasing.

Among the genotypes (Table 4), Marco Aurelio showed the highest yield value even if associated with lower thousand kernel weight. Instead, Redidenari was the genotype with the best technological quality performance showing the highest protein content and gluten index values. However, the behavior of the genotypes changed in relation to growing seasons (Table 5) and nitrogen levels adopted (Table 6). In particular, the yield decrease observed in the second year was different among the genotypes (Table 5); it was 5% and 9% for Marco Aurelio and Redidenari, and 14% and 17% for Pietrafitta and Quadrato, respectively. Moreover, Marco Aurelio in addition to presenting lower yield decrease in the second year also showed an increase in the protein content that was double compared to the other genotypes (3.1% vs 0.4, 1.36 and 1.07% for Pietrafitta, Quadrato and Redidenari, respectively). Finally, as for gluten index, Marco Aurelio and Redidenari showed a significant decrease in the second year, more marked for Redidenari (Table 5).

The nitrogen fertilization did not significantly affect the grain yield response in Marco Aurelio, while for both Pietrafitta and Redidenari, the highest values were observed under N90 level; for Quadrato the highest value was observed under N120 even if not significantly different from N36 (Table 6). On the contrary, for all genotypes a positive effect of the nitrogen level on grain protein content was evident with the highest values observed under N120. The effect of nitrogen fertilization on gluten index was not clear; only Quadrato showed a significant decrease under N120 level (Table 6).

Table 4. Effect of the year, nitrogen level and genotype on grain yield, thousand kernel weight, grain protein content and gluten index.

Experimental factors	Grain yield ($t\ ha^{-1}$)	Thousand kernel weight (g)	Grain protein content (%)	Gluten index (-)
Year				
2017	6.66 a	60.91 a	14.53 b	64.44 a
2018	5.91 b	50.21 b	16.00 a	58.50 b
Nitrogen level				
N36	6.20 b	55.16 a	14.25 c	63.83 a
N90	6.36 a	55.90 a	15.33 b	62.71 a
N120	6.28 ab	55.62 a	16.23 a	57.88 b
Genotype				
Marco Aurelio	7.11 a	50.62 d	15.74 b	57.72 bc
Pietrafitta	5.75 c	64.47 a	15.29 c	56.50 c
Quadrato	6.42 b	54.56 b	14.08 d	61.39 b
Redidenari	5.85 c	52.60 c	15.97 a	70.28 a

For each experimental factor, values in column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Table 5. Effect of the year x genotype interaction on grain yield, thousand kernel weight, grain protein content and gluten index.

	2017				2018			
	Marco Aurelio	**Pietrafitta**	**Quadrato**	**Redidenari**	**Marco Aurelio**	**Pietrafitta**	**Quadrato**	**Redidenari**
Grain yield (t ha^{-1})	7.29 a	6.20 c	7.02 ab	6.12 c	6.92 b	5.31 e	5.82 d	5.57 de
Thousand kernel weight (g)	54.82 c	71.21 a	59.25 b	58.39 b	46.42 e	57.74 b	49.88 d	46.80 e
Grain protein content (%)	14.19 f	15.11 d	13.40 g	15.43 c	17.29 a	15.47 c	14.76 e	16.50 b
Gluten index (-)	60.78 b	56.44 b	60.89 b	79.67 a	54.67 c	56.56 b	61.89 b	60.89 b

In each row, values followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's tes

Table 6. Effect of the genotype x nitrogen interaction on grain yield, thousand kernel weight, grain protein content and gluten index.

	Marco Aurelio	Pietrafitta	Quadrato	Redidenari
Grain yield (t ha^{-1})				
N36	7.16 a	5.6 f	6.46 bc	5.61 ef
N90	7.00 a	5.99 de	6.21 cd	6.25 bcd
N120	7.17 a	5.68 ef	6.60 b	5.68 ef
Thousand kernel weight (g)				
N36	50.18 e	64.67 a	53.80 bcd	51.99 cde
N90	50.76 de	65.38 a	54.22 bc	53.26 bcde
N120	50.91 de	63.37 a	55.67 b	52.53 bcde
Grain protein content (%)				
N36	15.33 c	14.1 e	12.8 f	14.75 d
N90	15.60 c	15.33 c	14.03 e	16.33 b
N120	16.28 b	16.43 ab	15.40 c	16.82 a
Gluten index (-)				
N36	57.67 bcde	56. 00 cde	67.33 ab	74.33 a
N90	54.33 de	61.17 bcd	66.83 abc	68.50 ab
N120	61.17 bcd	52.33 de	50. 00 e	68. 00 ab

For each parameter, values in each row and column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Measurement of %UPP and analysis of gluten protein molecular size distribution

SE-HPLC was used to compare the molecular size distribution of the semolina proteins by a quantitative comparison of elution profiles.,

The analysis of variance performed on the percentage of SDS-insoluble protein fraction (F1*), SDS-soluble protein fraction (F1-F4), monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP) showed a general significant effect of the year (Y), genotype (G), nitrogen level (N) and their interactions (Table S2). A significant decrease of F1* and %UPP was observed in 2018 compared to 2017. Moreover, in 2018 a significant increment of the polymeric fraction, due to an increase of both F1 and F2 was observed. On the contrary, in the same year, a decrease of the monomeric fraction, due to a decrease of F4 was evident, determining also a lower mon/pol ratio with respect to 2017 (Table 7). As for the nitrogen levels, a general positive effect of N90 compared with N36 was observed for F1*, %UPP and for the monomeric fraction, while there have never been significant differences between N36 and N120 (Table 7). Finally, as for genotypes, Marco Aurelio showed higher values of %UPP and polymeric fraction, due to higher values of F1* and F2, and lower value of mon/pol ratio. On the contrary Redidenari and Pietrafitta showed lower values of polymeric fraction (again mainly due to lower F1* and F2 values) and higher values of monomeric fraction and mon/pol ratio (Table 7). Finally, Quadrato showed intermediate values for all the fraction considered. The behavior of the genotypes changed in relation to growing seasons (Table 8). A significant decrease of F1* in the second year was evident for Marco Aurelio and Pietrafitta, more marked for the former. As consequence also %UPP significantly decrease in 2018 for Marco Aurelio (13.7%) and Pietrafitta (4.2%). On the contrary, a significant increase of F1* and %UPP was observed in the second year for Quadrato. All genotypes showed the increase of F1 values in the second year and only Marco Aurelio and Pietrafitta the increase of F2 values. Also for the polymeric and monomeric fraction the effect of the growing season was observed only for Quadrato and Redidenari. In particular, in 2018 these two genotypes showed higher polymeric and lower monomeric fraction values than 2017. The increase in polymeric fraction was due mainly to the significant increase in 2018 of both F1* and F1 for Quadrato, and of F1 for Redidenari, while the decrease of the monomeric fraction was due mainly to the F4 decrease.

Relative to the effect of the genotype x nitrogen level interaction (Table 9), a significant effect of nitrogen level on F1* was evident for Marco Aurelio and Redidenari; in particular for the former the F1* values increased with N level increasing, while for Redidenari the highest value was observed under N90. Both of these genotypes showed also highest %UPP values under N90. Moreover, only Redidenari showed a significant effect of the nitrogen level on the polymeric and the monomeric fraction, showing under N120 lower polymeric and higher monomeric fraction values.

Table 7. Effect of the year, genotype and nitrogen level on SDS insoluble (F1*) and soluble protein fraction (F1-F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP).

Experimental factors	F1*	F1	F2	F3	F4	F1*+F1	Polymeric fraction (F1*+F1+F2)	Monomeric fraction (F3+F4)	%UPP	mon/pol
					(%)					(-)
Year										
2017	10.66 a	24.16 b	11.52 b	22.99 a	30.68 a	34.82 b	46.34 b	53.66 a	30.20 a	1.17 a
2018	9.71 b	26.99 a	12.07 a	23.24 a	27.99 b	36.70 a	48.77 a	51.23 b	26.26 b	1.06 b
Nitrogen level										
N36	10.07 b	26.22 a	11.75 a	22.59 a	29.38 a	36.29 a	48.04 a	51.96 b	27.66 b	1.09 b
N90	10.68 a	24.69 b	11.76 a	23.46 a	29.42 a	35.37 b	47.13 b	52.87 a	30.04 a	1.13 a
N120	9.80 b	25.81 a	11.88 a	23.30 a	29.21 a	35.61 ab	47.49 ab	52.51 ab	27.00 b	1.12 ab
Genotype										
Marco Aurelio	12.32 a	25.71 a	13.22 a	22.73 b	26.02 d	38.04 a	51.26 a	48.74 c	32.18 a	0.96 c
Pietrafitta	8.46 c	25.82 a	10.91 c	24.31 a	30.50 b	34.28 c	45.20 c	54.80 a	24.64 c	1.21 a
Quadrato	10.28 b	25.38 a	11.92 b	22.97 ab	29.45 c	35.66 b	47.58 b	52.42 b	28.69 b	1.11 b
Redidenari	9.68 b	25.37 a	11.12 c	22.46 b	31.37 a	35.05 bc	46.17 c	53.83 a	27.43 b	1.17 a

For each experimental factors, values in column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Table 8. Effect of the year x genotype interaction on SDS insoluble (F1*) and soluble protein fraction (F1-F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP).

	2017				2018			
(%)	**Marco Aurelio**	**Pietrafitta**	**Quadrato**	**Redidenari**	**Marco Aurelio**	**Pietrafitta**	**Quadrato**	**Redidenari**
F1*	14.95 a	9.18 c	8.93 cd	9.56 c	9.70 c	7.74 d	11.62 b	9.80 c
F1	23.11 d	24.98 c	24.00 cd	24.53 c	28.31 a	26.67 b	26.76 b	26.21 b
F2	12.76 b	10.32 f	12.09 c	10.90 ef	13.68 a	11.50 cde	11.76 cd	11.34 de
F3	22.50 b	23.72 ab	23.27 ab	22.45 b	22.96 ab	24.89 a	22.66 ab	22.47 b
F4	26.68 d	31.79 a	31.70 a	32.56 a	25.36 e	29.21 c	27.19 d	30.19 b
F1*+F1	38.1 a	34.2 c	32.3 c	34.1 c	38 a	34.4 bc	38.4 a	36 b
Polymeric fraction (F1*+F1+F2)	50.83 a	44.49 c	45.03 c	45.00 c	51.68 a	45.9 bc	50.14 a	47.34 b
Monomeric fraction (F3+F4)	49.17 c	55.51 a	54.97 a	55.00 a	48.32 c	54.1 ab	49.86 c	52.66 b
UPP	39.0 a	26.7 cd	27.1 cd	28.0 bc	25.3 d	22.5 e	30.3 b	26.9 cd
mon/pol (-)	0.97 c	1.25 a	1.22 a	1.22 a	0.94 c	1.18 ab	0.99 c	1.12 b

Values in each row followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test.

Table 9. Effect of the genotype x nitrogen level interaction on SDS insoluble (F1*) and soluble protein fraction (F1-F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP).

(%)		Marco Aurelio	Pietrafitta	Quadrato	Redidenari
F1*	N36	11.18 bcd	9.25 efg	10.26 cde	9.58 def
	N90	12.70 ab	8.33 fg	9.80 def	11.91 abc
	N120	13.10 a	7.80 g	10.77 cde	7.55 g
F1	N36	25.86 ab	26.51 ab	25.81 ab	26.72 a
	N90	25.07 b	25.10 b	25.17 b	23.42 c
	N120	26.21 ab	25.86 ab	25.18 b	25.98 ab
F2	N36	13.01 a	10.73 de	11.71 bc	11.54 bcd
	N90	13.45 a	11.02 cde	12.05 b	10.50 e
	N120	13.20 a	10.98 cde	12.02 b	11.32 bcde
F3	N36	23.10 abc	23.38 abc	22.97 abc	20.9 c
	N90	23.28 abc	24.10 ab	23.36 abc	23.09 abc
	N120	21.8 bc	25.45 a	22.57 abc	23.39 abc
F4	N36	26.85 e	30.13 bcd	29.26 d	31.26 ab
	N90	25.51 f	31.45 a	29.63 d	31.09 abc
	N120	25.70 ef	29.91 cd	29.46 d	31.77 a
F1*+F1	N36	37.04 abc	35.76 bcde	36.07 bc	36.29 bc
	N90	37.76 ab	33.43 f	34.96 cdef	35.32 cdef
	N120	39.31 a	33.66 def	35.95 bcd	33.53 ef
Polymeric fraction	N36	50.04 ab	46.49 cd	47.77 bc	47.88 bc
(F1*+F1+F2)	N90	51.22 a	44.46 d	47.01 cd	45.82 cd
	N120	52.51 a	44.64 d	47.97 bc	44.84 d
Monomeric fraction	N36	49.95 cd	53.51 ab	52.23 bc	52.17 bc
(F3+F4)	N90	48.78 d	55.54 a	52.99 ab	54.17 ab
	N120	47.49 d	55.36 a	52.03 bc	55.15 a
UPP	N36	30.2 bc	25.8 def	28.5 cd	26.2 def
	N90	33.8 a	24.9 efg	27.7 cde	33.7 a
	N120	32.5 ab	23.2 fg	29.9 bc	22.4 g
mon/pol (-)	N36	1.00fg	1.15 b-e	1.10 def	1.10 de
	N90	0.96 g	1.25 a	1.14 cde	1.19 abcd
	N120	0.91 g	1.24 ab	1.09 ef	1.23 abc

For each parameter, values in each row and column followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test.

PCA analysis

Principal component analysis (PCA) was performed on the correlation matrix. The results of PCA allowed two factors to be identified explaining 51% and 20.9% of total variance, respectively (Table 10). The first factor (PC1) was highly and positively associated with the largest insoluble polymers (F1*), the medium size soluble polymers (F2), the largest glutenin polymers (both insoluble and soluble; F1*+F1) and with the polymeric fraction (F1*+F1+F2). Moreover, it was highly and negatively related with the small oligomers fraction (F3), the monomeric gliadin fraction (F4), the total monomeric fraction (F3+F4) and mon/pol ratio. Thus, PC1 could be considered a factor linked to the degree of polymerization, mostly depending on the capacity to form covalent bonds. The second factor (PC2) was positively associated with gluten index (depending on the interactions among gluten proteins, both gliadins and glutenins), with the largest insoluble polymers (F1*) and with %UPP (depending on glutenin polymers size and amount) and negatively related with grain protein content (that can affect mostly gliadin accumulation) and the large size soluble polymers (F1) (that affect negatively %UPP). Thus, PC2 could be considered as a "gluten proteins assembly" factor, including the different interactions occurring in the gluten network. Both the factors linked to the degree of polymerization and the gluten proteins aggregation are major determinants of technological quality.

Table 10. Loading matrix values for the first two principal components (PC1 and PC2), considering the original variables. The corresponding percentages of accounted variation are also reported.

Original variables	Loading matrix values	
	PC1	PC2
Grain protein content	0.09	-0.57
Gluten index	-0.21	0.47
F1* (%)	0.72	0.64
F1 (%)	0.29	-0.80
F2 (%)	0.54	-0.35
F3 (%)	-0.57	-0.26
F4 (%)	-0.81	0.29
F1*+F1 (%)	0.94	0.04
F1*+F1+F2 (%)	0.99	-0.09
F3+F4 (%)	-0.99	0.09
UPP (%)	0.55	0.77
mon/pol	-0.99	0.10
Percentage explained variation	**51**	**20.9**
Percentage cumulative variation	**71.9**	

In figure 1 the biplot relative to the principal component analysis is reported. Based on the barycenter of the categorical variables (Fig. 1, yellow marks), the nitrogen level did not show a clear separation along the two factors considered. On the contrary, the separation between the two years was observed mainly along the "gluten proteins assembly" factor (PC2) with the 2018 in the lower part. However, the separation between the crop seasons has to be interpreted also considering the genotype behaviors. Only for Quadrato the two years were separated mainly along the PC1 (polymerization degree factor), with the 2018 showing the positive and higher values. No clear separation was observed for the early maturing genotype Redidenari along the two PC factors. On the other hand, Marco Aurelio and Pietrafitta showed a clear separation of the two years only along the PC2, more marked for Marco Aurelio, with the 2018 showing the lower values. Finally, only the two genotypes Marco Aurelio and Pietrafitta were clearly separated along PC1, presenting Marco Aurelio positive values and RDD negative values.

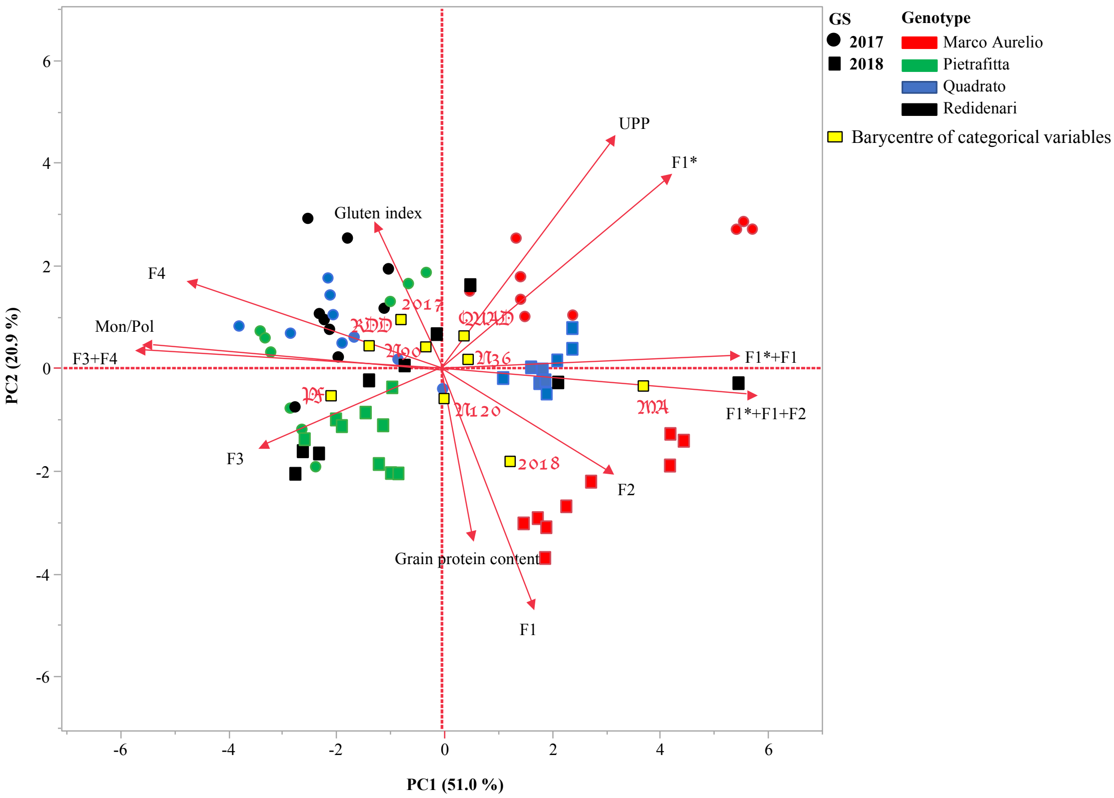


Figure 1. Biplot relative to the principal component analysis performed on grain protein content, gluten index, SDS insoluble (F1*) and soluble protein fraction (F1-F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein. In yellow, the barycenter of the categorical variables, growing season (2017 and 2018), genotype (MA, Marco Aurelio; PF, Pietrafitta; QUAD, Quadrato; RDD, Redidenari) and nitrogen level (N36, N90 and N120) are reported.

DISCUSSION

In the Mediterranean climate, the rainfall variability together with the frequency of high temperature during the grain filling period, may cause large fluctuations in durum wheat grain yield and technological quality aspects [3, 37]. In semi-arid regions a further increase in temperatures together with reduced rainfall are expected following the ongoing climate change [38-39]. This trend will influence also the crop responses to nitrogen fertilization, which depend on rainfall amount and distribution during the crop cycle, to the amount and timing of nitrogen applications as well as to the initial soil nitrogen levels [40-41]. Moreover, Malik et al. [33] highlighted that the combinations of cultivars, nitrogen and temperature are needed to explain the variation in the quantity and size distribution of the polymer proteins and their effect on the quality of the end-product. To the best of our knowledge, for durum wheat, this type of information is still lacking. The results obtained in this study represent a tile of the complex mosaic depicting the interactions among environment, fertilization and genotype.

Glutenin polymers are among the major determinants of wheat quality. Polymers are formed by different types of subunits that are functionally divided into chain terminators, chain extenders, and chain branches, according to their possibility to form one, two, or three (or more) intermolecular bonds, respectively (reviewed in [23]). The combination of these three functional glutenin classes gives rise to a range of glutenin polymers with different sizes and structures, that contributes to dough rheological properties. In general, the higher the size and amount of glutenin polymers, the better dough strength, that can be predicted by the %UPP value [7].

In our experimental condition, the two growing seasons showed a different climatic trend in terms of rainfall distribution and temperatures. Significant lower yield and thousand kernel weight, together with higher protein concentration were observed for all the genotypes in 2018, characterized by higher temperatures during the grain filling with respect to the first growing season. Moderate high temperature during grain filling, between 25 °C and 35 °C, and short periods of very high temperature (>35 °C) at the end of grain filling phase, as those we observed in the second growing season, are frequently associated with a decrease in grain yield and an increase in grain protein concentration [42, 8]. However, the genotypes Marco Aurelio and Redidenari (released in

2010 and 2015, respectively) were less influenced by the growing season with respect to Quadrato and Pietrafitta (both released in 1999). The positive effect of nitrogen fertilization was clearer for the protein content than for grain yield as also reported in literature under Mediterranean climate [43-46]. However, the high yield response observed for Redidenari under N90 level was particularly interesting, indicating the possibility of limiting nitrogen inputs by adopting genotypes capable to optimize the use of nitrogen.

The growing season differently affected the gluten index, an indicator of gluten strength for durum wheat, in relation to the genotypes, showing only Marco Aurelio and Redidenari lower values in the warmer year. In bread and soft wheat, dough strength has been often positively correlated with the proportion of UPP [15, 18, 47-49]. As for durum wheat, the relation between %UPP and gluten index has been less investigated. In our experimental condition, this relation was genotype dependent, since only Marco Aurelio and Redidenari showed simultaneous decrease of gluten index and %UPP in the second year.

The composition and functionality of storage proteins have been significantly affected by growing season and genotype, while the effect of N fertilization level was rather small (Table S2) [50] as also resulted by PCA analysis. Several studies reported an increase in the proportions of the monomeric gliadins with increasing N availability [26-27]. In our experimental conditions, this was true only for the genotype Redidenari due to an increase of F4 component represented mainly by α/β type gliadin. An interesting result was the increase of %UPP for both Marco Aurelio and Redidenari under N90 level due to the increase of the F1*. The significant decrease of the larger insoluble polymers fraction (F1*) and %UPP observed in the second growing season for Marco Aurelio and Pietrafitta has to be discussed in relation to their earliness. Indeed, the very high temperature recorded at the end of the crop cycle (3 days with T >35°C) could have negatively influenced these two genotypes that are medium and medium-late maturing genotypes. This result is probably due to the fact that the assembly of the storage proteins takes place at the end of the grain filling phase [10, 51-52]. Shewry et al. [53] proposed that, at the end of the cycle, the loss of water favors the polymer chains contact inducing the assembly through disulphide crosslinking or through inter-chain hydrogen bonding. The effect of the temperatures on gluten protein assembly, have been studied mostly in bread wheat and only few studies are available for durum wheat. In common wheat, several researches suggested that moderate high temperature or few days of very high temperature resulted in a significant reduction in the proportion of the SDS-insoluble protein fraction [15, 17, 47]. Other studies showed that the size of the glutenin polymers increased in response to short periods of very high temperature [18]. Ferreira et al. [8], in durum wheat, reported also a positive effect of the high temperature during the whole grain filling period on gluten protein assembly. Thus, the relationship between the gluten protein assembly and high temperatures is still not clear and needs more investigation. In our experimental conditions, in the second growing season, the two late maturing genotypes (Marco Aurelio and Pietrafitta), together with the decrease in F1* and %UPP showed an increase of both F2 and F1 fraction, the latter together with the other genotypes, confirming that the synthesis of the SDS soluble polymers continued also under high temperature condition [14, 47]. Due to the concurrent decrease in F1* and increase in F1 and F2 fractions, Marco Aurelio and Pietrafitta did not significantly change their polymeric fraction between the two years. The increase of both %UPP and polymeric fraction observed in Quadrato and only of polymeric fraction observed in Redidenari in the second growing seasons is also linked to their earliness. Indeed, it seems like that on these genotypes, which are medium-early and early maturing, respectively, only the moderately high temperatures occurring during the grain filling acted, but not the extreme ones recorded at the end of the crop cycle. Indeed, also the results of the PCA highlighted the negative effect of the extreme temperatures on the gluten proteins assembly properties (PC2) only for Marco Aurelio and Pietrafitta, while for Quadrato a separation of the values only along the polymerization degree factor (PC1) was observed, with the warmer year showing the positive and higher values.

Because %UPP depends on protein distributions among the four areas typically used for its calculation, with the chain branchers and extenders mostly present in the fractions F1 (in particular F1*) and F2, it is important not only to select durum wheat varieties with proper glutenin compositions able to give rise to polymers of adequate size and amounts, but also that are synthetized in periods less susceptible to environmental changes, such it has occurred here for the medium early and early maturing varieties.

CONCLUSIONS

In the two growing seasons, the four durum wheat genotypes showed different capacity of the gluten proteins to assembly in a visco-elastic structure in relation to their earliness. In particular, in the second warmer year the late maturing genotype, Marco Aurelio and Pietrafitta showed a significant decrease of larger insoluble polymers fraction (F1*) and %UPP with a negative effect on their protein assembly level, despite Marco Aurelio always showed higher degree of polymerization. On the contrary, the medium-early and early maturing genotypes Quadrato and Redidenari, probably due to their earliness, did not change their "protein assembly level" in relation to the growing season.

The effect of N fertilization on the gluten protein polymerization and assembly was rather small, but among the N levels utilized the increase of

F1*, %UPP and monomeric fraction under N90 was observed. Moreover, also the highest yield and gluten index values were obtained under N90. This was true especially for Redidenari.

In general, the effect of the growing season on the parameters evaluated was more evident than those of genotype and nitrogen level.

The results obtained in this study regarding four durum wheat genotypes, clearly indicate different patterns of protein assembly in relation to the growing season, a factor that has a great influence on quality characteristics, thus contributing to the rational selection of the durum wheat genotypes, in particular those to include in supply chains, suitable for facing the ongoing climate changes in Mediterranean area.

SUPPLEMENTARY MATERIALS

Table

Table S1. Mean square of effects (year, Y; genotype, G; nitrogen level, N) resulting from analysis of variance (ANOVA) performed on yield and technological parameters

Factors	DF	GY	TKW	GPC	GI
Y	1	10.19**	2063.07**	38.87**	636.0**
G	3	7.01**	681.67**	12.76**	697.6**
N	2	0.15**	3.38ns	23.76***	240.5**
YxG	3	0.61**	23.22**	6.11**	374.3**
GxN	6	0.38**	3.99ns	1.01**	202**
YxN	2	1.61**	2.42ns	0.74**	93.5ns
YxGxN	6	0.29**	15.10**	0.76**	40.6ns

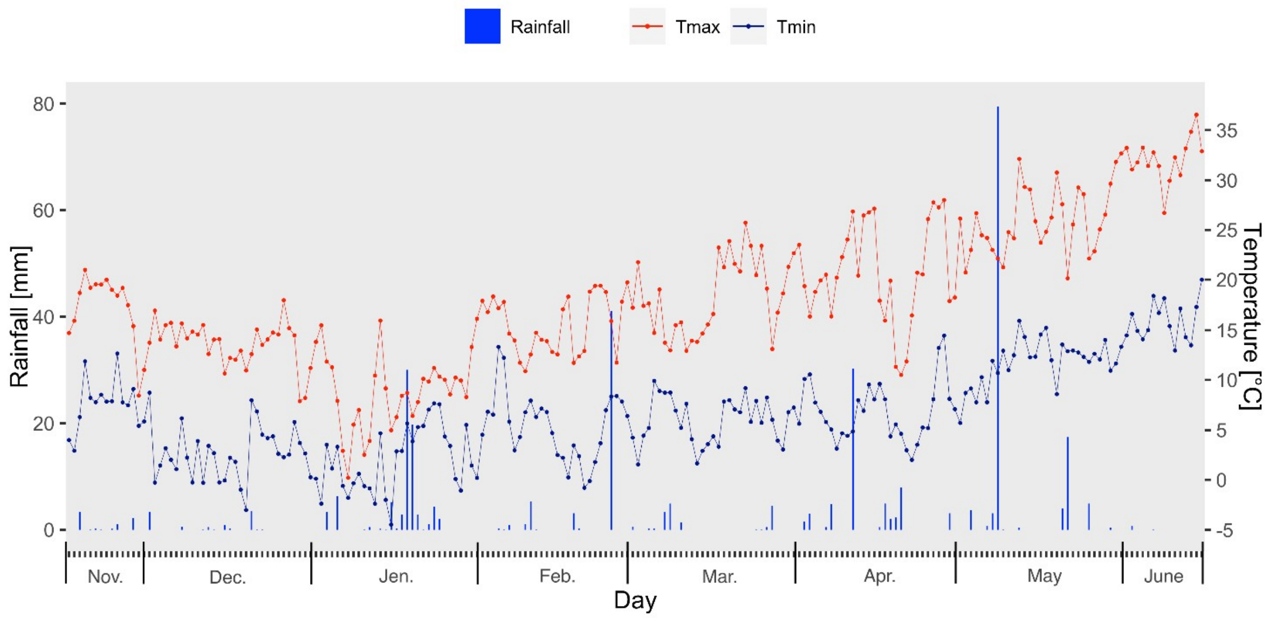
DF: degree of freedom; GY: grain yield; TKW: thousand kernel weight; GPC: grain protein content; GI: gluten index. ** P ≤ 0.01; * P ≤ 0.05; n.s. not significant.

Table S2. Mean square of effects (year, Y; genotype, G; nitrogen level, N) resulting from analysis of variance (ANOVA) performed on sonicated protein fraction (F1*) and SDS-soluble protein fraction (F1-F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (UPP).

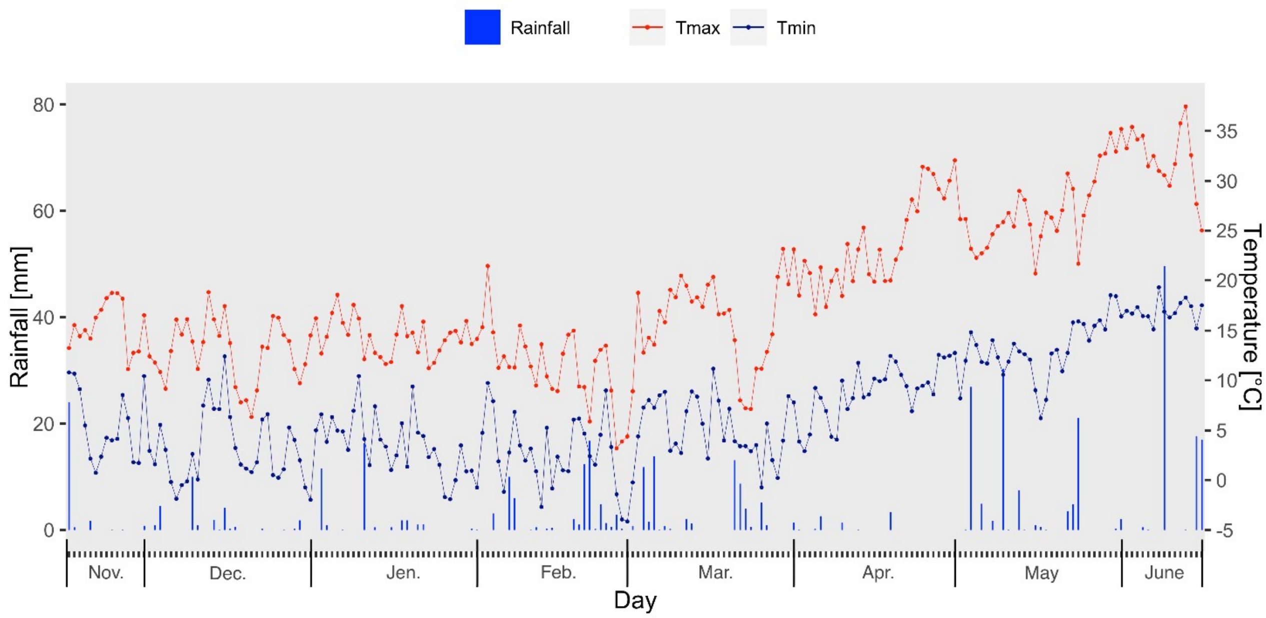
Factors	F1*	F1	F2	F3	F4	F1*+F1	F1*+F1+F2	F3+F4	mon/pol	UPP
Year (Y)	16.02**	144.05**	5.44**	1.21n.s.	130.30**	63.75**	106.39**	106.39**	0.21**	279.78**
Genotype (G)	46.89**	0.96n.s.	19.71**	12.13**	99.14**	47.26**	127.15**	127.15**	0.23**	175.93**
Nitrogen (N)	4.87**	15.14**	0.13n.s.	5.15n.s.	0.29n.s.	5.45*	5.03*	5.03*	0.013*	61.28**
YxG	50.20**	12.38**	1.99**	2.51n.s.	7.97**	28.86**	16.12*	16.12**	0.032**	230.54**
YxN	16.72**	13.04**	3.00**	10.30*	6.80**	45.06**	28.33**	28.33**	0.049**	43.34**
GxN	11.48**	2.90**	0.77**	5.8*	2.67**	8.84**	9.04**	9.04**	0.36**	58.00**
YxGxN	15.74**	4.12**	0.72**	7.09**	1.81n.s.	8.15**	6.39**	6.39**	0.009**	72.91**

** P ≤ 0.01; * P ≤ 0.05; n.s. not significant.

Figure

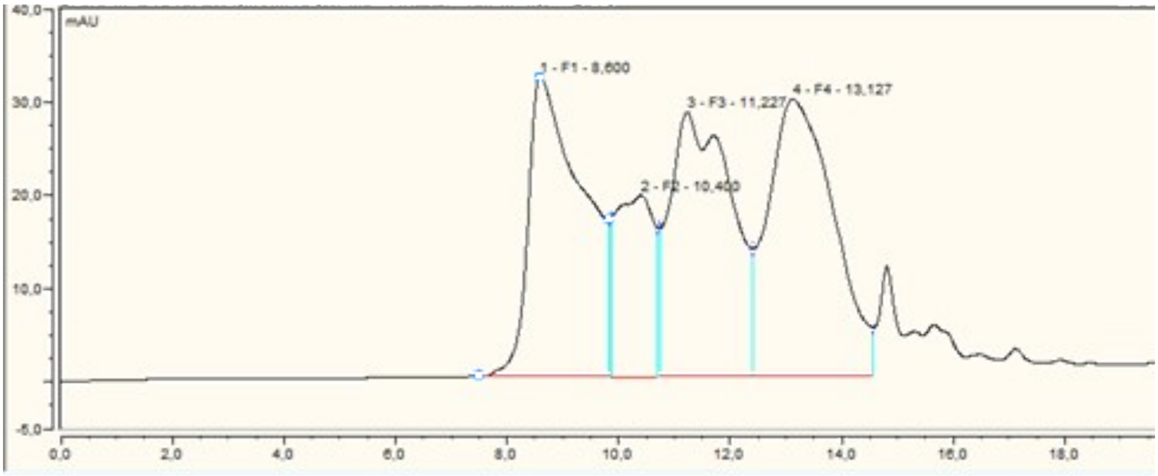


(a)

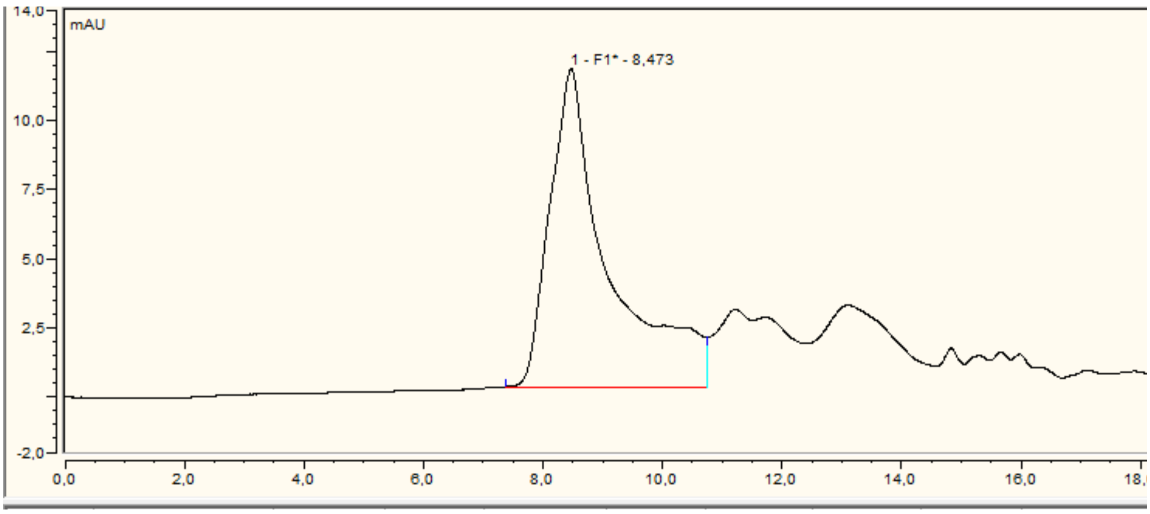


(b)

Figure 1S. Rainfall distribution and maximum and minimum mean temperatures for the two growing seasons 2017 (a) and 2018 (b).



(a)



(b)

Figure S2. SE-HPLC chromatograms of SDS-extractable protein fraction (a) and of SDS-unextractable protein fraction (b).

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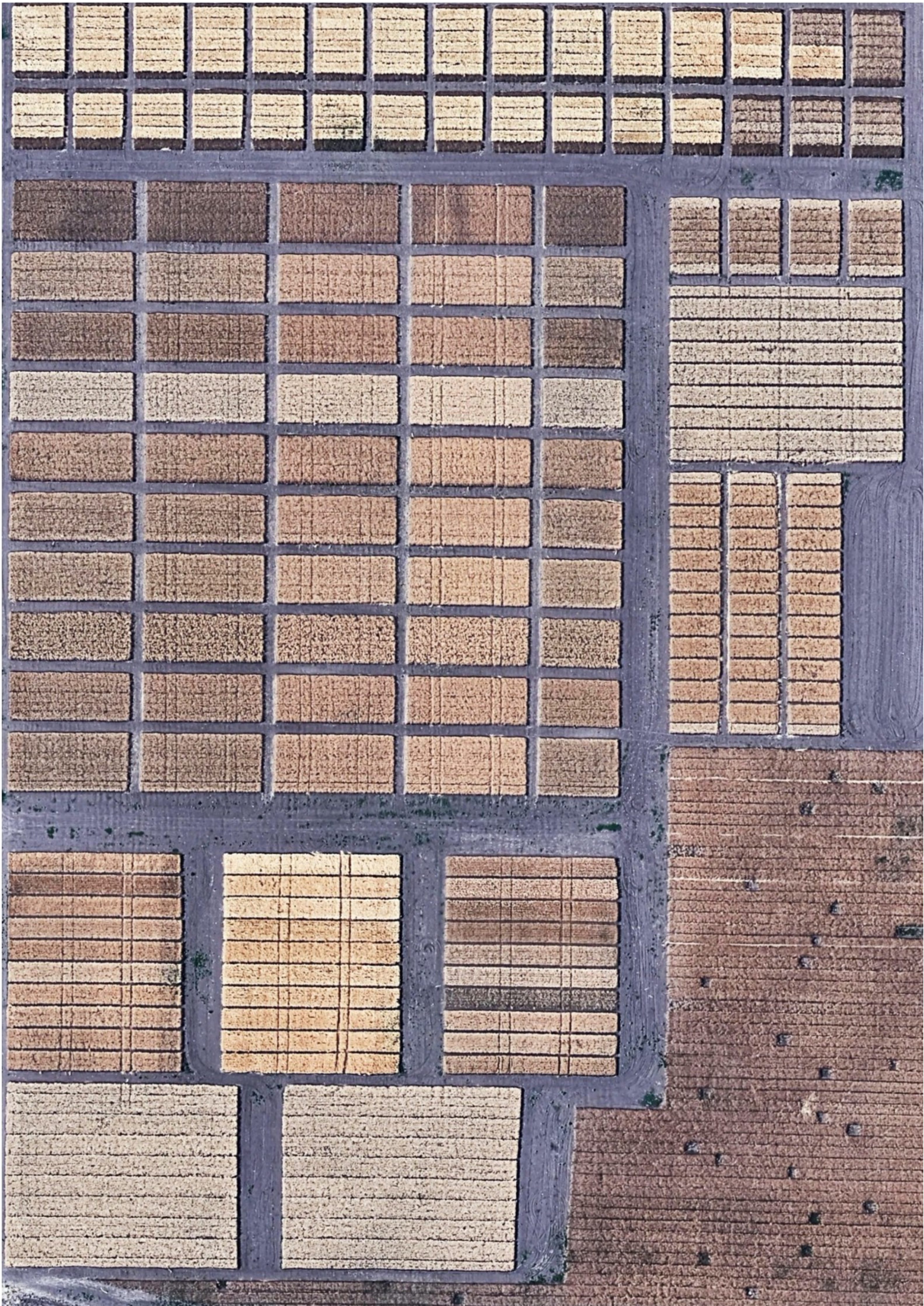
V. Foliar Application Strategies to Improve Grain Yield and N-Related Traits in Old and Modern Durum Wheat Varieties Grown under Organic Management in Mediterranean Area



ABSTRACT

Organic farming systems are often characterized by limited nitrogen (N) availability. Fertility management is a crucial factor in limiting yield and grain protein content that often characterizes the organic wheat systems. The present study aimed to evaluate foliar N, sulphur (S), and selenium (Se) application effect on durum wheat N uptake and utilization efficiency, grain yield, and protein, in a Mediterranean organic system. Experimental field trials were conducted during two consecutive growing-seasons on four Italian durum wheat varieties, including two old (Old Saragolla and Cappelli) and two moderns (Marco Aurelio and Nadif). Four organic fertilization managements were evaluated, including a control (CTR) fertilized with dry blood meal at seeding and the application of foliar N (CTR+N) and S (CTR+S) also combined (CTR+NS). Also, the selenium (Se) application effect was evaluated by comparing (i) Se0, control without selenium, and (ii) Se60, with one foliar application of sodium selenate. The modern variety Marco Aurelio showed the highest N utilization efficiency (NUtE) and grain yield, also in the drier year. Soil N fertilization with blood meal at seeding was the primary determining factor for the parameters considered. S and N had a positive and synergic effect, especially under drought conditions, on pre-anthesis N uptake, N translocation, NUtE, and grain yield, only when both were applied as foliar fertilizer (CTR+NS). Se treatment had a positive effect on post-anthesis N uptake and NUtE, determining a yield increase of 17% for the old variety Cappelli mainly attributed to post-anthesis N uptake increase, and of 13% and 14% for the modern varieties Marco Aurelio and Nadif mainly attributed to NUtE increase. This study provided evidence that by exploiting the synergistic effect of multiple nutrients in foliar applications, it was possible

to positively influence organic durum wheat response and improve production standards even in dry year conditions.

KEYWORDS

Durum wheat, Organic farming, Nitrogen, Sulphur, Selenium, Foliar application, NUtE, N uptake

INTRODUCTION

Organic farming systems are often characterized by limited nitrogen (N) availability that depends on the soil fertility and the organic N sources (Dawson et al., 2008). The N fertilization management in organic wheat cultivation is usually based only on the application of exogenous organic matter before sowing (Petersen et al., 2013; Mazzoncini et al., 2015); however, plants uptake N mainly under the inorganic form, and thus soil organic matter's mineralization rate and timing are essential for organic systems. As the organic N accumulated in the soil undergoes complex and dynamic transformations, there was contrasting information in the literature relative to the soil N availability in Mediterranean regions. Indeed, Fagnano et al. (2012) reported that the low soil organic matter content, low temperatures, and high autumn–spring rainfall can cause deficient levels of available mineral N in the soil during most of the wheat crop cycle. On the contrary, Rossini et al. (2018) highlighted that soil temperature and water availability during the winter season in the Mediterranean area can sustain the soil microorganisms' mineralization capacity. Although it is well-known that splitting mineral N application in conventional agriculture increases fertilization efficiency (Blandino et al., 2015; Fuertes-Mendizábal et al., 2010; Carucci et al., 2020), topdressing or foliar fertilization is not commonly used in the organic farming system. Fertility management is a crucial factor in limiting yield and grain protein content that often characterizes the organic wheat systems (Mason and Spaner, 2006).

Consequently, the evaluation of the nitrogen use efficiency (NUE) traits could be more attractive for low input and organic systems than the conventional method, even if more complicated. Indeed, the complexity of the N cycle linked to the organic sources and the limited availability of mineral N make the measurement of NUE in organic systems complicated and sometimes misleading (Dawson et al., 2008). For this reason, in the organic system, it could be more suitable to evaluate the N utilization efficiency (NUtE), which is an NUE component (Moll et al., 1982). In some conventional studies, higher NUtE was correlated with a higher yield, with modern varieties having higher grain yield and NUtE due to a more significant partitioning of dry matter and N to grain (Singh and Arora, 2001; Carucci et al., 2020). On the other hand, Foulkes et al. (1998) suggested that old varieties may be more suitable for organic growing systems being more efficient in recovery N from the soil than modern varieties. Thus, the choice of wheat variety is an essential management practice in the organic system.

Sulfur (S) is another critical factor in durum wheat nutrition. Since S is an essential constituent of enzymes involved in N metabolism, as nitrate reductase and nitrite reductase (Mendel, 1997; Campbell et al., 1999; Swamy et al., 2005), its deficiency could lead to a decrease in N assimilation. Salvagiotti et al. (2009), studying different combinations of N and S fertilizer rates in bread-wheat grown under a conventional system, showed that S addition increased NUE mainly by increasing the N recovery from the soil. Although many studies have been conducted on the influence of N and S fertilizer on wheat yield and quality under the conventional system (Raming et al., 1975; Moss et al., 1981; Tea et al., 2004; Tea et al., 2007; Salvagiotti et al., 2007), a few information is available about the interaction of S fertilization with organic N source. Rossini et al. (2018) reported that S application to the soil might have a synergistic effect with organic N fertilization of durum wheat, determining higher yields and better quality. To the best of our knowledge, no information is available in the literature about the foliar application of sulfur in the durum wheat organic system.

Besides macronutrient as N and S, in the Commission Regulation (EC) No 889/2008, also trace microelements as boron (B), cobalt (C), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn) may be used in organic production and only to the extent necessary. Selenium is not included in this list, although its action on plants has been studied for almost 70 years (Lyons et al., 2009), and several studies have shown that adequate concentrations of Se can determine the increasing plant productivity (Li and

Gao, 2009; Nawaz et al., 2014; Ducsay et al., 2016, Lara et al., 2019). Also for wheat was demonstrated a higher yield with Se application (Ducsay et al., 2016). Moreover, Se improves plant tolerance to drought stress (Yao et al., 2009) and may also protect plants from fungal infection and invertebrate phloem-feeders (Hanson et al., 2004). For these reasons, the use of Se in the organic system could be relevant. Furthermore, it has been found that foliar Se application in the form of selenate as an alternative to soil distribution minimizes the impact on soil microbiology, improving the effectiveness even at a low application rate (Kapolna et al., 2012).

The present study aimed to evaluate the effect of different organic fertilization strategies based on foliar application of N, S, and Se on yield, grain protein, and N-related traits in 4 different durum wheat varieties grown under the organic system in the Mediterranean area. Although numerous scientific studies have independently discussed the effect of N and S fertilization on wheat, there is a lack of key information on the potential synergistic effects of these fertilizers in organic systems. Moreover, the study of the effect of Se in the durum wheat organic system, never done before, can give essential information for its use and integration in the European Organic Production Regulation.

MATERIALS AND METHODS

Field Trials

Experimental field trials were conducted during two consecutive growing seasons, 2017-2018 and 2018-2019 (namely 2018 and 2019, respectively), in a field managed according to the criteria of organic farming at Research Centre for Cereal and Industrial Crops (CREA-CI) in Foggia, Southern Italy (41°46′ N, 16°54′ E). Four Italian durum wheat (*Triticum turgidum* spp. *durum*) varieties, including two old (Old Saragolla, landrace, released in 1900 and Cappelli released in 1915, De Santis et al., 2017) and two moderns (Marco Aurelio and Nadif, released in 2010 and 2016, respectively) were grown on a clay soil (United States Department of Agriculture Classification, Washington, DC, USA) with the following main chemical characteristics (in 2018 and 2019, respectively): organic matter (Walkley-Black method) 2.5 and 2.6%; available phosphorus (Olsen method) 62.0 and 68.0 mg kg^{-1}; exchangeable potassium (ammonuim acetate method) 422 and 450 mg kg^{-1}; total nitrogen (Dumas method) 1.3 and 1.1 ‰. The fields chosen for experimental trials were homogeneous and without preceding crop (set-aside). The sowing date was on 1st December and 24th November 2018 and 2019, respectively, with a seeding rate of 350 germinable seeds per square meter.

Four organic fertilization managements were evaluated (Table S1; Supplementary material): 1) control (CTR) fertilized with dry blood meal in a single application at seeding; 2) CTR plus foliar S application at flag leaf sheath opening stage (CTR+S); 3) CTR plus N foliar application at the beginning of heading (CTR+N); 4) CRT plus the combination of N and S foliar applications at flag leaf sheath opening stage and at the beginning of the heading, respectively (CRT+NS). The selenium (Se) application effect was evaluated by comparing (i) Se0, control without selenium, and (ii) Se60, with one foliar application of sodium selenate (Na_2SeO_4; BioXtra), at the rate of 60 g ha^{-1} (De Vita et al., 2017) at the booting stage (BBCH stage 41) (Lancashire et al., 1991).

The foliar fertilizer applications were made using a hand-held knapsack sprayer. All agricultural practices applied to the durum wheat crop during the two years were performed according to the organic agronomic technique commonly adopted by local farmers, following the Council Regulation (EC) No 834/2007 (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32007R0834).

In each year, the experiment was set up in a split-split plot design with three replicates: the variety was the main plot, the organic fertilization management was the plot, and the selenium application was the sub-plot (10.2 square meters).

Durum wheat grain was machine-harvested at full maturity on 29 and 18 June in 2018 and 2019. During the experimental period, the daily rainfall and temperature were recorded by a weather station near the experimental area. Soil samples for the analysis of mineral nitrogen content (Nmin) were taken at a depth of 0.3 m at sowing (before the distribution of organic fertilizer) and at harvest. From each sample, a subsample of 30 g fresh weight was taken to obtain an extract in 1 M KCl (soil/solution ration 1:2). Soil extracts were filtered and analyzed for nitrate and ammonium following Keeney and Nelson (1982). The Nmin was determined as the sum of NO_3^- and NH_4^+ content.

Grain yield, grain protein content, N translocation and N utilization efficiency

At harvest time, the grain yield was assessed for each plot. Grain protein content was also determined by near-infrared reflectance spectroscopy, using an Infratec 1229 grain analyzer (Foss Tecator, Hillerød, Denmark).

In each experimental year, at anthesis (BBCH stage 69) and physiological maturity (BBCH stage 87) (Lancashire et al., 1991), plant samples were taken from 0.5 linear meters of two adjacent rows, cutting-off the shoots at the crown level; at physiological maturity, plant samples were separated into straw and grain. Dry matter was determined by drying the samples in an oven at 65 °C until a constant weight was reached. Subsequently, all samples were ground using a Cyclotec Sample Mill 1093 (Foss Tecator, Hillerød, Denmark). The nitrogen concentration of all samples was determined in triplicate using Leco CHNS 628 Analyzer (Leco corporation, St. Joseph, Michigan). N content was calculated by multiplying the dry weight by the concentration of N of each sample; the plant's N content at physiological maturity was the sum of the N content of the straw and grain.

The N translocation during grain filling was calculated following Giuliani et al. (2011) as a difference between plant N content at anthesis and straw N content at maturity. For this estimate, it was assumed that N was totally translocated from the vegetative part to the developing grain since losses of N due to volatilization during grain filling were not determined (Arduini et al., 2006; Masoni et al., 2007). Plant N content at anthesis was considered a measure of the pre-anthesis N uptake, while post-anthesis N uptake was estimated as a difference between plant N content at maturity and plant N content at anthesis (Bogard et al., 2010). Finally, nitrogen utilization efficiency (NUtE) was calculated as grain yield/plant N content at maturity, and nitrogen harvest index (NHI) as grain N content/ plant N content at maturity (Kubota et al., 2019).

Statistical Analysis

The dataset was tested according to the basic assumptions of analysis of variance (ANOVA). The normal distribution of the experimental error and the common variance of the experimental error were verified through Shapiro–Wilk and Levene's tests, respectively. When required, Box–Cox transformations (Box and Cox, 1964) were applied before analysis. The ANOVA model was performed according to a split–split-plot design with three replicates considering year, variety, organic fertilization management, Se application, and their interactions. All the factors were considered fixed, while the replicates as random. The statistical significance of the difference among the means was determined using Tukey's honest significance post hoc test at the 5% probability level. ANOVA analysis was performed using the "nlme package" of the "R" statistical software, version 3.6.3 (R Core Team, 2018).

RESULTS

Weather conditions

According to the Köppen classification, the study site's climate is the Mediterranean, and the De Martonne aridity index indicates that it is characterized by a semiarid climate. Rainfall is concentrated between autumn and winter, and the mean annual rainfall value is about 450 mm. The annual mean wet days value ranges from 60 to 95 days, while the annual mean rainfall intensity value ranges from 7 to 11.5 mm day^{-1}. The mean annual temperature values range from 12 to 17 °C (Polemio and Lonigro, 2015).

Total rainfall in 2018 and 2019 was 401 mm and 299 mm (Figure 1), respectively. The average rainfall recorded in both growing seasons was below the long-term average, especially for the second year. During the two growing seasons, rainfall was well distributed, even if in 2019, during the grain filling period (from the end of April to the middle of June), there was 104 mm less rainfall than in 2018.

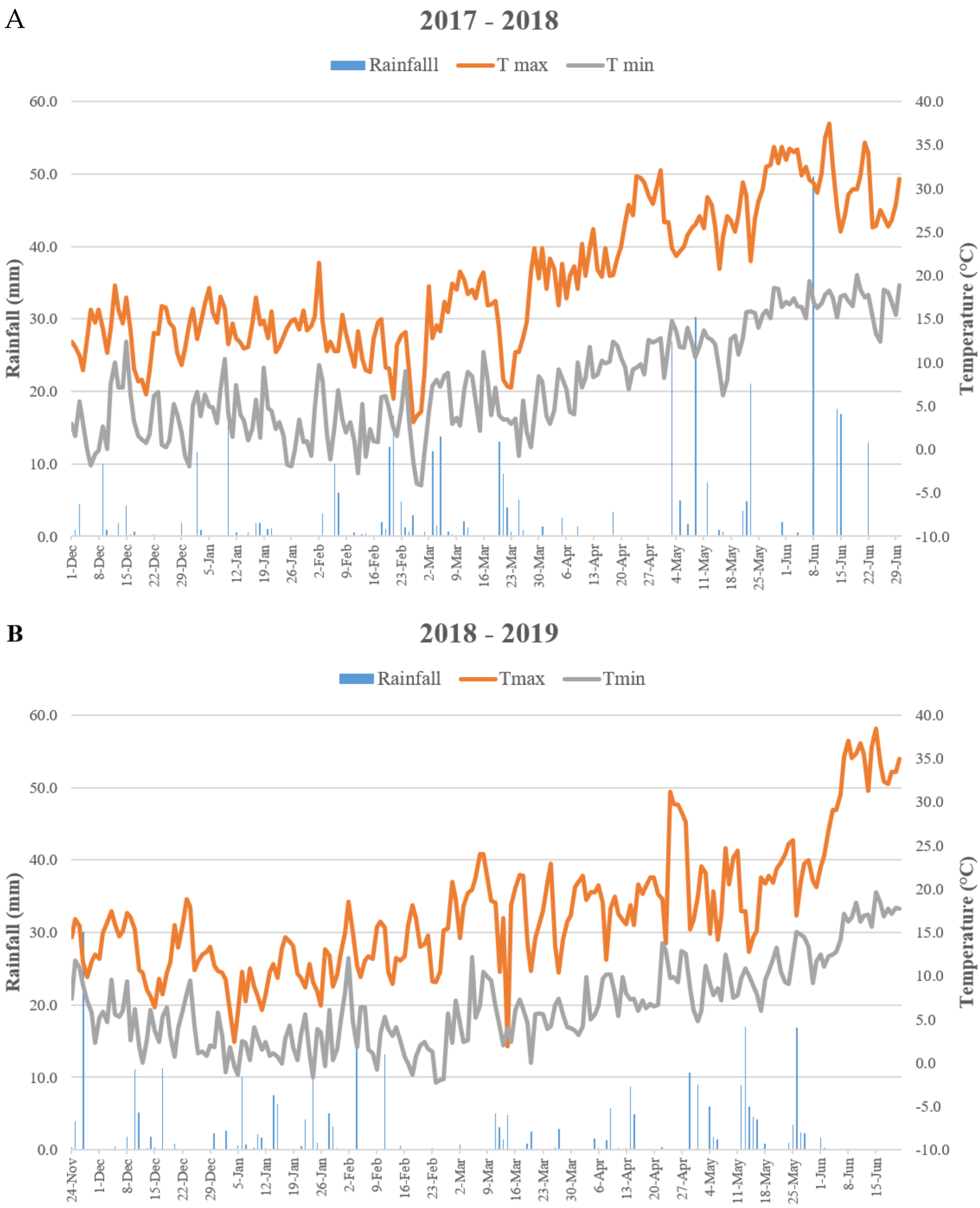


Figure 1. Daily rainfall and maximum and minimum temperatures for the two study-years 2018 (A) and 2019 (B).

During the two years, the mean temperatures were also different. The first year was characterized by a higher number of days with a maximum temperature between 30 °C and 35 °C (23 days in 2018 and 6 days in 2019) during the grain filling period, whereas in the 2019 more days with a temperature upper to 35 °C were recorded (3 vs 8 in the first and second year, respectively).

Variety effects on N-related traits, grain yield, and grain protein content

Analysis of variance for N-related traits, grain yield, and grain protein content revealed significant differences for most of the variables considered (Table S2; Supplementary material).

Cappelli was the variety with the highest pre-anthesis N uptake, N translocation, grain protein content, and the lowest NUtE and grain yield. On the contrary, the landrace Old Saragolla showed the lowest values for pre and post-anthesis N uptake, N translocation, and N grain content. Marco Aurelio showed the highest post-anthesis N uptake, NUtE, and grain yield values. Finally, Nadif showed the highest NHI and N grain content and the lowest grain protein content values (Table 1). The varieties' behavior changed with the growing season (Figure 2). Cappelli and Marco Aurelio showed values significantly lower of pre-anthesis N uptake in the second year, while Old Saragolla and Nadif did not show significant differences between the two years. On the contrary, in the second year, Cappelli showed a significant increase in post-anthesis N uptake, while the two modern varieties showed an evident decrease. N translocation, NHI, and N grain content decreased in the second year for all varieties excepted for Old Saragolla. The variation of NUtE between the two years was evident only for Cappelli and Marco Aurelio, which showed an opposite trend: the old variety Cappelli showed NUtE decrease in the second year and the modern variety Marco Aurelio an increase. All the varieties showed a decrease in grain yield and grain protein content in the second drier year.

Table 1. Effect of year, variety, fertilization strategies and selenium application on pre-anthesis N uptake (kg ha^{-1}), post-anthesis N uptake (kg ha^{-1}), N translocation (kg ha^{-1}), nitrogen utilization efficiency (NUtE; kg kg^{-1}), nitrogen harvest index (NHI; kg kg^{-1}), N grain content (kg ha^{-1}), grain yield (t ha^{-1}) and grain protein content (%).

Experimental Factor	Pre-anthesis N uptake	Post-anthesis N uptake	N translocation	NUtE	NHI	N grain content	Grain yield	Grain protein content
Year								
2018	82.5±2.39^{a}	19.3±1.5^{a}	39.3±1.9^{a}	30.3±0.7^{a}	0.573±0.007^{a}	58.6±1.7^{a}	2.98±0.06^{a}	12.9±0.05^{a}
2019	69.4±2.16^{b}	15.0±1.5^{b}	28.3±1.4^{b}	29.8±1.0^{a}	0.512±0.007^{b}	43.3±1.4^{b}	2.36±0.06^{b}	10.7±0.08^{b}
Variety								
Cappelli	89.0±4.2^{a}	16.1±2.8^{ab}	37.8±2.9^{a}	22.1±0.9^{c}	0.51±0.01^{c}	53.9±2.7^{a}	2.21±0.08^{d}	12.5±0.1^{a}
Old Saragolla	65.4±2.43^{d}	15.7±1.4^{b}	25.4±1.7^{c}	30.8±1.0^{b}	0.51±0.01^{c}	41.1±1.4^{b}	2.42±0.06^{c}	12.0±0.2^{b}
Marco Aurelio	76.9±2.40^{b}	18.8±2.1^{a}	34.5±1.9^{b}	36.2±1.1^{a}	0.56±0.01^{b}	53.3±2.4^{a}	3.33±0.07^{a}	11.5±0.2^{c}
Nadif	72.5±3.14^{c}	18.1±2.2^{ab}	37.5±2.8^{a}	31.0±1.1^{b}	0.61±0.01^{a}	55.6±2.7^{a}	2.71±0.08^{b}	11.3±0.1^{d}
Fertilization strategies								
CTR	81.0±3.51^{a}	14.2±1.3^{b}	36.6±2.4^{a}	30.0±1.5^{a}	0.534±0.012^{b}	50.8±2.0^{ab}	2.71±0.10^{a}	11.9±0.2^{a}
CTR+N	72.8±3.65^{b}	20.9±2.8^{a}	31.7±2.7^{b}	29.5±1.4^{a}	0.559±0.011^{a}	52.6±2.6^{a}	2.58±0.09^{b}	11.9±0.2^{a}
CTR+S	71.2±3.54^{b}	19.7±2.2^{a}	30.7±2.8^{b}	30.4±1.1^{a}	0.540±0.012^{b}	50.4±3.3^{b}	2.65±0.10^{ab}	11.6±0.1^{c}
CTR+NS	78.8±2.44^{a}	13.9±2.0^{b}	36.3±1.9^{a}	30.2±1.1^{a}	0.538±0.012^{b}	50.0±1.7^{b}	2.74±0.09^{a}	11.8±0.1^{b}
Selenium application								
Se_0	79.3±2.3^{a}	14.1±1.0^{b}	36.1±1.7^{a}	28.2±0.8^{b}	0.532±0.008^{b}	50.2±1.7^{b}	2.52±0.05^{b}	11.9±0.1^{a}
Se_{60}	72.6±2.4^{b}	20.2±1.9^{a}	31.5±1.8^{b}	31.8±0.9^{a}	0.553±0.008^{a}	51.7±1.8^{a}	2.82±0.08^{a}	11.7±0.1^{b}

CTR: control fertilized with dry blood meal at seeding; CTR+N; control plus N foliar application at the beginning of heading stage (BBCH stage 51); CTR+S; control plus S foliar application at flag leaf sheath opening stage (BBCH stage 47); CTR+NS: control plus the combination of S and N foliar applications at flag leaf sheath opening stage and at the beginning of heading (BBCH stage 47 and 51), respectively (for more details see Table S1, supplementary material). Se0, no selenium application; Se60, selenium application. For each experimental factor, values in a column followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test. Data are reported as means±standard errors.

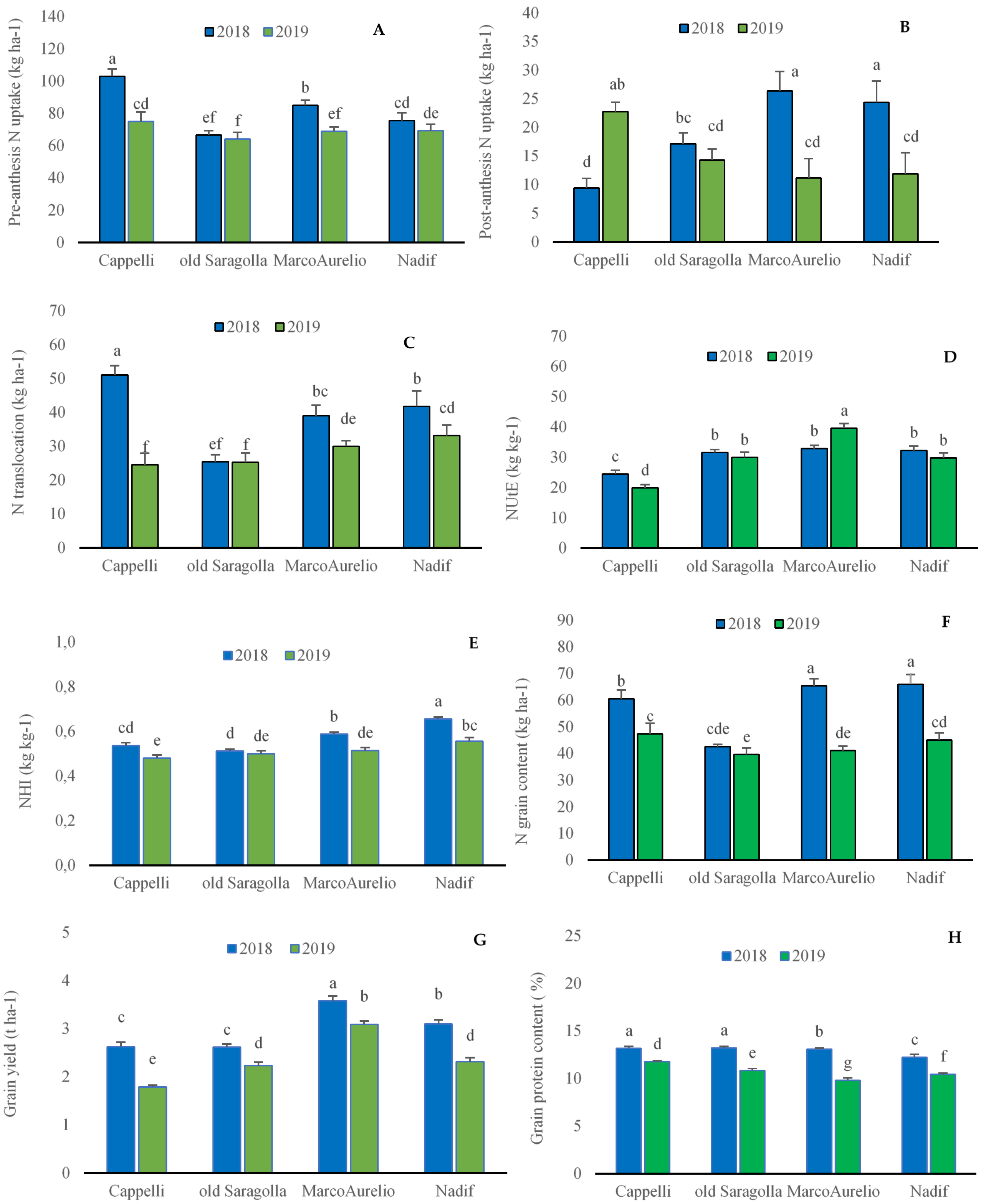


Figure 2. Effect of the interaction year x variety on pre-anthesis N uptake (A), post-anthesis N uptake (B), N translocation (C), nitrogen utilization efficiency (NUtE; D), nitrogen harvest index (NHI; E), N grain content (F), grain yield (G) and grain protein content (H). Values followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test. Vertical bars indicate standard errors (n=24).

Fertilization strategies effects on N-related traits, grain yield, and grain protein content

Regarding the fertilization strategies (Table 1), the control condition (CTR), fertilized with dry blood meal in a single application at seeding, and the CTR+NS fertilization showed higher pre-anthesis N uptake, N translocation, and grain yield values. On the contrary, post-anthesis N uptake was higher under CTR+N and CTR+S fertilization. CTR+N also showed the highest NHI and N grain content values but lower grain yield. Grain protein content values were higher under CTR and CTR+N fertilization treatment, while no significant differences among the fertilization treatments were observed for NUtE. The fertilization strategies investigated differently influenced N parameters, grain yield, and grain protein content in relation to the growing seasons (Table 2). In the second drier year, only the CTR+NS treatment caused an increase of pre-anthesis N uptake and N translocation, more evident for pre-anthesis N uptake. On the contrary, CTR+NS caused a decrease of post-anthesis N uptake in the second year, which increased only under CTR+N fertilization. For NUtE, significant differences between the two years were observed only under CTR+N and CTR+S fertilization with an opposite trend: indeed, CTR+N caused a NUtE decrease in the second year and CTR+S a significant increase. Finally, NHI, N grain content, grain yield, and grain protein content showed lower values in the second year under all the fertilization strategies adopted. For the grain yield, it is important to note that while in the first year no significant differences were observed among the fertilization treatments, in the second year the best result was obtained under CTR+NS. Further N fertilization (CTR+N) with respect to the CTR positively influenced more the old varieties than the modern ones (Table 3). In particular, organic N foliar application caused an increase of pre-anthesis N uptake, N translocation, N grain content, grain yield for Old Saragolla, and post-anthesis N uptake, NHI, and N grain content for Cappelli. The positive effect of the CTR+N fertilization for the two modern varieties was only on NHI for Marco Aurelio and grain protein content for Nadif. On the contrary, CTR+N caused a decrease in pre-anthesis N uptake

Table 2. Effect of interaction year x fertilization strategies on pre-anthesis N uptake (kg ha^{-1}), post-anthesis N uptake (kg ha^{-1}), N translocation (kg ha^{-1}), nitrogen utilization efficiency (NUtE; kg kg^{-1}), nitrogen harvest index (NHI; kg kg^{-1}), N grain content (kg ha^{-1}), grain yield (t ha^{-1}) and grain protein content (%).

Experimental factor	**2018**				**2019**			
	CTR	**CTR+N**	**CTR+S**	**CTR+NS**	**CTR**	**CTR+N**	**CTR+S**	**CTR+NS**
Pre-anthesis N uptake	85.7±4.2^{ab}	78.8±6.6^{cd}	88.8±3.5^{a}	76.8±4.0^{cd}	76.3±5.5^{d}	66.8±2.8^{e}	53.6 ±3.4^{f}	80.8±2.0^{bc}
Post-anthesis N uptake	15.71±2.15^{e}	17.7±2.33^{b-e}	22.70±4.05^{abc}	21.27±3.39^{a-d}	12.75±1.33^{e}	24.16±4.97^{a}	16.75±1.75^{cde}	6.44±0.69^{f}
N translocation	41.0±0.7^{a}	38.9±4.0^{a}	42.2±4.1^{a}	35.2±3.5^{bc}	32.2±3.3^{c}	24.4±2.8^{d}	19.1±2.1^{e}	37.3±1.6^{ab}
NUtE	30.5±1.5^{bcd}	33.4±1.9^{abc}	27.3±0.7^{efg}	29.9±0.9^{b-f}	29.5±2.6^{c-f}	25.7±1.7^{fg}	33.5±1.8^{ab}	30.5±1.9^{b-e}
NHI	0.561±0.016^{bc}	0.591±0.009^{a}	0.568±0.019^{b}	0.573±0.014^{ab}	0.507±0.015^{de}	0.526±0.017^{cd}	0.512±0.011^{de}	0.504±0.018^{e}
N grain content	56.7±2.5^{b}	56.6±3.6^{b}	64.9±4.7^{a}	56.3±2.2^{b}	44.9±2.8^{d}	48.6±3.7^{c}	35.9±2.1^{e}	43.8±1.9^{d}
Grain yield	3.04±0.14^{a}	2.96±0.10^{a}	3.01±0.12^{a}	2.92±0.11^{a}	2.38±0.10^{c}	2.20±0.10^{d}	2.29±0.13^{cd}	2.56±0.12^{b}
Grain protein content	13.00±0.10^{a}	13.06±0.11^{a}	12.68±0.07^{b}	13.00±0.09^{a}	10.81±0.19^{c}	10.72±0.16^{d}	10.62±0.11^{e}	10.70±0.15^{d}

CTR: control fertilized with dry blood meal at seeding; CTR+N; control plus N foliar application at the beginning of heading stage (BBCH stage 51); CTR+S; control plus S foliar application at flag leaf sheath opening stage (BBCH stage 47); CTR+NS: control plus combination of S and N foliar applications at flag leaf sheath opening stage and at the beginning of heading (BBCH stage 47 and 51), respectively (for more details see Table S1, supplementary material). Se0, no selenium application; Se60, selenium application. Values in row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors (n=24).

Table 3. Effect of interaction variety x fertilization strategies on pre-anthesis N uptake (kg ha^{-1}), post-anthesis N uptake (kg ha^{-1}), N translocation (kg ha^{-1}), nitrogen utilization efficiency (NUtE; kg kg^{-1}), nitrogen harvest index (NHI; kg kg^{-1}), N grain content (kg ha^{-1}), grain yield (t ha^{-1}) and grain protein content (%).

	N uptake pre-anthesis	**N uptake post-anthesis**	**N translocation**	**NUtE**	**NHI**	**N grain content**	**Grain yield**	**Grain protein content**
CTR								
Cappelli	**106.3±6.40^{a}**	**9.3±1.34^{h}**	**45.7±3.39^{ab}**	**20.1±1.94^{h}**	**0.482±0.022^{h}**	**55.1±2.80^{de}**	**2.25±0.17^{e}**	**12.86±0.18^{a}**
Old Saragolla	**62.1±4.20^{h}**	**20.1±1.65^{cde}**	**20.3±2.18^{i}**	**28.1±0.57^{fg}**	**0.496±0.009^{fgh}**	**40.3±1.00^{ijk}**	**2.31±0.12^{e}**	**12.07±0.35^{e}**
M. Aurelio	**71.0±5.77^{fg}**	**19.6±2.92^{cde}**	**29.9±4.02^{fg}**	**39.3±2.92^{a}**	**0.527±0.020^{efg}**	**49.4±6.00^{efg}**	**3.36±0.17^{a}**	**11.48±0.48^{g}**
Nadif	**84.5±4.32^{cde}**	**8±1.76^{h}**	**50.5±3.11^{a}**	**32.5±2.51^{cdef}**	**0.632±0.010^{a}**	**58.5±3.09^{cd}**	**2.91±0.16^{bc}**	**11.20±0.30^{i}**
CTR+N								
Cappelli	**94.3±11.26^{b}**	**33.1±9.08^{a}**	**38.0±9.35^{cde}**	**18.7±1.76^{h}**	**0.539±0.031^{cde}**	**71.1±7.84^{a}**	**2.26±0.21^{e}**	**12.74±0.27^{b}**
Old Saragolla	**69.3±5.54^{g}**	**18.5±2.88^{c-f}**	**27.7±3.90^{fgh}**	**31.5±2.29^{defg}**	**0.530±0.011^{def}**	**46.3±2.40^{fgh}**	**2.65±0.08^{cd}**	**12.00±0.39^{e}**
M. Aurelio	**66.3±1.96^{gh}**	**14.2±2.77^{d-h}**	**32.2±2.13^{efg}**	**37.9±0.70^{ab}**	**0.577±0.013^{bc}**	**46.4±2.12^{fghi}**	**3.04±0.13^{b}**	**11.48±0.51^{g}**
Nadif	**61.2±2.69^{h}**	**18±3.51^{c-g}**	**28.7±2.60^{fgh}**	**30.0±2.34^{defg}**	**0.588±0.022^{b}**	**46.6±2.08^{fghi}**	**2.37±0.17^{de}**	**11.40±0.24^{gh}**
CTR+S								
Cappelli	**64.7±6.82^{gh}**	**10.9±2.88^{fgh}**	**26.9±4.88^{gh}**	**27.4±0.59^{g}**	**0.501±0.008^{fgh}**	**38.7±2.10^{jk}**	**2.08±0.13^{e}**	**11.96±0.19^{ef}**
Old Saragolla	**59.6±5.95^{h}**	**13.3±3.54^{d-h}**	**22.2±3.79^{hi}**	**34.2±2.70^{bcd}**	**0.490±0.013^{gh}**	**35.6±3.00^{k}**	**2.38±0.14^{de}**	**11.85±0.31^{f}**
M. Aurelio	**82.5±3.35^{de}**	**29.6±5.65^{ab}**	**34.1±3.88^{def}**	**31.6±1.47^{defg}**	**0.568±0.019^{bcd}**	**63.7±4.53^{bc}**	**3.46±0.14^{a}**	**11.50±0.48^{g}**
Nadif	**78.0±9.40^{ef}**	**30±3.12^{abc}**	**39.4±8.23^{bcd}**	**27.4±2.57^{g}**	**0.579±0.032^{bc}**	**69.4±9.23^{ab}**	**2.69±0.17^{c}**	**11.31±0.27^{hi}**
CTR+NS								
Cappelli	**90.6±2.42^{bc}**	**11±1.12^{fgh}**	**40.7±1.95^{bcd}**	**22.4±1.36^{h}**	**0.510±0.017^{efgh}**	**51.7±1.84^{efg}**	**2.25±0.11^{e}**	**12.33±0.22^{c}**
Old Saragolla	**70.6±3.05^{fg}**	**10.9±2.51^{gh}**	**31.2±3.12^{fg}**	**29.3±1.24^{efg}**	**0.508±0.023^{efgh}**	**42.1±3.25^{hij}**	**2.36±0.09^{e}**	**12.20±0.39^{d}**
M. Aurelio	**87.7±4.79^{bcd}**	**11.9±3.59^{e-h}**	**41.8±4.20^{bc}**	**36.1±2.15^{abc}**	**0.534±0.022^{def}**	**53.6±4.26^{de}**	**3.49±0.10^{a}**	**11.40±0.52^{gh}**
Nadif	**66.2±4.80^{gh}**	**21.2±6.50^{bcd}**	**31.5±4.78^{efg}**	**33.1±1.21^{cde}**	**0.604±0.028^{ab}**	**52.6±3.05^{ef}**	**2.86±0.09^{bc}**	**11.46±0.27^{g}**

CTR: control fertilized with dry blood meal at seeding; CTR+N; control plus N foliar application at the beginning of heading stage (BBCH stage 51); CTR+S; control plus S foliar application at flag leaf sheath opening stage (BBCH stage 47); CTR+NS: control plus the combination of S and N foliar applications at flag leaf sheath opening stage and at the beginning of heading (BBCH stage 47 and 51), respectively (for more details see Table S1, supplementary material). Se0, no selenium application; Se60, selenium application. Values in a row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors (n=12) and N translocation in Cappelli, NHI, N grain content, and grain yield in Nadif and of grain yield in Marco Aurelio.

Sulfur fertilization (CTR+S) had a positive effect on the modern varieties with respect to CTR, causing an increase of pre and post-anthesis N uptake and NHI for Marco Aurelio and post-anthesis N uptake for Nadif. Moreover, S fertilization also caused an increase of N grain content for the two modern varieties and a decrease for the two older ones; on the contrary, S decreased NUtE of the two modern varieties and increased that of the two older ones. Finally, CTR+S caused a decrease of pre-anthesis N uptake and N translocation for Cappelli and grain protein content for Old Saragolla.

Finally, the combination of N and S fertilization (CTR+NS) differently influenced the four varieties under study for the parameters considered without a clear trend between old and modern varieties.

Selenium effects on N-related traits, grain yield, and grain protein content

Selenium application caused an increase of post-anthesis N uptake, NUtE, NHI, N grain content, and grain yield values and the decrease of pre-anthesis N uptake, N translocation, and grain protein content (Table 1). Relative to the growing season, the Se treatment caused a decrease of pre-anthesis N uptake and N translocation in the second year, while it increased post-anthesis N uptake, NUtE, and NHI in both years. Finally, only in the first year, Se caused an increase of N grain content and grain yield and a decrease of grain protein content, while in the second year, not significant differences were observed (Table 4).

Table 4. Effect of interaction year x selenium application on pre-anthesis N uptake (kg ha^{-1}), post-anthesis N uptake (kg ha^{-1}), N translocation (kg ha^{-1}), nitrogen utilization efficiency (NUtE; kg kg^{-1}), nitrogen harvest index (NHI; kg kg^{-1}), N grain content (kg ha^{-1}), grain yield (t ha^{-1}) and grain protein content (%).

Experimental factor	2018		2019	
	Se_0	**Se_{60}**	**Se_0**	**Se_{60}**
Pre-anthesis N uptake	83.3±3.9^a	81.7±2.8^a	75.3±2.5^b	63.5±3.4^c
Post-anthesis N uptake	16.8±1.5^{bc}	21.9±2.6^a	11.5±1.0^c	18.6±2.7^{ab}
N translocation	40.2±2.7^a	38.5±2.6^a	32.1±1.9^b	24.4±2.0^c
NUtE	27.8±1.0^b	32.7±0.8^a	28.7±1.3^b	30.9±1.6^a
NHI	0.57±0.01^b	0.58±0.01^a	0.50±0.01^d	0.53±0.01^c
N grain content	57.0±2.6^b	60.3±2.2^a	43.6±1.8^c	43.0±2.3^c
Grain yield	2.6±0.06^b	3.3±0.07^a	2.4±0.08^c	2.3±0.09^c
Grain protein content	13.0±0.07^a	12.9±0.07^b	10.7±0.11^c	10.7±0.11^c

Se0, no selenium application; Se60, selenium application. Values in a row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors (n=48).

Se caused a decrease of pre-anthesis N uptake for all the varieties under study excepted for Cappelli, which was not affected by Se treatment; on the contrary, the Se treatment caused a significant increase of post-anthesis N uptake for all varieties excepted for Nadif, particularly evident for Cappelli and Marco Aurelio (Figure 3A). Moreover, Se treatment caused an N translocation decrease excepted than for Cappelli, and an increase of NUtE only for the modern varieties Marco Aurelio and Nadif (Figure 3B). Se caused a significant increase of N grain content only for Cappelli, while grain protein content slightly decreased for the two modern varieties without affecting the old ones (Figure 3C). NHI increased under Se treatment in Cappelli and Nadif and decreased in Marco Aurelio; no Se effect on Old Saragolla was evident. Grain yield increased under Se60 in all varieties excepted for Old Saragolla (Figure 4D). Under CTR+N, CTR+S, and CTR+NS the Se treatment caused a significant decrease of pre-anthesis N uptake and a significant increase of post-anthesis N uptake. Both CTR+S and CTR+NS combined with selenim treatment negatively affected N translocation, while CTR and CTR+NS together with the Se60 caused the increase of NUtE. NHI values increased under Se60 combined with CTR+N and with CTR+S. Moreover, Se positively interacted with CTR+N for N grain content and with CTR+N and CTR+NS, for grain yield. Finally, no combined effect of selenium and fertilization strategies was observed for the grain protein content excepted for CTR+NS that showed under Se60 a significant decrease (Table 5).

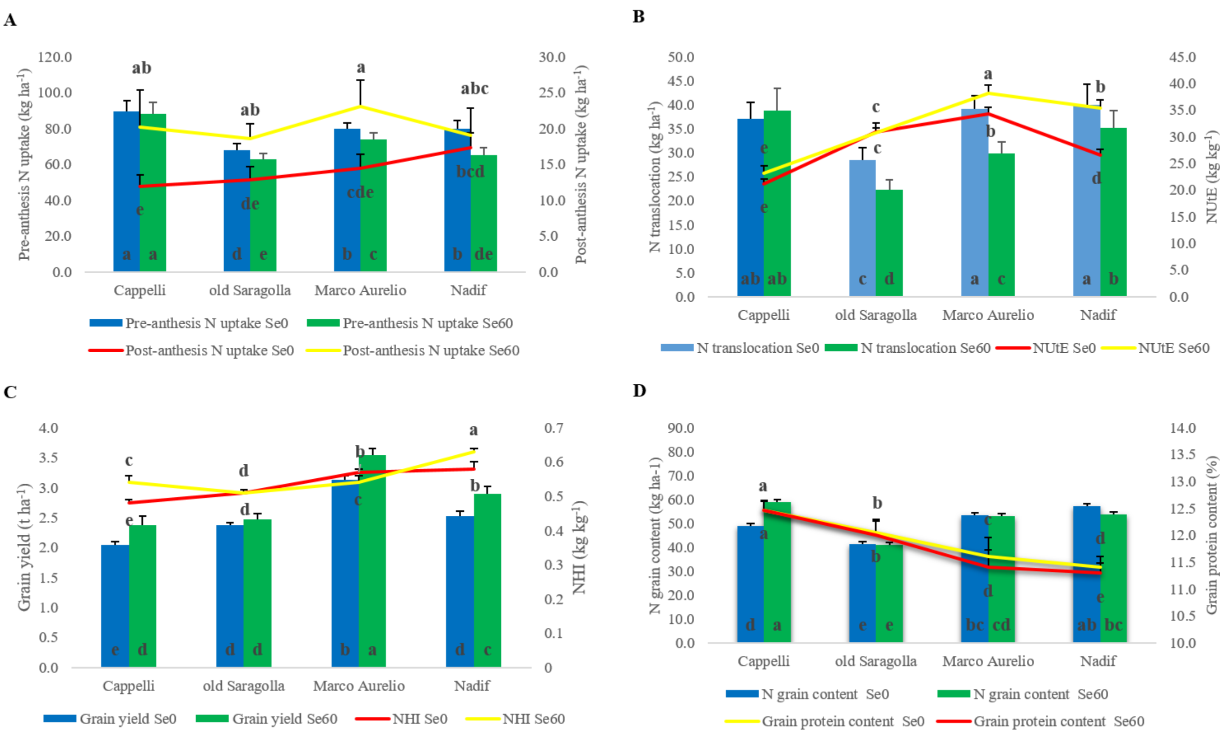


Figure 3. Effects of interaction variety x selenium application on pre and post-anthesis N uptake (A), on N translocation and NUtE (B), on N grain content and grain protein content (C) and on grain yield and NHI (D). Se0, no selenium application; Se60, selenium application. Values followed by different letters are significantly different at P ≤ 0.05 according to Tukey’s test. Vertical bars indicate standard errors (n=24).

Table 5. Effect of interaction fertilization x selenium application on pre-anthesis N uptake (kg ha^{-1}), post-anthesis N uptake (kg ha^{-1}), N translocation (NT; kg ha^{-1}), nitrogen utilization efficiency (NUtE; kg kg^{-1}), nitrogen harvest index (NHI; kg kg^{-1}), N grain content (NGC; kg ha^{-1}), grain yield (GY; t ha^{-1}) and grain protein content (GPC; %).

Experimental factor	**Se0**				**Se60**			
	CTR	**CTR+N**	**CTR+S**	**CTR+NS**	**CTR**	**CTR+N**	**CTR+S**	**CTR+NS**
Pre-anthesis N uptake	80.8±4.5^{b}	74.9±5.9^{c}	75.5±4.8^{c}	86.0±2.9^{a}	81.2±5.52^{b}	70.7±4.4^{de}	66.9±5.2^{e}	71.6±3.4^{cd}
Post-anthesis N uptake	14.35±1.7^{c}	15.42±2.1^{cd}	17.42±2.2^{c}	9.31±1.2^{d}	14.12±1.9^{c}	26.43±4.9^{a}	22.04±3.9^{ab}	18.41±3.7^{bc}
N translocation	36.0±3.2^{bc}	33.1±3.5^{cd}	33.0±4.5^{cd}	42.4±2.0^{a}	37.1±3.6^{b}	30.3±4.1^{de}	28.3±3.5^{e}	30.2±2.8^{de}
NUtE	26.5±1.4^{d}	28.4±1.8^{cd}	29.5±1.7^{bc}	28.5±1.6^{cd}	33.4±2.4^{a}	30.7±2.1^{bc}	31.4±1.2^{ab}	31.8±1.3^{ab}
NHI	0.53±0.01^{c}	0.53±0.02^{c}	0.51±0.02^{d}	0.54±0.02^{c}	0.54±0.02^{c}	0.58±0.01^{a}	0.56±0.01^{b}	0.54±0.02^{c}
N grain content	50.4±2.5^{b}	48.5±3.7^{b}	50.42±4.7^{b}	51.7±2.4^{b}	51.3±3.3^{b}	56.7±3.6^{a}	50.3±4.8^{b}	48.4±2.4^{b}
Grain yield	2.45±0.10^{ef}	2.37±0.09^{f}	2.59±0.11^{de}	2.66±0.11^{cd}	2.96±0.16^{a}	2.78±0.14^{bc}	2.71±0.18^{bcd}	2.83±0.13^{ab}
Grain protein content	11.92±0.27^{a}	11.88±0.29^{a}	11.69±0.24^{c}	11.93±0.27^{a}	11.89±0.28^{a}	11.90±0.27^{a}	11.62±0.22^{c}	11.76±0.27^{b}

CTR: control fertilized with dry blood meal at seeding; CTR+N; control plus N foliar application at the beginning of heading stage (BBCH stage 51); CTR+S; control plus S foliar application at flag leaf sheath opening stage (BBCH stage 47); CTR+NS: control plus the combination of S and N foliar applications at flag leaf sheath opening stage and at the beginning of heading (BBCH stage 47 and 51), respectively (for more details see Table S2, supplementary material). Se0, no selenium application; Se60, selenium application. Values in a row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors (n=24).

DISCUSSION

Nitrogen is one of the essential nutrients for plant growth, development, and reproduction as it plays an essential role in the processes of protein, nucleic acid, and chlorophyll synthesis and is a crucial factor in the photosynthesis cycle (Bloch et al., 2020). Although numerous studies were conducted to evaluate the effect of N and S fertilization on wheat performances in the past, fewer studies were conducted to evaluate the foliar application of organic fertilizers. In this study, we discussed the potential of the synergistic effect of N, S, and Se fertilizers on N-related traits, yield grain protein content of durum wheat varieties grown under organic management.

In the study period, the two growing seasons differed for the rainfall distribution and amount during the crop cycle, the second year being drier than the first one. This climatic trend caused a different soil N mineralized content (Nmin), evaluated as the sum of $NO3^-$ and $NH4^+$ content. In particular, both at seeding and harvest, Nmin was higher in the second drier year than in the first one (seeding: 62 and 70 kg ha^{-1} in 2018 and 2019, respectively; harvest: 47 and 62 kg ha^{-1} in 2018 and 2019, respectively), but the Nmin difference between the beginning and the end of the cycle was much lower in the second year (about 47% less than 2018), indicating lower N mineralization during the crop cycle and thus lower N available for the crop. This result agrees with the literature that reported minor net N mineralization in dry areas with respect to areas with higher rainfall and warmer temperatures (Dawson et al., 2008).

Variety effects on N-related traits, grain yield, and grain protein content

Since N is a limiting factor in organic cereal production, the use of varieties capable of utilizing N efficiently is of great importance in organic wheat cultivation (Dawson et al., 2008). In literature is often reported that the modern high-yield varieties are not suitable for the organic production (Murphy et al., 2007). To test this hypothesis, also under Mediterranean condition, we choose to evaluate one landrace (Old Saragolla), one old (Cappelli), and two modern durum wheat varieties (Marco Aurelio and Nadif). The four varieties under study differed for their ability to uptake N also in relation to the growing season. The highest pre-anthesis N uptake values observed for the old variety Cappelli was due to its well-developed root system, characterized by high length (Iannucci et al., 2021). The higher capacity attributed to older varieties and landraces to scavenge N deeper pools (Hawkesford, 2014) is an important aspect of organic agriculture management practice. In our study, this was true for the old variety Cappelli but not for the landrace Old Saragolla, which showed pre-anthesis N uptake lower than the two modern varieties considered. Due to the highest pre-anthesis N uptake, Cappelli also showed the highest N translocation value, since, in cereals, the amount of nitrogen available for translocation depends mainly on the amount of nitrogen stored in the plant's vegetative organs at flowering and, therefore, on N pre-anthesis uptake (Barbottin et al., 2005). Indeed, in our experimental condition, N translocation and pre-anthesis N uptake were highly and positively correlated ($r = 0.85$; $P \leq 0.001$). In the second drier year, all the varieties showed lower pre-anthesis N uptake values, probably due to the lower N mineralization detected in the soil with respect to 2018, also causing a decrease of N translocation.

In this study, the post-anthesis N uptake also considers the foliar N application at the beginning of the heading stage; for this parameter, the opposite trend with respect to pre-anthesis N uptake was observed in the first year, since the modern varieties showed higher values and Cappelli the lowest, with an intermediate behavior of the landrace Old Saragolla. These differences could be attributed to the high sink strength of the more productive modern varieties that may have enhanced the foliar N adsorption (Mi et al., 2000). However, when the condition of low rainfall during grain filling occurs, as in 2019, Cappelli showed a marked post-anthesis N uptake increase (more than double respect to 2018) while the two modern varieties showed an evident decrease. It seems like that, in the second year, Cappelli compensated the decrease of pre-anthesis N uptake via root system with an increase of the post-anthesis N uptake via foliar adsorption. After flowering and during grain filling, any further N uptake is likely to be allocated directly to the grain (Hawkesford, 2014). Indeed, we found that post-anthesis N uptake was positively correlated with N grain content ($r = 0.43$; $P \leq 0.001$) and negatively with N translocation ($r = -0.43$; $P \leq 0.001$), as also reported by

Bogard et al. (2010). For this reason, in the first wetter year, the modern varieties, which showed the highest post-anthesis N uptake, also showed the higher N grain content values. In contrast, in the second drier year, together with the decrease of post-anthesis N uptake, the modern varieties also showed an N grain content decrease (37% for Marco Aurelio and 32% for Nadif), more evident than for Cappelli (22%). The evaluation of nitrogen use efficiency (NUE) in the organic system is challenging and can also be inappropriate because the applied organic N fertilizer is not all available to the plant, and it is not the only source of available N (Dawson et al., 2008). For this reason, the evaluation of nitrogen utilization efficiency (NUtE) and the nitrogen harvest index (NHI) seems to be more useful in the organic management system (Dawson et al., 2008). NUtE reflects the plant's ability to transform the N uptake into the grain (Hawkesford, 2014), while NHI has significance for maximizing grain protein content for a given amount of plant N (Dawson et al., 2008). The higher NUtE and NHI values observed for the modern varieties and the lowest observed for old variety Cappelli, in both years, agrees with the progress in NUtE observed by Ortiz-Monasterio et al. (1997) among several cultivars released between 1950 and 1985 by the CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo). Elite European winter wheat germplasm has also demonstrated a trend toward improved NUtE and NHI over the last 25 years, under high N applied fertilizer level (Cormier et al. 2013). This study demonstrated that the better N utilization efficiency of the modern varieties is also evident under the organic system. In particular, the most interesting differences were observed between the modern Marco Aurelio and the old Cappelli. Under water stress conditions, NUtE decreased in Cappelli while increased in Marco Aurelio, demonstrating the latter's ability to efficiently use plant nitrogen to make yield even under stressful conditions. In fact, the reduction in yield in 2019 was only 14% for Marco Aurelio and 32% for Cappelli. Thus, Marco Aurelio cultivated under an organic system, even under water stress conditions, behaved very well and even better than the old variety Cappelli. This result was surprising since, in literature, it is reported that the modern varieties yielded more than the old varieties and landrace but with lower stability across the environments and the years (Migliorini et al., 2016). This distinct cultivar's behavior in response to climatic fluctuations is crucial for Mediterranean environments characterized by high year-to-year variability of rainfall and temperatures (Rossini et al., 2018) amplified by the ongoing climate change. The highest grain yield value showed by Marco Aurelio, which is a high-yield cultivar (Gagliardi et al., 2020), was not in agreement with the hypothesis that varieties that perform well under conventional management may not perform well in organic conditions (Murphy et al., 2007). Accordingly, Kubota et al. (2019), studying modern Canadian cultivars, reported that they performed relatively well in conventional and organic management systems for grain yield and NUtE.

Fertilization effects on N-related traits, grain yield, and grain protein content

Winter crops generally suffer significant yield reduction under organic conditions due to slow mineralization during their growth cycle. However, commercial organic fertilizers with a low C/N ratio may contain this reduction since they are quickly mineralized in soil, increasing nutrient availability (Sacco et al., 2015). In this study, the CTR treatment was represented by blood meal (Table S2) with a C/N ratio of 3.03 and can be considered a rich source of N and relatively fast (Ravindran et al., 2013; Tosti et al., 2016). Moreover, in our experimental conditions, the frequency and the amount of rainfall in the two crop seasons led us to assume the lack of leaching phenomena; on the contrary, the limited water availability during the second growing season caused a lower N mineralization and thus lower N availability for the crop (Dawson et al., 2008). This was confirmed by the lower pre-anthesis N uptake observed under CTR fertilization in the second drier year with respect to the first one. Since the literature reported that in wheat, using organic fertilization, N availability is limited during the grain filling period (Fagnano et al., 2012; Tosti et al., 2016), in this study, we also wanted to test the effect of organic foliar N application at heading stage on durum wheat. Thus, the pre-anthesis N uptake considers the N uptake by the roots, while the post-anthesis N uptake also considers the N absorbed by the leaf. Surprisingly, the organic N foliar fertilizer (CTR+N) addition caused a marked decrease of pre-anthesis N uptake with respect to the CTR. It seems like that the addition of foliar element at the beginning of the heading momentary stopped the root absorption processes. Moreover, in the first year, CTR+N caused a slight significant increase of NHI without effect on the other parameters considered, suggesting that in an organic system, soil N fertilization with blood meal is a major determining factor for the other parameters (Tea et al., 2007). On the contrary, under water-stressed conditions, leaf N application caused a significant increment of post-anthesis N uptake and N grain content. Also, Martre et al. (2006) showed that the N accumulation in the wheat grain is sink-regulated, especially under low N supply. In our organic condition, it can be assumed that the additional N, taken up after foliar N application at heading, was accumulated primarily in the grain but was not utilized efficiently for the protein synthesis because it did not cause a positive variation of grain protein content with respect to CTR. These results obtained under organic condition are opposite to those obtained in wheat grown under conventional systems in which, after foliar fertilization at anthesis stage, the efficient remobilization of N to the grain has been shown to increase grain protein content (Luo et al., 2000; Rawluk et al., 2000; Tea et al., 2007). Moreover, under the conventional system, applying fertilizer near to heading has been often shown to increase grain protein content without any reduction in grain yield (Bogard et al., 2010); this seems to be not true in the organic system as the addition of N foliar at heading (CTR+N) did not determine an increase both in grain protein content and in grain yield in the first wetter year, and even their decrease in the drier year.

Regarding the S foliar fertilization, a different effect on the parameters examined in relation to the growing season was observed. In the first wetter year, CTR+S increased post-anthesis N uptake and N grain content with respect to CTR, in agreement with Tea et al. (2007), who demonstrated that S applied by foliar spray was mainly assimilated in the grain favoring N accumulation. However, once again, this N accumulation was not efficiently used for protein synthesis nor for yield since both NUtE and grain protein content were lower under S fertilization, and yield was not affected. On the other hand, under the conventional system, sulfur's effect on wheat protein concentration and yield is controversial. Karamanos et al. (2013) reported the lack of consistent S fertilization effects on grain protein concentration, while Tea et al. (2007) reported that foliar S application significantly increased it. Relative to yield, in literature is often reported a positive effect of the S fertilization on wheat grain yield (Archer, 1974; Ramig et al., 1975; Withers et al., 1997; Zhao et al., 1999), while Grant et al. (2004) and Malhi et al. (2009) found that S fertilization did not increase wheat yield. Although several studies reported a positive effect of the S fertilization on plants under water stress conditions (Rausch & Wachter, 2005; Nazar et al., 2011; Chan et al., 2013), we found a decrease in pre-anthesis N uptake, N translocation, N grain content, and grain protein content in the second drier year under CTR+S. On the contrary, the foliar fertilization with N and S together (CTR+NS) caused an increase of pre-anthesis N uptake, N translocation, NUtE, and grain yield showing a positive and additive effect of N and S (Klikocka, et., 2016), under water stress condition. Thus, under our organic condition, S and N had a positive and synergic effect only when both of them were applied as foliar fertilizer at the end of the vegetative stage, while lack of interaction between foliar S and N derived by the mineralization of blood meal (CTR) was observed.

Selenium effects on N-related trait, grain yield, and grain protein content

Currently, in the Commission Regulation (EC) No 889/2008, microelements in organic cultivation are allowed. However, to the best of our knowledge, no studies are available on selenium's effect on durum wheat cultivated under organic management. Selenium is not an essential element for plants, but its beneficial effects on stress tolerance have been frequently reported (Terry et al. 2000, Germ et al. 2007). Se improves plant tolerance to drought stress by regulating water status (Yao et al., 2009), increasing chlorophyll in plant leaves (Dong et al., 2013), and may also protect plants from fungal infection and invertebrate phloem-feeders (Hanson et al., 2004). For these reasons, the use of Se in the organic system could be relevant. In this study, we

applied foliar Se as sodium selenate at the booting stage (De Vita et al., 2017). As also discussed for N and S foliar application, the foliar Se treatment always caused a decrease of pre-anthesis N uptake. Only the old variety Cappelli did not change its pre-anthesis N uptake in relation to the Se treatment, probably due to its well-developed root system, as discussed above (Iannucci et al., 2021). Due to the highly significant correlation between pre-anthesis N uptake and N translocation, the latter decreased under Se treatment, especially in the second drier year, except for Cappelli.
On the contrary, Se caused a significant increase of post-anthesis N uptake in both growing seasons and all varieties except for Nadif. The increase of post-anthesis N uptake under Se treatment was more evident when nitrogen was also added at heading as liquid blood meal and when both foliar N and S were added. Also, the literature reported the increase of the nitrogen absorption under Se treatment due to a positive influence of selenium on amino acids metabolism (Owusu-Sekyere et al., 2013; Guerrero et al., 2014; Hajiboland and Sadeghzade, 2014; Nawaz et al., 2014; Zhang et al., 2014; Bocchini et al., 2018; Yuan et al., 2018). With post-anthesis N uptake, Se increased NUtE under all the fertilization strategies applied, especially in modern varieties Marco Aurelio and Nadif. Under conventional management, several authors reported a positive effect of Se on N assimilation in wheat plants (Hajiboland and Sadeghzade, 2014; Ježek et al., 2011), probably due to the increase of the nitrate reductase activity (Hajiboland and Sadeghzade, 2014). Thus, these results obtained under the organic system are of relevant interest, since part of the mechanism for the slowing down N metabolism under low N availability is due to the reduction of nitrate reductase activity (Hajiboland and Sadeghzade, 2014). So, the use of Se in combination with modern varieties seems to improve this relevant aspect.
The Se together with foliar N application favored the N accumulation in the grain; however, this increase was not accompanied by an increase in grain protein content. On the contrary, the Se treatment caused a decrease of grain protein content in the first year and for the two modern varieties. Probably, the absorbed nitrogen was not efficiently converted in protein, according to Lara et al. (2019), who found N in

inorganic forms such as nitrate, nitrite, and ammonium in *Triticum aestivum* grain under Se treatment.

The effect of Se on wheat yield is still controversial. Several authors reported increases in wheat grain yield after selenate foliar applications (Li and Gao, 2009; Nawaz et al., 2014; Ducsay et al., 2016, Lara et al., 2019), while Grant et al. (2007), Broadley et al. (2010) and Tang et al. (2011) reported no significant effect of Se application on the yield in winter wheat. In our experimental condition, we observed a positive effect of Se treatment on grain yield in the first growing season characterized by higher rainfall, and particularly under CTR+N fertilization with a yield increase of 17%. Moreover, among the four varieties under study, the old one Cappelli showed a grain yield increase of 17%, mainly linked to post-anthesis N uptake, while modern varieties Marco Aurelio and Nadif showed a yield increase of 13% and 14%, respectively, probably due to their higher NUtE values. The landrace Old Saragolla grain yield was not affected by Se treatment.

CONCLUSIONS

In this study, we demonstrated the better N utilization efficiency of the modern varieties grown under the organic system and also under water stress conditions with respect to the old one. Marco Aurelio showed the highest ability to use efficiently plant nitrogen to make yield even under stressful conditions. Relative to the fertilization strategies, soil N fertilization with blood meal at seeding was the primary determining factor for the parameters considered since, in the organic system, the addition of N foliar at heading did not determine an increase in grain protein content nor in grain yield in the first year and even their decrease in the drier year.

Foliar application of S and N had a positive and synergic effect, on pre-anthesis N uptake, N translocation, NUtE, and grain yield, especially under drought conditions, while lack of interaction between foliar S and N derived by the mineralization of blood meal was observed.

We observed a positive effect of Se treatment on post-anthesis N uptake and NUtE, also determining a yield increase of 17% for the old variety Cappelli mainly attributed to post-anthesis N uptake increase, and of 13% and 14% for the modern varieties Marco Aurelio and Nadif mainly attributed to NUtE increase.

In conclusion, this study provided evidence that by exploiting the synergistic effect of multiple nutrients in foliar applications, it was possible to positively influence organic durum wheat response and improve production standards even in dry year conditions. The Se application's effectiveness opened new perspectives to improve crop productivity under Mediterranean climate conditions, where drought events are predicted due to the ongoing climate change. Further studies are needed to validate the results obtained with experiments conducted under different conditions.

SUPPLEMENTARY MATERIALS

Table

Table S1. Organic fertilization management.

Organic fertilizer	Sowing	Flag leaf sheath opening (BBCH stage 47)	Beginning of Heading (BBCH stage 51)
kg ha^{-1}	**Dry blood meal (N14.5%; C44%; C/N3.03)**	**Bio Sulphur (S=50%)**	**Liquid bood meal (N=4%)**
CTR	50	-	-
CTR+N	50	-	20
CTR+S	50	45	-
CTR+NS	50	45	20

Table S2. F-value relative to year (Y), variety (V), fertilization strategies (Fs), Selenium application (Se) and their interactions resulting from analysis of variance (ANOVA) performed on pre-anthesis N uptake, post-anthesis N uptake, N translocation, nitrogen utilization efficiency (NUtE), nitrogen harvest index (NHI), N grain content, grain yield and grain protein content.

	DF	Pre-anthesis N uptake	Post-anthesis N uptake	N translocation	N grain content	NUtE	NHI	Grain Yield	Grain protein content
Year (Y)	1	419.7***	232.6**	224.7***	527.4***	0.7 ns	266.1***	569.7***	20436.5***
Variety (V)	3	237.4***	46.2**	111.8***	119.5***	243.0***	158.0***	349.9***	2295.3***
Fertilization strategies (Fs)	3	53.5***	273.9***	31.2***	4.3**	1.1 ns	10.6***	7.4***	112.1***
Selenium application (Se)	1	108.7***	765.3***	71.2***	6.6*	91.4***	62.1***	161.8***	30.2***
Y x V	3	79.9***	848.1***	100.6***	60.5***	43.9***	24.6***	18.0***	1390.2***
Y x Fs	3	160.4***	393.3***	91.8***	72.7***	58.3***	1.19 ns	11.8***	32.4***
V x Fs	9	93.0***	357.1***	47.5***	87.9***	21.7***	10.4***	13.8***	103.5***
Y x Se	1	63.6***	20.0 ns	29.3***	12.4***	12.7***	10.0**	241.8***	17.4***
V x Se	3	18.8***	51.7**	17.5***	28.7***	26.6***	66.0***	8.4**	6.0***
FT x Se	3	24.2***	128.1***	25.5***	19.6***	9.4***	19.7***	15.9***	13.8***
Y x V x Fs	9	104.6***	105.0***	79.0***	51.7***	15.8***	17.2***	4.6***	20.9***
Y x V x Se	3	59.8***	475.4***	54.8***	29.6***	21.6***	54.8***	3.4*	6.2***
Y x Fs x Se	3	22.4***	559.9***	36.0***	46.2***	5.4**	65.4***	3.1*	1.1 ns
V x Fs x Se	9	25.7***	142.2***	33.8***	16.6***	11.0***	30.7***	2.9**	13.9***
Y x V x Fs x Se	9	2.5*	4.2***	54.7***	231.5***	40.5***	32.6***	20.5***	49.0***

DF, degree of freedom; *** P ≤ 0.001; ** P ≤ 0.01; * P ≤ 0.05; ns, not significant

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VI. Selenium Agronomic Biofortification of Organic Fertilized Wheat: Se Grain Content and Selenium Speciation

Box 4. BIOFORTIFICATION

Globally, around 3 billion people have inadequate diets and are often malnourished (Haddad et al., 2016). They are suffering from either undernutrition, micronutrient deficiencies, and/or are overweight. Most of these people are living in low- and middle-income countries. To achieve the sustainable development goal of ending malnutrition by 2030 (Assembly, 2015), the overall diet quality and inadequate intake of people affected should be improved. Biofortification ranked fifth in the top ten list of solutions for global problems ranked by the Copenhagen Consensus 2008 and is the highest-ranked nutrition-sensitive agricultural intervention (Horton et al., 2008). Biofortification is a sustainable and cost-effective approach. An analysis of cost-effectiveness, which is expressed in the cost of one Disability Adjusted Life Year saved, ranks biofortification as highly cost-effective, defined as < 200 $ for one DALY saved (Meenakshi et al., 2010). Biofortification can be achieved through three different methods: 1) conventional plant breeding, 2) transgenic approaches or, 3) using fertilizers (Figure B5).

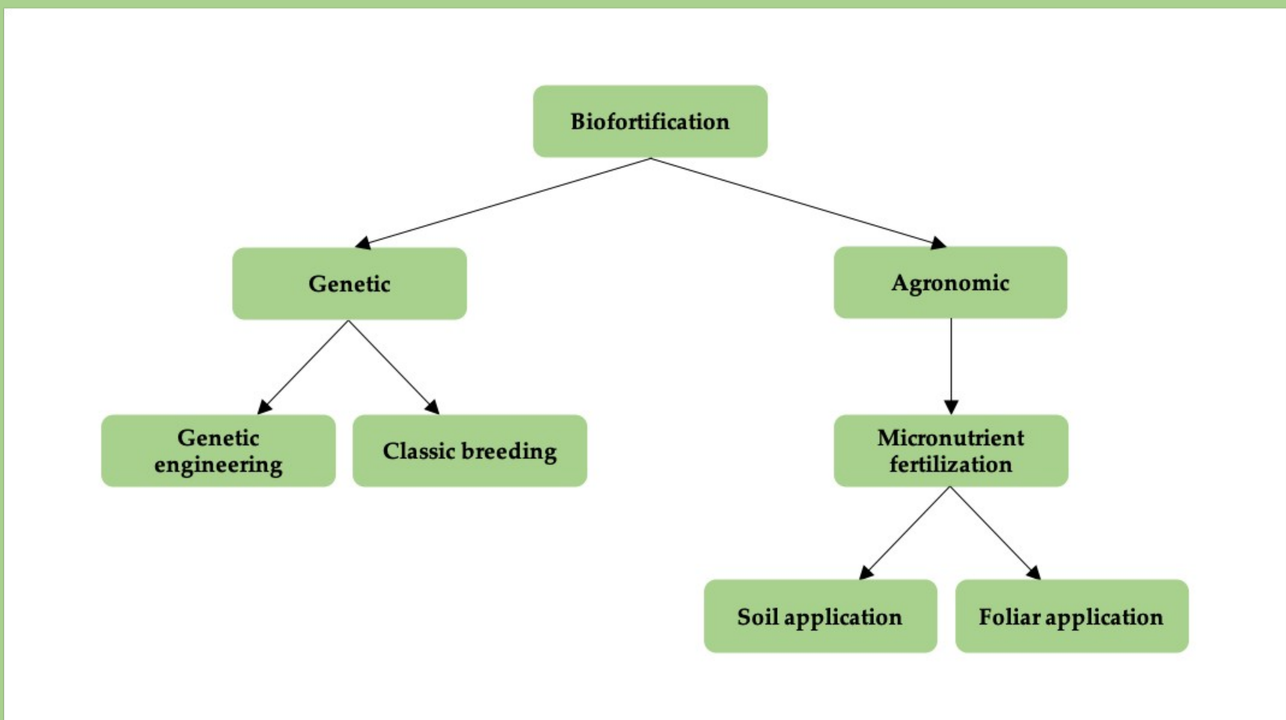


Figure B5. Biofortification management

The regular consumption of biofortified crops will lead to measurable improvements in human health and nutrition (Bouis et al., 2017). The third method for biofortification is using either soil or foliar fertilizers. Foliar fertilization has shown to be more effective than soil fertilization, but the disadvantages of high costs, leaf damage, low uptake, and wash-off by rainfall make it unattractive for smallholder farmers (Khoshgoftarmanesh et al., 2010). The advantage of using fertilizers is that farmers do not have to change the seeds they are using but only apply them. However, these fertilizers must be applied regularly and are expensive (Hirschi et al., 2009), making them less fit within the original philosophy of biofortification of reaching the poor through a sustainable measure. As one of the world's major staple food crops, wheat is consumed by 35% of the human population, contributing almost 20% of dietary energy and protein to developing countries' diets. Due to its significant role in ensuring food security, wheat is an ideal candidate for biofortification (Singh and Govidan, 2017)

This chapter briefly reports the results obtained during the abroad research period at the Department of Analytical Chemistry-Faculty of Chemical Sciences of Complutense University of Madrid, in collaboration with the research group “Determination of traces, speciation and proteomics (trep)” under the supervision of Prof. Yolanda Madrid Albarrán. The research activity was relative to the Se grain quantification and speciation and determined interesting preliminary results that need further investigation.

INTRODUCTION

Plants make up 80% of our food (FAO, 2020) and provide macronutrients for energy and growth and essential micronutrients that protect us from chronic diseases, making substantial contributions to our health (Martin and Li, 2017). However, micronutrient deficiencies, known as “hidden hunger,” affect the health of the world’s people in many ways (Muthayya et al., 2013). In developing countries, the diet depends on a few staple crops, which are calorie-rich but nutritionally deficient, while, in developed countries, the pervasiveness of fast-food industries and the rising costs of fruit and vegetables have contributed to a reduction of the nutritional value of Western diets (Martin and Li, 2017; Gearhardt et al., 2009; Gearhardt et al., 2011).

On the global scale, the selenium (Se) deficiency and its negative impact on health have progressively increased over recent decades (Schiavon et al., 2020). One billion people have an inadequate Se dietary intake (Combs 2001; Tan et al. 2002) and, generally, this scanty Se is associated with areas where its concentration in the soil is low. Nowadays, selenium-deficient soils are common in many regions (Lyons et al., 2003), and in the future, this scenario is likely to worsen due to the ongoing climate changes that will cause a reduction of soil Se content, principally in cultivated areas (Jones et al. 2017).

Biofortification of cereals and other staple crops is a potential solution for increasing Se levels in the human diet. In general, biofortification stimulates the accumulation of microelements in the edible parts of the plant, consumed by humans (Cakmak 2008). Durum wheat (*Triticum turgidum* var. *durum* Desf.) is the primary cereal grown in several countries of the Mediterranean basin and mainly used for bread, couscous, and pasta production (De Vita et al., 2017), which are foods consumed daily by many people (Giacco et al., 2016).

Research investigating Se biofortification in durum wheat has been traditionally focused on defining the optimal Se fertilizer source, timing, and rate related to the accumulation in grain and its derived products (Galinha et al., 2013; Poblaciones et al., 2014; De Vita et al., 2017). Far less research has, however, explored whether the supply of macronutrients as nitrogen (N) and sulfur (S) influences the biofortification process of this trace element (Duncan et al., 2017).

An essential consideration in the agronomic biofortification of Se concerns the effect of S because they compete for uptake in higher plants (Gupta and Gupta, 2000, Lyons et al., 2005; Lyons et al., 2004; Adams et al., 2002). S is essential to durum wheat crops for optimal yield and grain quality (Pompa et al., 2009; Ercoli et al., 2011). Se, in the form of selenate (SeO_4^{2-}), is structurally similar to sulfate (SO_4^{2-}), for this reason, this latter may influence SeO42- uptake through two different plant physiological processes: i) competition for the membrane transport, since SeO_4^{2-} is taken up by the plants via sulfate transports (Gupta and Gupta, 2000; Läuchli, 1993); and ii) regulation of the expression of genes encoding sulfate transports by S status of the plant (Bucher et al., 2004; Smith et al., 1997; Li et al., 2008). Also, the assimilation of selenate follows the sulfate pathway, including some of the same enzymes and their binding sites (Sors et al., 2005; Terry et al., 2000). Furthermore, many Se species (e.g., selenocysteine - SeCys, selenomethionine - SeMet) are analogs of S-containing amino acids (e.g., cysteine, methionine) (Ellis and Salt, 2003; Terry et al., 2000; Chan et al., 2010) and, in the end, Se-amino acids can be integrated into proteins, compromise their function (Sabbagh and Van Hoewyk 2012; Van Hoewyk 2013).

N represents the primary constraint in obtaining adequate yield and quality in durum wheat (Ryan et al., 2009), especially in the organic system. Considering its importance, it is also interesting to understand the possible effect of this macronutrient on Se biofortification in durum wheat. Previous studies on *Triticum aestivum* (winter/spring wheat), carried out in a conventional system, showed that mineral N availability promotes Se accumulation in grain (Chen et al., 2016; Klikoka et al., 2017). The interaction between N and Se is mainly due to the effect of N on the increasing of S assimilation and metabolization. Because Se and S use the same metabolic pathway in plants, N can also promote Se absorption and its metabolization into selenoproteins (Zhou et al., 2020). However, to the best of our knowledge, no study is still available about the effect of fertilization with organic N and S sources on Se biofortification.

Besides evaluating the total Se grain content, another critical issue is assessing the individuation and concentration of Se species to provide information about how grain Se is bioavailable to end-users and its possible toxicological effects. Indeed, the inorganic Se species such as selenate (Se^{VI}) and selenite (Se^{IV}) are generally considered to be the most toxic Se species (Fordyce, 2013), while the organic Se species are considered less toxic (Rayman et al., 2008; Zhu et al., 2009; Dumont et al., 2006). Among the studies investigating the Se species in wheat grain, no one assesses the influence of organic fertilizer management on the production of Se organic species in wheat grains.

This research activity focused on whether the application of selenate and organic N and S sources via foliar, individually and in combination, alters the content of total Se in grains of old and modern Italian durum wheat varieties under Mediterranean conditions. Moreover, a possible alteration in the production of Se species in the grains of durum wheat cultivated under organic fertilization management was also evaluated.

MATERIALS AND METHODS

Field Experiments

The field experiments conducted in two consecutive growing seasons 2017-2018 and 2018-2019 (namely 2018 and 2019, respectively), have been described previously (chapter V). Briefly, one old (Cappelli), one landrace (old Saragolla) and two moderns (Marco Aurelio and Nadif) Italian durum wheat (*Triticum turgidum* spp. *durum*) genotypes were grown in southern Italy (Foggia, 41°46′ N, 16°54′ E) under four different organic fertilization management: 1) control (CTR) fertilized with dry blood meal in a single application at seeding; 2) CTR plus foliar S application at flag leaf stage (BBCH stage 47) (Lancashire et al., 1991) (CTR+S); 3) CTR plus N foliar application at the beginning of heading (BBCH stage 51) (Lancashire et al., 1991) (CTR+N); 4) CRT plus the combination of N and S foliar applications at flag leaf stage and the beginning of the heading, respectively (CRT+NS). Furthermore, two different selenium (Se) application: 1) Se0, control without selenium; 2) Se60, with one application of sodium selenate (Na2SeO4), at the rate of 60 g ha^{-1} (De Vita et al., 2017) at the booting stage (BBCH stage 41) (Lancashire et al., 1991). A split split-plot design with three replicates was adopted: the genotype was the main plot, the organic fertilization management was the plot, and the selenium application was the sub-plot (10.2 square meters).

Climatic details have been reported previously in section RESULTS - Weather conditions of chapter **V**.

Determination of the total Se content by ICP-MS

The Se grain content was determined using ICP-MS after acid digestion in a microwave oven (MSP 1000, CEM, Matheus, NC) procedure described by Moreno-Martin et al. (2020), with minor modifications. About 0.5 g of whole grain flour were weighted in an Xpress tube (volume 10 mL), and 5 mL of concentrated nitric acid (Merck, Madrid, Spain), and 1.0 mL of 30% (v/v) hydrogen peroxide (Panreac, Barcelona, Spain) were added. The mixture was held for 30 min at room temperature and then heated at maximum power 800 W, first at 130 °C for 5 min, after a temperature ramp of 15 $°C·min^{-1}$, and finally at 160 °C for 15 min increasing the temperature to 10 $°C·min^{-1}$. After digestion, the mixture was diluted with Milli-Q water to 25 mL, filtered through a 0.22 μm syringe Nylon filter (Scharlab, Barcelona, Spain), and analyzed. Se content in the digests was measured by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies Inc., USA) by monitoring ^{77}Se, ^{78}Se, and ^{80}Se isotopes under continuous acquisition mode.

The experimental conditions were as follows: RF power of 1550 W, sample depth of 8 mm, plasma gas flow rate 15.0 L·min^{-1}, auxiliary gas flow rate 0.90 L·min^{-1}, and conical nebulizer with a flow rate 1.01 L·min^{-1}. Se quantification was performed by external calibration with the isotope ^{78}Se.

The whole grain flour used in the analysis has been obtained by ground kernels using a Cyclotec Sample Mill 1093 (Foss Tecator, Hillerød, Denmark).

2.3 Extraction Se-species and HPLC-ICP-MS analysis

After evaluation of total Se in grain, its speciation was performed on Cappelli and Nadif genotypes with Se60 application, throughout HPLC-ICP-MS analysis after extraction of Se species with enzymatic hydrolysis, according to the method reported in Moreno-Martin et al. (2020), with minor modifications. Briefly, 0.1 g of whole grain flour were treated with 20 mg of non-specific protease Streptomyces griseus (Protease XIV) (Sigma-Aldrich, Madrid, Spain) and 5 mL of 30 mmol·L^{-1} tris-(hydroxymethyl)-aminomethane (Sigma-Aldrich, Madrid, Spain) at pH 7.5 (adjusted with 0.05 M HCl) in a polypropylene tube for 24 h at 37 °C and without shaking. The reaction mixture was then centrifuged at 11000 rpm for 15 min (Eppendorf 5804 F34-6-38 centrifuge, Hamburg, German), filtered through 0.22 μm syringe Nylon filter. The different enzymatic extracts were analyzed by ICP-MS to determine the total extracted Se content, and HPLC-ICP-MS to detect and identify the Se species. An evaluation of Se species in Protease XIV was also performed to check for impurities. With respect to the HPLC-ICP-MS analysis, the HPLC unit consisted of a PU-2089 HPLC pump (JASCO, Tokyo, Japan) fitted with a six-port injection valve (model 7725i, Rheodyne Rohner Park, CA, USA) and with an injection loop whose volume varied depending on the chromatographic column employed. In order to get an unambiguous identification of the Se species, the extracts were run through two different columns. The first column was a Hamilton PRP-X100 (250 × 4.1 mm, 10 μm) with an anion exchange mechanism and a 100 μL injection loop, with a mobile phase consisting of 10 mM citric acid at pH 5.0 adjusted with ammonia and 2% methanol (Scharlab, Barcelona, Spain). The elution mode was isocratic with a flow rate of 1 mL ·min^{-1}. The second column was a Phenomenex Kintex EVO C18 (150 X 3.0 mm, 5 μm) with a reversed-phase mechanism, and a 20 μL injection loop was employed. The mobile phase consisted of 0.1% formic acid at pH 3.2 and 0.5% methanol. The elution mode was also isocratic with a flow rate of 0.5 mL·min^{-1}. The outlet of both columns was connected directly into the conical nebulizer of ICP-MS with PEEK tubing. Analyses were performed by ICP-MS in time-resolved analysis mode by monitoring ^{77}Se, ^{78}Se, and ^{80}Se isotopes and by employing the operating conditions summarized in the Section Determination of the total Se content by ICP-MS. Se species were identified after separation in both chromatographic columns by comparing their retention time with those of standard solutions: Seleno-L-methionine (Sigma-Aldrich, Madrid, Spain) and Selenomethylseleno-L-cysteine (Sigma-Aldrich, Madrid, Spain). Se quantification was performed by external calibration with the isotope ^{78}Se.

Finally, the confirmation of the identity of Se species was carried out by HPLC-ESI-MS/MS. The analysis was performed using a Shimadzu LC-MS-8030 triple quadrupole system (Shimadzu Scientific Instrument, Columbia, MD, USA) equipped with a Nexera LC-30AD solvent delivery unit, a Nexera SIL 30AC autosampler with temperature-controlled tray, and a CTO-20AC column oven. Separation was performed with a Phenomenex Kinetex EVO C18 column. The analyses were carried out at room temperature, using a mobile phase consisting of a mixture of 0.1% formic acid aqueous solution at pH 3.2 and methanol. Isocratic elution was performed using 0.5% methanol, the flow rate was 0.30 mL·min−1, and the injection volume was set at 10 μL. The instrument operated in positive electrospray ionization (ESI) mode. Nitrogen was used as both nebulizing (1.5 L·min^{-1}) and drying (15.0 L·min^{-1}) gas. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 230 kPa in the collision cell, and the collision energy voltage applied was in the range 10–33 eV. The ionization voltage for ESI was set at 4.5 kV; the interface current was fixed at 4.4 μA, and the detector voltage at 2.10 kV.

Full-scan spectra and MS/MS spectra were acquired to obtain the available transitions for each Se species identified by ICP-MS.

Statistical Analysis

The dataset was tested according to the basic assumptions of analysis of variance (ANOVA). The normal distribution of the experimental error and the common variance of the experimental error were verified through Shapiro–Wilk and Levene's tests, respectively. When required, Box-Cox transformations (Box and Cox, 1964) were applied before analysis. The grain Se content and Se speciation data were processed by analysis of variance (ANOVA), according to a split split-plot design, using a linear mixed model (LMM). The effects of year, variety, organic fertilization treatment, Se application (only for grain Se content) and their interactions were considered as fixed. The "blocks within years" was included as a random effect, together with the residual error terms.

The statistical significance of the difference among the means was determined using Tukey's honest significance post hoc test at the 5% probability level. All the analyses were performed using the R statistical software (R Core Team, 2018), with the lme4 package for ANOVA (Bates et al., 2015).

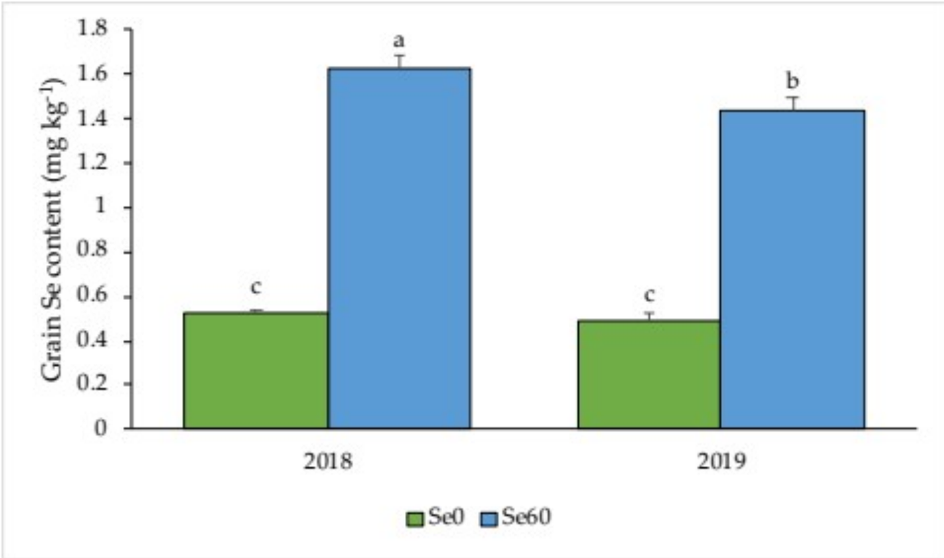
RESULTS AND DISCUSSION

Before seeding, the total Se in soil was 0.056 ± 0.0085 mg kg^{-1} (mean ± SE) in 2018 and 0.087 ± 0.004 mg kg^{-1} in 2019. So, according to the classification by Hawkesford and Zhao (2007), the soils can be considered deficient in total Se.

The ANOVA results relative to the total grain Se content are reported in Table 1.

Table 1. F-values relative to year (Y), variety (V), fertilization strategy (Fs), Selenium application (Se) and their interactions resulting from analysis of variance (ANOVA) performed on grain Se content.

	DF	Grain Se content
Year (Y)	1	12.0832*
Variety (V)	3	42.9841***
Fertilization strategies (Fs)	3	39.4836***
Selenium application (Se)	1	3599.0316***
YxV	3	22.5878***
YxFs	3	10.0963***
VxFs	9	6.8474***
YxSe	1	19.5236***
VxSe	3	18.7205***
FsxSe	3	18.8835***
YxVxFs	9	4.5424***
YxVxSe	3	10.2129***



YxFsxSe	3	1.5649$^{n.s.}$
VxFsxSe	9	0.7306$^{n.s.}$
YxVxFsxSe	9	1.0679n.s.

In both years, the grain Se content increased after biofortification (Se60) three times more than no supplemented plants (Se0), even if in the second drier year, a lower Se60 value was observed (Figure 1).

Figure 1. Effect of interaction year x selenium application on grain Se content. Se0, no selenium application; Se60, selenium application. Values followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Vertical bars indicate standard errors (n=48).

Thus, in both years, Se foliar application increased Se grain content to levels that meet the Se requirements of humans and animals (food and fodder should contain >50–100 μg Se kg^{-1}) (Gissel-Nielsen et al., 1984), indicating that foliar application of selenate was effective in raising durum wheat grain Se concentration. Concerning the no supplemented plants (Se0), higher Se grain contents in both the years were observed with respect to De Vita et al. (2017), that in the same cultivation area (Apulia, Foggia) reported a Se grain content of 0.16 mg kg^{-1}, under a conventional fertilization system and without Se application. Our result is in accordance with AL-Ghumaiz et al. (2020), who found a higher Se concentration on spring wheat cultivated under organic fertilization with respect to conventional fertilization treatments. This may be due to low soil pH values often associated with organic fertilizers, which could conditionate micronutrient availability to plants (Marschner, 1995; Teng and Timmer, 1990), including Se availability.

Moreover, grain Se content was significantly influenced by the different wheatear conditions of the two years. In particular, under Se foliar application (Se60), the highest value was observed in the first year, while no significant differences were observed between the two years when the Se was absorbed from the indigenous soil source (Se0). The higher grain yield in 2018 with respect to 2019 (see chapter **V** – Table 1) excluded a possible dilution effect; so, it seems clear that the rainfall was a crucial factor influencing the absorption of foliar Se and its accumulation into the grain. In particular, Rodrigo et al. (2014) found an inverse correlation between precipitation during the days before Se foliar spray fertilization and the Se grain accumulation. Thus, probably also in our study, the lower rain amount occurred in 2018 in the period before the Se foliar application, led to a higher Se accumulation in the grain. Indeed, during the ten days before the Se application, rainfalls were 3.5 mm and 21.2 mm in 2018 and 2019, respectively (Figure 2).

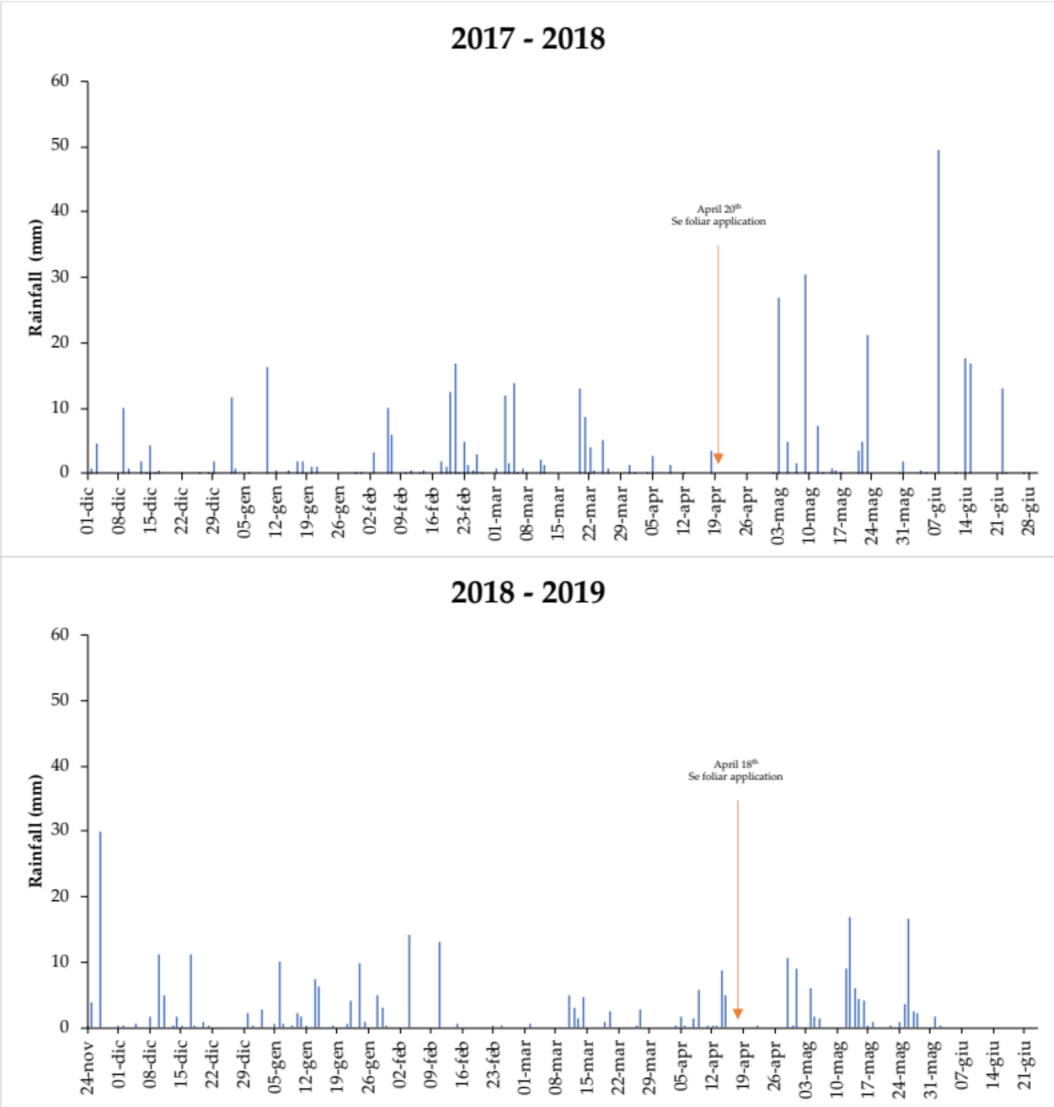


Figure 2. Daily rainfall for the two study-years.

This result was also in accordance with Poblaciones et al. (2014), which under dry conditions found that the plant may absorb eagerly and very efficiently through the stomata the Se fertilizer applied. Therefore, due to the irregular precipitation distribution typically of the Mediterranean environment (Symeonidis et al., 2013), it is necessary to pay special attention to the Se fertilizer management to get a Se biofortification as effective as possible. Considering the higher Se grain content reported in the literature under organic fertilization with respect to conventional fertilization (AL-Ghumaiz et al., 2020), we tested the interaction of Se application with different organic fertilization programs. The Se foliar application caused a significant increase of the grain Se content under all the fertilization strategies adopted. However, adding the foliar S alone (CTR+S) or in combination with organic N (CTR+NS), a decrease of Se grain content with respect to CTR was observed.

Indeed, a reduction of 24% and 25% with respect to the CTR was observed for CTR+S and CTR+NS, respectively (Table 2).

Table 2. Effect of the selenium application x fertilization strategy on Se grain content.

	Se0	Se60
	mg kg^{-1}	
Fertilization strategies		
CTR	0.55±0.04^{c}	1.70±0.08^{a}
CTR+N	0.54±0.04^{c}	1.71±0.08^{a}
CTR+S	0.48±0.04^{c}	1.36±0.07^{b}
CTR+NS	0.44±0.05^{c}	1.37±0.06^{b}

Se0, no selenium application; Se60, selenium application; CTR: control fertilized with dry blood meal at seeding; CTR+N; control plus N foliar application at the beginning of heading stage (BBCH stage 51); CTR+S; control plus S foliar application at flag leaf sheath opening stage (BBCH stage 47); CTR+NS: control plus the combination of S and N foliar applications at flag leaf sheath opening stage and at the beginning of heading (BBCH stage 47 and 51), respectively. For each experimental factor, values in each column and row followed by different letters are significantly different at P ≤ 0.05 according to Tukey's test. Data are reported as means±standard errors (n=24).

These results confirm the negative effect of S on Se accumulation in durum wheat grains that were also found in other studies conducted using conventional fertilizer system (Lyons et al., 2005; Adams et al., 2002), implying that there was a competitive inhibition by S of Se uptake via the primary S transporter. Considering that the fertilizers were applied via foliar in our study, our results let us suppose that the relationship between S and Se in plants is not confined to root uptake by the soil, but other physiological mechanisms might be involved. The lack of effect of organic N foliar application (CTR+N) on grain Se concentration observed in our study was also found in previous studies (Soltanpour et al., 1982; Lyons et al., 2004). Another notable observation is that the different organic fertilization strategies have no effect on the uptake of Se from the indigenous soil source (Se0), so we can assume that the foliar application of organic N and S fertilizer did not interact with the uptake of Se from the soil.

The four durum wheat varieties under study differently accumulated Se in the grain with the Se application. No difference was observed among the varieties without Se application, excepted for the landrace old Saragolla that showed the lowest value. On the contrary, under Se application, Nadif showed the highest value between the two modern varieties, and Cappelli the highest value between the two old varieties (Table 3).

Table 3. Effect of selenium application x variety interactions on Se grain content.

	Se0	Se60
	mg kg^{-1}	
Variety		
Cappelli	0.56±0.03^{d}	1.57±0.05^{b}
old Saragolla	0.31±0.04^{e}	1.24±0.08^{c}
Marco Aurelio	0.51±0.02^{d}	1.46±0.05^{b}
Nadif	0.62±0.05^{d}	1.87±0.07^{a}

Se0, no selenium application; Se60, selenium application; CTR: control fertilized with dry blood meal at seeding; CTR+N; control plus N foliar application at the beginning of heading stage (BBCH stage 51); CTR+S; control plus S foliar application at flag leaf sheath opening stage (BBCH stage 47); CTR+NS: control plus the combination of S and N foliar applications at flag leaf sheath opening stage and at the beginning of heading (BBCH stage 47 and 51), respectively. For each experimental factor, values in each column and row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test. Data are reported as means±standard errors (n=24).

Our results confirmed the variety-dependent response for Se accumulation under Se fertilization, in agreement with Galinha et al. (2015) and Lee et al. (2011), who observed genetic variation in Se concentration and uptake among different winter wheat and spring wheat cultivars. However, other studies suggested slight genetic variation in grain Se concentrations across bread wheat genotypes (Lyons et al., 2005; Zhao et al., 2009). De Vita et al. (2017) and Zhao et al. (2009) highlighted that the old varieties had significantly higher grain mineral concentrations than the modern varieties. Our results partially agree with these authors since even if the old variety Cappelli showed higher values than the modern Marco Aurelio, the highest value was observed for the modern Nadif.

After crop uptake, Se is transformed into different species by the plant. The bioavailability of Se for humans and animals largely depends on the species of Se consumed rather than the total Se content (Premarathna et al., 2012); for this reason, we have also investigated the Se speciation in an old (Cappelli) and modern (Nadif) genotypes.

Selenomethionine (SeMet), Se-methylselenocystine (SeMetSeCys), and uncharacterized Se were found in grains' extracts without significant difference in their content between the two cultivars cultivated, under different organic fertilizer management, and in the two growing seasons. However, the notable observation was that SeMetSeCys was the major characterized Se species in all samples accounting between 0.77 and 0.84 mg kg^{-1} grain, while the concentration of SeMet ranged from 0.42 and 0.55 mg kg^{-1} grain. Generally, the SeMet is the dominant organic form of Se in cereals grown under the conventional system (Stadlober et al., 2001; Poblaciones et al., 2014; Cubadda et al., 2010; Ogra et al., 2009; Olson et al., 1970) and SeMetSeCys in vegetables (Zhu et al., 2009). This new evidence emerging from our study could be explained by the organic fertilizer management of our field trial rather than the conventional fertilization of previous studies available in the literature.

In order to confirm the Se species identity, the HPLC-ICP-MS/MS analysis was carried out, confirming the results obtained by HPLC-ICP-MS.

CONCLUSIONS

The foliar selenium biofortification applied to durum wheat (*Triticum turgidum* spp. *durum*) grown under organic fertilization management increased the grain Se's values up the values requested by humans and animals. However, our results showed that it is necessary to pay special attention to the wheatear conditions before the Se foliar application to get the Se biofortification as effective as possible, particularly under the variable condition typically of the Mediterranean area. Concerning the fertilization management, the negative effect of organic foliar S on Se accumulation was observed, probably due to competitive inhibition of Se uptake via the primary S transporter. The Se foliar application led to a variety-dependent response for Se accumulation. The modern Nadif showed the highest value, followed by the old Cappelli; thus, in our study, it is not so closely demonstrated that the old varieties had significantly higher grain mineral concentrations than the modern ones. Finally, in our experimental condition, SeMetSeCys was the major characterized Se species in all samples, followed by SeMet. This is a surprising result, considering that SeMet is generally the dominant organic form of Se in cereals, while SeMetSeCys in vegetables.

Appear clear that further researches are necessary to deep inside some aspects, including the investigation of the physiological mechanism that inhibits the Se enrichment in combination with organic S fertilization, when both of them were applied via foliar, and the possible interaction relative to the aggregation level of the gluten protein which is essential for durum wheat quality.

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VII. Conclusions

The main objective of this Ph.D. dissertation was to examine the management of nitrogen fertilizers combined with different agronomic strategies to improve the efficiency of nitrogen use by different Italian durum wheat genotypes, accompanied by improvements in grain yield, grain quality and nutritional value, considering low-input and organic agricultural systems in the Mediterranean area. The objective was pursued through the four original studies reported in the doctoral thesis.

The results reported in **chapter III** provide new insights into the relationships between strobilurin and nitrogen use efficiency for yield and grain protein in durum wheat cultivars, also under N low-input conditions. Today the strobilurins are one of the most utilized fungicides and, besides the traditional utilization in plant protection, has been demonstrated their positive effects on plant physiological processes. Our findings showed that the effect of strobilurin on nitrogen uptake and remobilization efficiency depends on cultivars. In particular, strobilurin had a positive effect on UPE, NUEy, and NUEp in cultivar characterized by high yield and low grain protein content (cv Saragolla) and on NUtE and NUEy in cultivar characterized by low yield and high grain protein content (cv Sfinge). Moreover, our results improved the knowledge related to the complexity of managing durum wheat nitrogen fertilization in low-input agricultural systems under Mediterranean climate conditions. We observed that strobilurin could reduce the N rate application from 120 to 90 kg ha^{-1}, split in three times, with satisfactory grain N content results. Nevertheless, the most interesting results were observed with drought stress. In this condition, strobilurin determined higher NUEy and NUEp values under most of the N fertilization adopted. These results are very interesting since drought conditions are predicted in the Mediterranean area due to the ongoing climate change.

In **chapter IV**, the attention was moved to durum wheat gluten protein composition. The results reported in the chapter provide evidence that the effect of the growing season on the durum wheat quality parameters was more evident than those of genotype and nitrogen level. The results obtained regarding four durum wheat genotypes indicate different patterns of protein assembly concerning the growing season, thus contributing to the rational selection of the durum wheat genotypes suitable for facing the ongoing climate changes in the Mediterranean area. Indeed, the four durum wheat genotypes showed a different capacity of the gluten proteins to assembly in a visco-elastic structure in relation to their earliness with respect to the wheatear conditions. In particular, in the warmer year, the late-maturing genotype (Marco Aurelio and Pietrafitta) showed a significant decrease of larger insoluble polymers fraction (F1*) and %UPP with a negative effect on their protein assembly level, despite Marco Aurelio always showed a higher degree of polymerization. On the contrary, the medium-early and early maturing genotypes (Quadrato and Redidenari), probably due to their earliness, did not change their "protein assembly level" in relation to the wheatear conditions. In the Mediterranean areas, besides water deficit and high temperatures, nitrogen (N) availability represents the main environmental factors that affect yield and quality in durum wheat. However, in our study, the effect of N fertilization on the gluten protein polymerization and assembly was relatively small, but among the N levels utilized, the increase of F1*, %UPP and the monomeric fraction under N90 were observed. Moreover, also the highest yield and gluten index values were obtained under N90.

The results reported in **chapter V** provide evidence that by exploiting the synergistic effect of multiple nutrients in foliar applications, it was possible to positively influence grain yield and N-related traits in old and modern durum wheat varieties grown under organic management in the Mediterranean area, even in dry year conditions. We demonstrated the better N utilization efficiency of the modern varieties grown under the organic system and also under water stress conditions with respect to the old one. Marco Aurelio showed the highest ability to use efficiently plant nitrogen to make yield even under stressful conditions. Relative to the fertilization strategies, soil organic N fertilization with blood meal at seeding was the primary determining factor for the parameters considered since, in the organic system, the addition of organic N source via foliar application at heading did not determine an increase in grain protein content nor in grain yield in the first year and even their decrease in the drier year. Foliar application of organic S and N had a positive and synergic effect, on pre-anthesis N uptake, N translocation, NUtE, and grain yield, especially under drought conditions. In contrast, a lack of interaction between organic foliar S and N derived by the mineralization of blood meal was observed. Moreover, the effect of Se in the durum wheat organic system was investigated for the first time, taking into consideration that adequate concentrations of Se can determine the increasing plant productivity. We observed a positive effect of Se treatment on post-anthesis N uptake and NUtE, also determining a yield increase of 17% for the old variety Cappelli mainly attributed to post-anthesis N uptake increase, and of 13% and 14% for the modern varieties (Marco Aurelio and Nadif) mainly attributed to NUtE increase. The Se application's effectiveness opened new perspectives to improve crop productivity under Mediterranean climate conditions, where drought events are predicted due to the ongoing climate change. Further studies are needed to validate the results obtained with experiments conducted under different conditions.

In **chapter VI**, it was explored the possibility to improve, throughout agronomic biofortification, the nutritional value of durum wheat grain cultivated under different organic fertilization program. We demonstrated that the foliar selenium biofortification applied to durum wheat grown under organic fertilization management, increased the grain Se accumulation up the values requested by humans and animals. However, our results showed that under the Mediterranean condition, it is necessary to paid special attention to the wheatear conditions before the Se foliar application in order to get the Se biofortification as effective as possible. Respect to the fertilization management, the negative effect of organic foliar S on Se accumulation was confirmed also under organic fertilization management. Finally, the modern Nadif showed the highest value, followed by the old Cappelli; thus, in our study it is not so closely demonstrated that the old varieties had significantly higher grain mineral concentrations than the modern ones.

Finally, a very surprising results is that, under our research conditions, SeMetSeCys was the major characterized Se species in all samples, followed by SeMet. Further researches are necessary to deep inside some aspects including the investigation of the physiological mechanism that inhibit the Se enrichment in combination with organic S fertilization, when both of them were applied *via* foliar, and the possible interaction relative to the aggregation level of the gluten protein which are essential for durum wheat quality.

Considering that define the best farming system that alone can satisfy the world safe feeding is impossible, a blend of innovative farming system, including low-input and organic ones, will be necessary for future global food and ecosystem security. The results described in this Ph.D. advance the current state of the knowledge about improving the sustainability, productivity and different quality aspects of durum wheat under low-input and organic farming systems. However, several significant issues remain to be investigated in future research.

List of Abbreviations

ATP – Adenosine Triphosphate

B – Boron

BBCH – Biologische Bundesanstalt, Bundessortenamt and Chemical Industry

C – Cobalt

CO_2 – Carbon Dioxide

CTR – Control Plot

Cu – Copper

EC – European Commission

ECC – European Economic Community

EU – European Union

F1 – HPLC fraction corresponds to large and medium size polymers (mainly HMW-GS)

F1* – HPLC fraction corresponds to SDS-insoluble glutenin polymers

F2 – HPLC fraction corresponds to large and medium size polymers (mainly LMW-GS)

F3 – HPLC fraction corresponds to ω-gliadins and small oligomers enriched in C-type and D-type LMW-GS subunits

F4 – HPLC fraction corresponds to monomeric gliadins (α-type and β-type) and non-gluten proteins

Fe– Iron

Mn – Manganese

FNAB – FŽdŽration Nationale d'Agriculteurs Biologiques

GPC – Grain Protein Content

Gw – Grain Weight

GY – Grain Yield

HCl – Hydrochloric acid

HMW-GS – High Molecular Weight Glutenin Subunits

HPLC-ICP-MS – High Performance Liquid Chromatography in combination with Inductively Coupled Plasma Mass Spectrometry

ICC – International Association for Cereal Science and Technology

ICP-MS – Inductively coupled plasma mass spectrometry

IFOAM – International Federation of Organic Agriculture Movements

K – Potassium

LCA – Life Cycle Analysis

LMW-GS – Low Molecular Weight Glutenin Subunits

Mo – Molybdenum

N – Nitrogen

N120 – 120 kg ha^{-1} of nitrogen fertilizer applied in two times

N120T3 – 120 kg ha^{-1} of nitrogen fertilizer applied in three times

N36 – 36 kg ha^{-1} of nitrogen fertilizer applied

N60 – 60 kg ha^{-1} of nitrogen fertilizer applied

N90 – 90 kg ha^{-1} of nitrogen fertilizer applied

N90T3 – 90 kg ha^{-1} of nitrogen fertilizer applied in three times

Na_2SeO_4 – Sodium selenate

NaCl – Sodium chloride

NAD – Nicotinamide Adenine Dinucleotide

NADH – Reduced Forms of Nicotinamide Adenine Dinucleotide

NH_3 – Ammonia

NH_4^+ – Ammonium

NHI – Nitroge Harvest Index

NiR – Nitrate Reductase

Nmin – Mineral Nitrogen Content

NO_3^- – Nitrate

Ns – Nitrogen Supply

NUE – Nitrogen Use Efficiency

NUEp – Nitrogen Use Efficiency for Protein

NUEy – Nitrogen Use Efficiency for Yield

NUtE – Nitrogen Utilization Efficiency

OECD – Organization for Economic Co-operation and Development

P – Phosphorus

PC – Principal Component

PCA – Principal Component Analysis

PVDF – Polyvinylidene Difluoride

S – Sulphur

SDS – Sodium Dodecyl Sulfato

Se – Selenium

Se0 – no foliar application of sodium selenate

Se60 – one foliar application of sodium selenate

Se^{IV} – Selenite

SeMet – Selenomethionine

SeMetSeCys – Selenomethylselenocystine

Se^{VI} – Selenate

SO_3 – Sulfur trioxide

SPAD – Signal passed at danger

ST0 – No strobilurin application

STaz – One strobilurin application

T – Temperature

TKW – Thousand Kernel Weight

UHPLC – Ultra High Performance Liquid Chromatography

UPE – Nitrogen Uptake Efficiency

UPP – Sodium Dodecyl Sulfato insoluble polymers

Zn – Zinc

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