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Carbon/Nitrogen Relations in C_3 Cereal Crops Within a Climate Change Context: Implications on Grain Yield and Quality

PhD Thesis

Sinda Ben Mariem

Supervisor: Iker Aranjuelo Michelena

Co-Supervisor: Fermín Morales Iribas

Tutor: César Arrese-Igor Sánchez

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El Dr. **Iker Aranjuelo Michelena** y el Dr. **Fermín Morales Iribas**, como directores de la tesis doctoral,

CERTIFICAN:

Que el trabajo titulado “Carbon/Nitrogen Relations in C_3 Cereal Crops Within a Climate Change Context: Implications on Grain Yield and Quality” recogido en la presente memoria, ha sido realizado por Dña. Sinda Ben Mariem en el Instituto de Agrobiotecnología (CSIC/Gobierno de Navarra) bajo su supervisión y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctora. Dña. Sinda Ben Mariem ha disfrutado de una beca Predoctoral del Gobierno de Navarra “Doctorados Industriales 2017-2019”. Este trabajo ha sido financiado por el proyecto “Nuevos criterios de mejora aplicados a la mejora de trigo duro en un contexto de cambio climático” MINECO AGL2016-79868-R, financiado por el Ministerio de Economía y Competitividad.

Y para que así conste, firman la presente en Pamplona, a __ de _____de 2021.

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SUMMARY

In a context of the expanding world population and global climate change, food security is becoming a challenge for worldwide society. To meet the increasing global agricultural demands, crop yields enhancement has been attempted since the green revolution and cereals production, in particular wheat, has increased since then by releasing high yielding new cultivars. However, improvement in crop yields has slowed since the 1990s and the recent gains in global crop production fall short of the expected demands mainly due to global warming. At a global scale, the relatively decrease in wheat production is principally because of the adverse effects of abiotic stresses that are increasing in intensity and frequency under climate change scenario. Low water availability and extreme temperatures will negatively affect the growth and productivity of major crop species including durum wheat. In the Mediterranean area, the process of grain filling is coinciding with dry and hot environmental conditions affecting final yield quantitatively and qualitatively as well. Moreover, studies conducted recently remarked that grain mineral composition is shifted and total protein content in grains is reduced when durum wheat grows in the presence of high CO_2 concentration ([CO_2]). It is clear that commercialised wheat genotypes are becoming more vulnerable to global climate change which is affecting not only grain yield but also quality. Thus, the understanding of physiological mechanisms that enable plants to adapt to drought stress and increasing atmospheric [CO_2] could help in screening and selection of genotypes with suitable grain yield and quality, and using these traits in breeding programs. On the other hand, the increase in nitrogen fertilizers application in wheat crops is consequently stimulating plant growth and increasing grain yield, nitrogen and protein concentration in kernels ensuring, thereby, good bread/pasta making quality and mitigating the negative effect of changing climate on grain production. Nevertheless, the excessive nitrogen supply can lead to environment pollution and may probably accentuate climate warming by increasing nitrous oxide (N_2O) emission. For this reason, optimizing nitrogen use efficiency (NUE) is a tool to increase crop yields while preserving the environment.

Within this context, the main objective of this work is the use of new wheat selection criteria to identify, in an integrative manner, genotypes and crop management practices conferring high nitrogen use efficiency to reach higher yield and better grain quality under increasing [CO_2] and low water availability. For this purpose, in the **first chapter (I)**, a meta-analysis study was carried out to provide an overview of the effects and interactions of multiple climate factors, specifically high [CO_2], drought, and elevated temperature on the productivity and grain quality of C_3 cereals. Findings presented in this chapter showed that despite of the positive effect of elevate [CO_2] on grain yield, this trait seems to be mitigated by heat and drought stress. Grain quality was also impacted by changing climate, characterized by an increase in carbohydrates and decrease in protein and minerals.

In the **second chapter (II)**, we assessed the grain quality trait of wheat archived samples since 1850 collected from many countries to evaluate the nutritional quality changes in grain under changing climate. This study confirmed the results foundin the previous chapter and showed an imbalance in carbohydrate/protein content marked after the 60s, adding to an impoverishment in minerals. Yield results from Broadbalk wheat experiment in Rothamsted (UK) showed an improvement of wheat yield since the green revolution attributed mainly to the introducing of semi-dwarf high yielding genotypes.

In **chapter (III)**, to investigate the impact of nitrogen fertilization on yield and grain quality, an experiment was performed where 20 durum wheat genotypes were fertilized since anthesis with two N fertilization levels under greenhouse conditions. Within these genotypes, only 6 lines were selected with high and low nitrogen use efficiency to characterize agronomic and quality traits. As expected, nitrogen supply increased grain yield while no effect was detected in thousand-grain weight. Grain soluble sugars, gluten fractions, mineral composition, and polyphenol concentrations were also improved by N application. The comparison among genotypes revealed that high yielding genotypes had higher grain carbohydrate concentrations while higher concentrations in grain minerals, gluten fractions, and polyphenols were recorded in low yielding cultivars.

Finally, in **chapter (IV)**, 4 durum wheat genotypes and 6 tritordeum lines with higher and lower NUE were exposed to high [CO_2] and drought stress in greenhouses, in order to characterize post-anthesis nutrient remobilization from leaves and ears sustaining grain filling, together with agronomic characterization under such conditions. It seems that the increase of atmospheric [CO_2] could attenuate the negative effect of drought on grain yield. Carbon and nitrogen metabolism in leaves and ears were altered under high CO_2 enrichment and larger effect was observed when it was combined with drought, and the relative contribution of each organ to grain filling was strongly affected by growing conditions.

RESUMEN

En un contexto de población mundial en expansión y cambio climático global, la seguridad alimentaria se está convirtiendo en un desafío para la sociedad mundial. Para satisfacer las crecientes demandas agrícolas mundiales, se ha intentado mejorar el rendimiento de los cultivos con diferentes estrategias. Durante la revolución verde, se consiguió incrementar la producción de cereales, en particular el trigo, mediante el uso de nuevos cultivares de alto rendimiento. Sin embargo, a pesar de los esfuerzos de los diferentes programas de mejora, la tasa anual de incremento de rendimiento de los cultivos se ha desacelerado desde los años noventa. Estudios recientes indican que dicha ralentización se debe, en gran medida, a cambios en las condiciones ambientales. A escala mundial, la relativa disminución de la producción de trigo se debe principalmente a los efectos adversos de los estreses abióticos que están aumentando en intensidad y frecuencia en el escenario de cambio climático. La baja disponibilidad de agua y las temperaturas extremas afectan negativamente al crecimiento y la productividad de las principales especies de cultivos como el trigo duro. En la zona mediterránea, el proceso de llenado de grano coincide con condiciones ambientales secas y cálidas que afectan al rendimiento final cuantitativa y cualitativamente. Además, los estudios realizados recientemente señalaron que la composición mineral del grano cambia y el contenido de proteína total en los granos se reduce cuando el trigo duro crece en presencia de una alta concentración de CO_2 ($[CO_2]$). Está claro que los genotipos de trigo comercializados se están volviendo más vulnerables al cambio climático global que está afectando no solo al rendimiento del grano sino también a la calidad. Por lo tanto, la comprensión de los mecanismos fisiológicos que permiten a las plantas adaptarse a las condiciones ambientales cambiantes (estrés hídrico, térmico y el aumento de la concentración de CO_2 atmosférico) podría ayudar en la selección de genotipos mejor adaptados. Como estrategia para incrementar la producción y calidad de los cultivos, a partir de la revolución verde, se optó por incrementar la aplicación de fertilizantes nitrogenados en los cultivos. Junto con los aumentos de la producción, la aplicación de fertilizantes favorece la concentración de nitrógeno y proteínas en los granos que favorecen la calidad y la elaboración de los productos derivados (pan, pasta, etc.). No obstante, el suministro excesivo de nitrógeno puede provocar problemas relacionados con el incremento en las

emisiones de gases invernadero que, acentuarían el calentamiento climático al aumentar la emisión de óxido nitroso (N_2O). Por esta razón, optimizar la eficiencia en el uso de nitrógeno (EUN) es una herramienta determinante para aumentar el rendimiento de los cultivos y preservar el medio ambiente.

En este contexto, el objetivo principal de este trabajo es el uso de la aplicación de nuevos criterios de selección de trigo para identificar, de manera integradora, genotipos y prácticas de manejo de cultivos que confieran alta eficiencia en el uso de nitrógeno para alcanzar mayor rendimiento y mejor calidad de grano en condiciones ambientales cambiantes ([CO_2] elevado y baja disponibilidad de agua). Para ello, en el **primer capítulo (I)**, se llevó a cabo un estudio de meta-análisis con el objetivo de poder proporcionar una visión general de los principales factores ambientales asociados al cambio climático ([CO_2], la sequía y la temperatura elevada) sobre la productividad y calidad del grano de cereales C_3. Los resultados presentados en este capítulo mostraron que, a pesar del efecto positivo de la [CO_2] elevada sobre el rendimiento de grano, este incremento se vería atenuado si hay interacción con otros factores de estrés. La calidad del grano también se vio afectada por el cambio climático. De esta manera se constata un aumento de carbohidratos y una disminución de proteínas y composición mineral.

En el **segundo capítulo (II)**, evaluamos la producción y calidad del grano de muestras de trigo almacenadas en diferentes museos y centros de investigación desde 1850. Se trata de muestras recolectadas en diferentes países con objeto de evaluar los cambios en la calidad nutricional del grano durante 1850-2016. Este estudio confirmó los resultados encontrados en el capítulo anterior y mostró un desequilibrio en el contenido de carbohidratos/proteínas especialmente marcado a partir de los años 60, lo que se suma a un empobrecimiento de los minerales. Por otro lado, los resultados de rendimiento del experimento de trigo Broadbalk en Rothamsted (Reino Unido) mostraron una mejora del rendimiento del trigo desde la revolución verde atribuida principalmente a la introducción de genotipos semi-enanos de alto rendimiento.

El **capítulo (III)** se diseñó con objeto de investigar el impacto de la fertilización nitrogenada sobre el rendimiento y la calidad del grano. Para ello, se realizó un primer experimento en invernadero donde se trabajó con 20 genotipos de trigo duro que fueron fertilizados con diferentes niveles de fertilización. De este primer estudio se seleccionaron 6 líneas con alta y baja eficiencia de uso de nitrógeno para caracterizar los parámetros agronómicos y de calidad. Como se esperaba, el suministro de nitrógeno aumentó el rendimiento de grano, mientras que no se detectó ningún efecto en el peso de mil granos. Los azúcares solubles en grano, las fracciones de gluten, la composición mineral y las concentraciones de polifenoles también mejoraron mediante la aplicación de nitrógeno. La comparación entre genotipos reveló que los genotipos de alto rendimiento tenían una mayor concentración de carbohidratos de grano. Por otro lado, en las variedades de bajo rendimiento se registraron concentraciones más altas en minerales de grano, fracciones de gluten y polifenoles.

Finalmente, en el **capítulo (IV)**, 4 genotipos de trigo duro y 6 líneas de tritordeum seleccionados como variedades de mayor y menor EUN fueron expuestos a alta [CO_2] y estrés hídrico. Junto con la correspondiente caracterización agronómica, el estudio tuvo como objeto analizar el impacto de las citadas variables ambientales en la removilización de nutrientes post-antesis de hojas y espiga que participan en el llenado del grano. Los resultados obtenidos indican que el aumento de [CO_2] atmosférico podría atenuar el efecto negativo de la sequía en el rendimiento de grano. Por otro lado, el metabolismo del carbono y el nitrógeno foliar y de espiga se vio alterado en condiciones de [CO_2] elevada. Dicha alteración fue especialmente notoria cuando se combinó con la sequía.

INTRODUCTION

I. Climate change: Main causes and effects

Climate change has become a widespread issue in the last few decades and its implications for Earth's environmental processes have been of particular relevance. Climate change is defined as a change in the statistical distribution of weather patterns when that change lasts for an extended period of time (i.e., decades to millions of years) (Kumar, 2015). Similarly, according to the Intergovernmental Panel on Climate Change (IPCC, 2014), climate change refers to alterations in the state of the climate that can be identified (e.g., by using statistical tests) by changes in the mean and/or variability of its properties and that persist for an extended period, typically decades or longer. Beura (2016) enumerates climate change as long-term changes in average temperature and precipitation. The greenhouse effect plays an important role regulating the climate of the earth. Actually, without the natural atmospheric greenhouse effect (due to greenhouse gases of natural form and concentration present in the atmosphere), earth's surface temperature would be far below freezing (Beura, 2016). The terrestrial temperature is the result of the balance maintained by the earth and the atmosphere in terms of the absorption of solar radiation that reaches the earth and the emissions of long-wave radiation (infrared) emitted into space. The solar energy is absorbed by the earth's surface and then reflected back to theatmosphere as heat. Then, as the heat goes out to space, greenhouse gases absorb a part of the heat and radiate it back to the earth's surface, to another greenhouse gas molecule or to space, a phenomenon known as the "Greenhouse Effect" (Kaddo, 2016). The amount of long-wave radiation retained, and therefore responsible for the temperature increase, is depending on the atmospheric constituents (mainly greenhouse gases). Thus, the greenhouse gases are thought to be the main contributors to rising earth's surface temperature, and an increase of their concentration in the atmosphere is probably resulting in global warming. The main greenhouse gases are water (H_2O), carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), ozone (O_3), and halocarbons. Anthropogenic greenhouse gas (GHG) emissions since the pre-industrial era have driven large increases in the atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Figure 1).

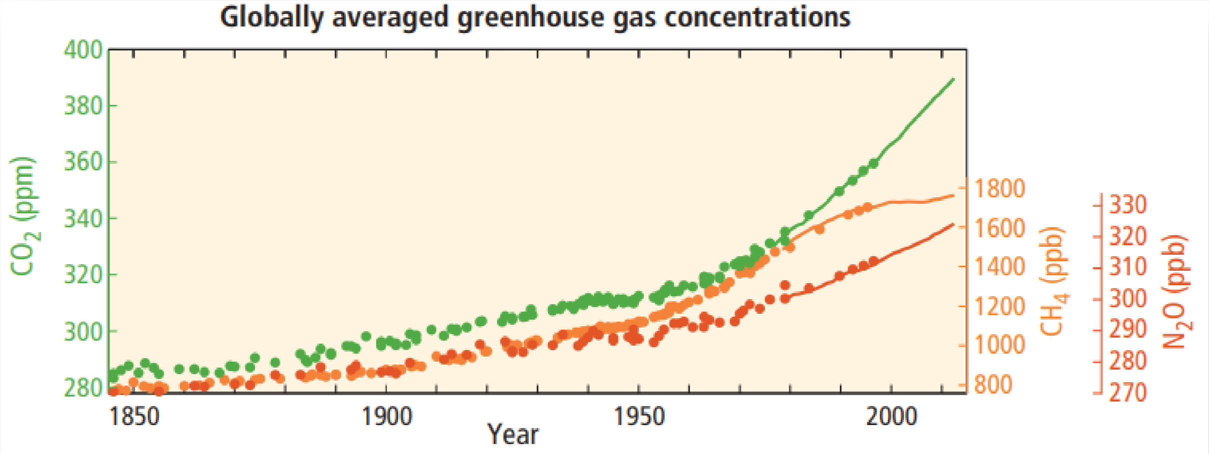

Figure 1: Observed changes in atmospheric concentrations of carbon dioxide (CO_2, green), methane (CH_4, orange) and nitrous oxide (N_2O, red) since 1850 (IPCC, 2014).

Globally, economic and population growth continues being the most important driver of increases in CO_2 emissions from fossil fuel combustion. In the same context, Paehler (2009) reported that the concentration of CO_2 ([CO_2]) has risen since the industrial revolution of the mid 1700s mainly due to human activities that release CO_2 into the atmosphere, such as burning fossil fuels, solid wastes, driving of vehicles and generation of electricity. Excluding emissions from non-energy sources, atmospheric CO_2 emissions would stabilize below 415 ppm. By comparison, scenarios that assume continued expansion of fossil fuel-based infrastructure predict cumulative emissions of 2986 to 7402Gt CO_2 during the rest of this century, leading to increase atmospheric [CO_2] that will be greater than 600 ppm by 2100 (Nakicenovic and Swart, 2000; Meehl et al., 2007). Agriculture has also contributed to increasing greenhouse gases concentrations by increasing nitrogen fertilization amount, submerging land in rice paddies, large herds of cattle and deforestation (Sivaramanan, 2015). In fact, the loss of forest mass causes the loss of an important carbon sink and alters chemical, physical, and biological conditions related to these forest ecosystems, which consequently modulate the general greenhouse gas emissions (Barrena et al., 2013; Stange et al., 2013). Futher, according to the IPCC (2014), agricultural practices are responsible for about 24% of CO_2 gas emissions. Since the "Green Revolution", there have been increases in grain yields through the introduction of external inputs such as irrigation, herbicides, pesticides or fertilisers, and reducing forestry areas for increasing agricultural areas (FAO, 2015).

In order to achieve increases in crop yields, modified land management strategies have been necessary and, therefore, soil biological and chemical processes, such as nitrification, de-nitrification or leaching, have been altered. In view of all these changes, continued emissions of these gases will cause further climate change, including substantial increases in global average surface temperature and important changes in regional climate.

Earth's average surface air temperature has risen by 1°C during last century since 1900 (Figure 2), with other half of increase occurring since the mid of 1970s. Relative to 1850-1900, the projected rise for the end of the 21st century (2081-2100) is ranging between 1.5 and 2°C as determined by different scenarios (IPCC, 2014). In the Mediterranean region, it has been observed that annual mean temperatures are now 1.4°C above late 19th century levels, particularly during the summer months (Cramer et al., 2018) and future warming is expected to exceed global rates by 25%, notably with summer warming at apace 40% larger than the global mean (Lionello and Scarascia, 2018).

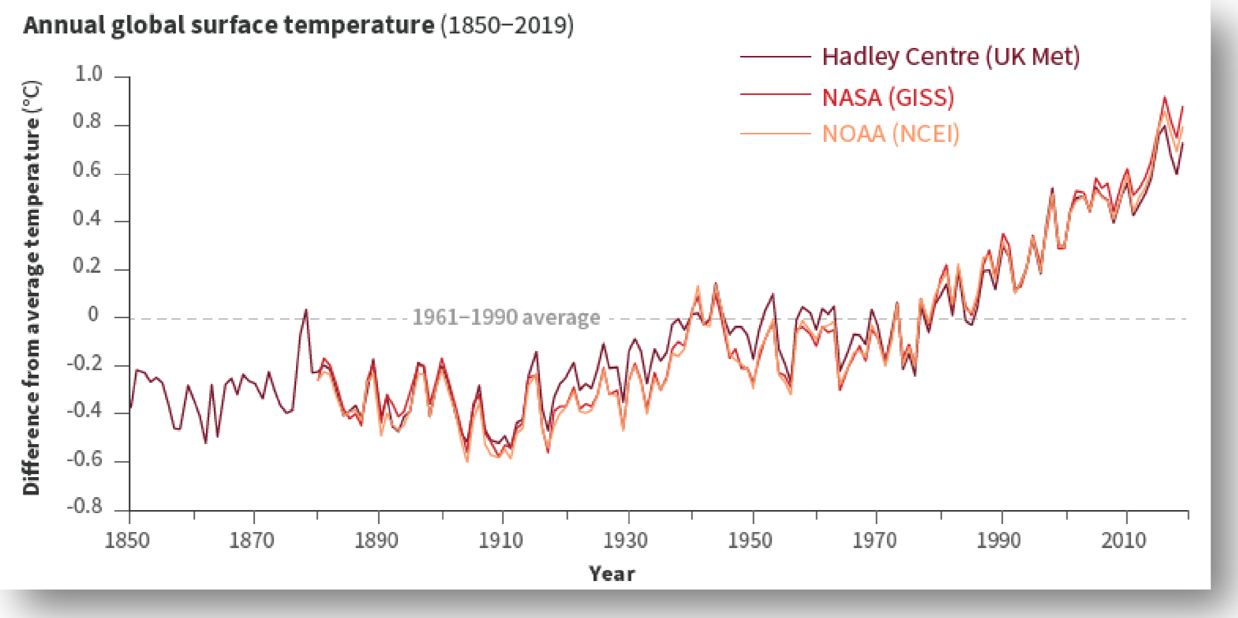

Figure 2: Earth's global averagesurface temperature has risen as shown in this plot of combined land and ocean measurements from 1850 to 2019. The temperature changes are relative to the global average surface temperature between 1961 and 1990 (The Royal Society and the US National Academy of Sciences, 2020).

It is inferred that global climate change induced by increases in temperature could lead to change precipitation patterns and probably raise the frequency of extreme events such as floods and droughts (Olofintoye et al., 2012). Changes in precipitation have already occurred and will not be uniform being dependent on the region (Figure 3). Many scenarios established by IPCC (2014) found that different precipitation patterns are expected to happen regarding to the latitude. Extreme precipitation events over most of the mid-latitude land masses and over wet tropical regions are foreseen to be more intense and more frequent. Saadi et al. (2015) reported that precipitation trends in the Mediterranean area are characterized by high variability in space and in time, but climate models clearly indicate a trend towards reduced rainfall in coming decades. The combination of warming and reduced rainfall generates strong trends towards drier conditions. In this context, Bates et al. (2008) claimed that climate projections suggest that one of the most significant impacts of climate change is likely to be on regional water resources. Principal variables affecting water availability are precipitation, temperature, potential evapotranspiration and strong increase of the population demand. Actually, the frequency and the intensity of droughts have already increased significantly in the Mediterranean basin since 1950 (Vicente-Serrano et al., 2014) and scenarios with 2-4°C temperature increases in the 2080s would imply stronger and more widespread decreases in precipitation of up to 30% (especially in spring and summer months) for southern Europe (Forzieri et al., 2014). On the other side, heavy rainfall events are likely to intensify by 10-20% in all seasons except for summer (Toreti et al., 2013; Toreti and Naveau, 2015). Flood risk, associated with extreme rainfall events, will increase due to climate changes (among other non-climatic factors) and is expected to increase in most areas in the Mediterranean basin with different intensity and timing among countries (Llasat et al., 2013; Gaume et al., 2016).

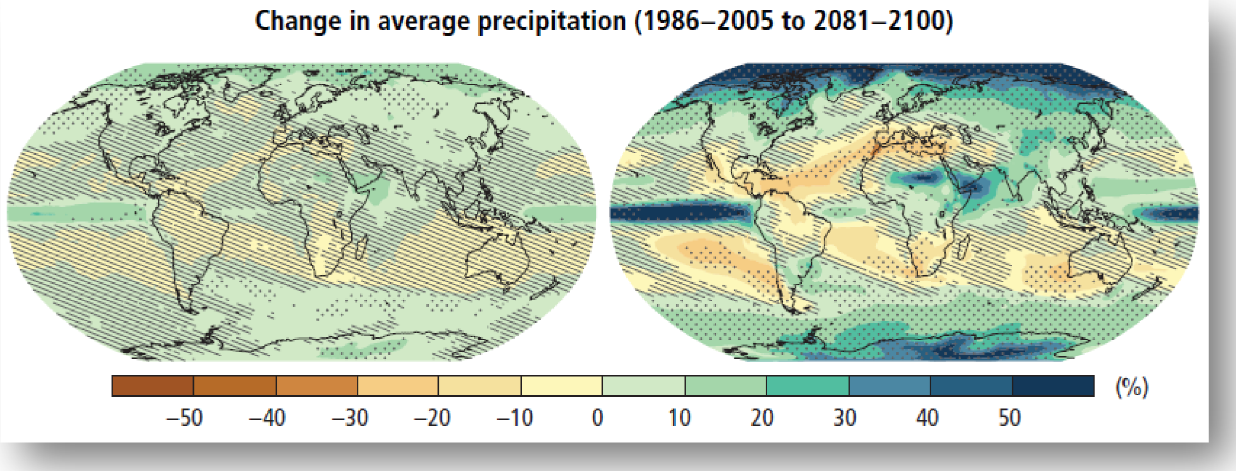

Figure 3: Change in average precipitation based on multi-model mean projections for 2081-2100 relative to 1986-2005 (IPCC, 2014).

Overall, current and expected climate changes may threaten food security of a worldwide-grown population. In fact, global warming is challenging plant growth under stressful environmental conditions impacting consequently crop yields and food quality. Alike other crops, cereal cultivation, in particular wheat, will face serious challenges in the next decades with a need to increase production to meet the predicted production demands of the global population.

II. Wheat: Origin and importance

Wheat is one of the most ancient crops and archaeological evidence of domesticated cereals is dated more than 9000 years BC (Nesbitt and Samuel, 1995). In fact, its domestication marks the transition from hunting-gathering to agrarian economy in western Asia, which marks the dawn of the evolution of western civilization (Dvorák et al., 2012). Wheat has always been considered as staple food for human kind, together with rice and corn, and because of its importance, its origin and evolution has received extensive attentions. Wheat was originated by a polyploidization process, and because of its importance was studied and discussed by several authors (Figure 4) (Bálint et al., 2000; Kilian et al., 2007; Goncharov, 2011; Matsuoka, 2011; Dvorák et al.,2012).

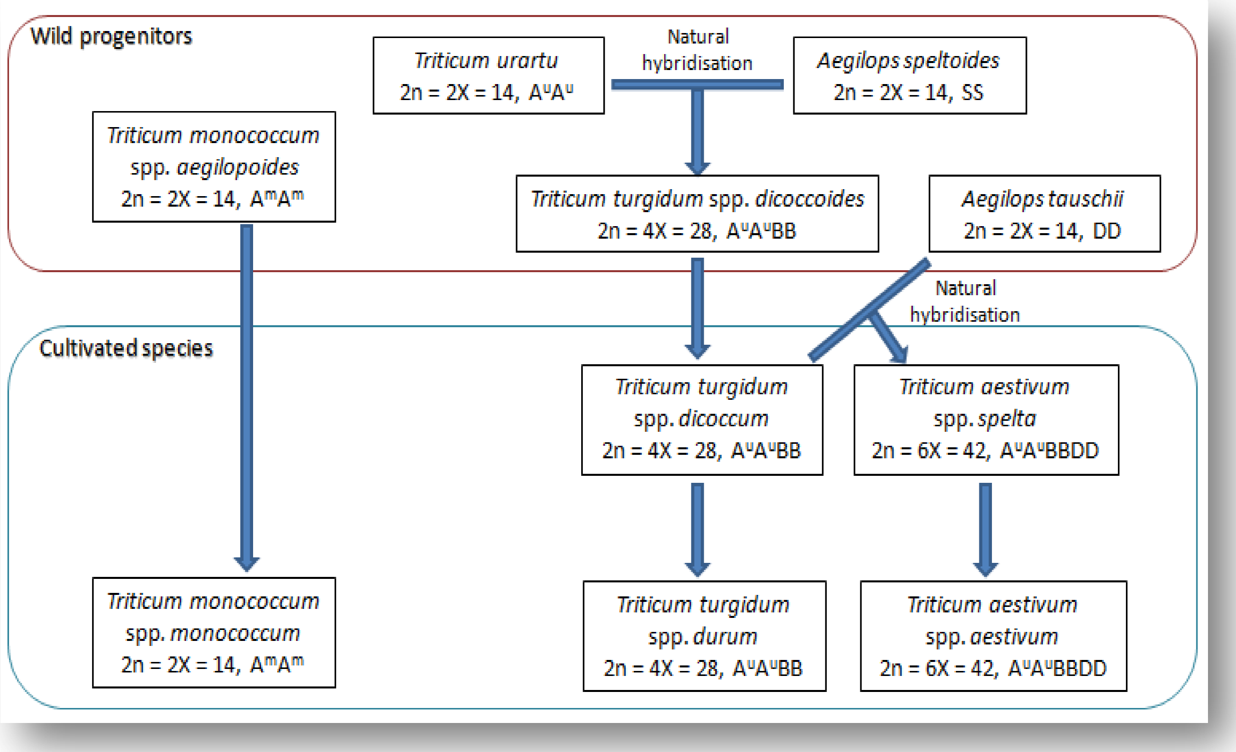

Figure 4: General scheme of the polyploid origin of wheat based on Bálint et al. (2000), Matsuoka (2011), and Dvorák et al. (2012).

Genus *Triticum* L. includes di- (2n=14), tetra- (2n=28) and hexaploid (2n=42) species (Goncharov, 2014). *Triticum monococcum* ssp. *Monococcum* is the only diploid species that has been cultivated and it derives from the domestication of *Triticum monococcum* spp. *aegilopoides* (Faris, 2014). *Triticum urartu* Thum. ex Gandilis the origin of the Agenome in polyploid wheat (Dvorák et al.,1993; Baum and Grant Bailey, 2004). The cross between *Triticum urartu* and *Aegilops speltoides* species originated the wild emmer (*Triticum turgidum* spp. *dicoccoides)* which then gave rise to cultivated emmer (*Triticum turgidum* spp. *dicoccum*). Many studies have suggested *Aegilops speltoides* (2n=2X=14, SS) as the probable donor of B genome and the cytoplasm of most tetra- and hexaploid species (Dvorák et al., 2012; Dvorák and Zhang, 1990, 1992). Then, durum wheat was originated from cultivated emmer (Matsuoka, 2011). Bread wheat was domesticated about 10000 years ago (Dubcovsky and Dvorák, 2007). It arose by spontaneous hybridization of cultivated tetraploid wheat (A^uA^uBB) with the wild species *Aegilops tauschii* (2n=2X=14, DD) in the Caspian Sea area (Dvorák et al., 1998; Wang et al., 2013).

At present, wheat is one of the most important food crops worldwide, which accounts for 30% of global grain production and 45% of cereal food, providing more than 20% of both calories and proteins consumed by world population (Braun et al., 2010; Weichert et al., 2017). Wheat is used to produce a wide number of products including bread, cookies, breakfast cereals, pasta, noodles, couscous, and beer, among others. Furthermore, wheat is utilized in animal feeding, in cosmetic and pharmaceutical industries and as raw material to produce bioethanol. Products derived from wheat grain are an important source of carbohydrates, protein and fiber. In addition, they provide a low amount of lipids (saturated, mono-unsaturated and poly-unsaturated) and a wide range of minerals (calcium, iron, magnesium, phosphorus, potassium, sodium, and zinc) and vitamins (thiamine, riboflavin, niacin, vitamin B-6, folate, vitamin E-alpha-tocopherol, and vitamin K-phylloquinone) as reported by United States Department of Agriculture Food Composition Databases (https://ndb.nal.usda.gov/ndb/). According to Food and Agriculture Organization (FAO, 2014), China is the major wheat producer reaching 17.3% of world production, followed by India (13.0%), Russia (8.2%), United States of America (7.6%) and France (5.4%). Wheat accounts for 31.5% of total cereal production in Spain, and in 2014, Spain ranked as the twenty-second wheat producer with a production level of 6,471,400 tonnes from a total harvested area of 2,171,200 ha. Despite wheat is a dominant crop in temperate areas, it exhibits a huge adaptability across different environments and it represents the most widely grown food, reaching aglobal harvested area of almost 222 Mha in 2014 (FAO, 2014). Almost 60% of its production is concentrated in the Mediterranean region due to its better adaptation to climates with warm temperatures and low rainfall, specifically in Italy, Spain, France and Greece (Lidon et al., 2014). Durum wheat (*Triticum turgidum* subsp. *durum* Desf.) is a minor cereal crop representing 5% of the total wheat crop cultivated worldwide (about 17 million ha) and the production was about 38 million tonnes in 2019 (Xynias et al., 2020). Currently, some hybrids between wheat and other cereals have presented some commercial interest, such as Triticale (*Triticum aestivum* × *Secale cereale*) or Tritordeum (*Triticum turgidum* × *Hordeum chilense*).

III. Tritordeum: Origin and importance

The development of crosses between wheat and barley has been a major goal for wheat breeders since it would allow the incorporation of favorable traits from barley to wheat. The first amphiploid between *Hordeum chilense* and *Tritirum aestivum* (cv. Chinese Spring) was obtained by Martín and Chapman at the former Plant Breeding Institute in Cambridge (UK) (Martín and Chapman, 1977), but the fertility of this octoploid tritordeum was very low ($2n=8X=56$, $H^{ch}H^{ch}AABBDD$). In contrast, hexaploid tritordeum ($2n=6X=42$, $H^{ch}H^{ch}AABB$) derived from the cross between *Hordeum chilense* and *Triticum turgidum* conv. *Durum* showed good fertility. Tritordeum (x *Tritordeum* Ascherson et Graebner) is a fertile amphiploid produced from hybridization between durum wheat (*Triticum durum*) and a wild barley species of Chilean origin (*Hordeum chilense*) used as a maternal parent, which in recent years has been introduced to European markets as an alternative to traditional cereals (Martín et al., 1999). Tritordeum has been subject of a breeding programme to become a novel cereal species crop, and it has also been used as a genetic bridge for transferring useful barley traits like storage proteins or carotene content to wheat (Martín et al., 1999; Ballesteros et al., 2005). It shows higher protein content than wheat parent and exhibits similar grain and flour nutritional characteristics as wheat (Cubero et al., 1986; Alvarez et al., 1992). It is also characterized by having 5.2 times more carotenoids than durum wheat inherited from wild barley (Atienza et al., 2007; Mellado-Ortega and Hornero-Méndez, 2015). Additionally, tritordeum shows high adaptability in the Mediterranean basin (Villegas et al., 2010). In summary, tritordeum was eventually created to replace wheat due to its higher nutritional value as well as its lower gluten levels (Vaquero et al., 2017). Regarding to grain yield, tritordeum has been found to have similar yield as wheat and especially to triticale under limiting water availability conditions. However, under better growing conditions, tritordeum yield was shown to be lower than wheat and triticale (Villegas et al., 2010). In addition, tritordeum revealed higher stomatal conductance and photosynthesis rate under water deficit conditions (Gallardo and Fereres, 1989). Therefore, this species may have interest to be cultivated in the Mediterranean region due to its drought resistance.

IV. Impact of environmental conditions related to global warming on wheat performance

The impacts of increasing [CO_2], elevated air temperature and changes in rainfall patterns resulting from climate change are expected to adversely affect crop growth and yields in many parts of the world (Travis and Daniel, 2010; Beddington et al., 2012; IPCC, 2013; Valizadeh et al., 2014). Therefore, assessing the impact of different abiotic stresses simultaneously on plant development is a matter of major concern in agriculture in order to estimate the potential impacts of projected climate conditions. Cereals, in particular wheat, are staple food crops that are widely cultivated in the Mediterranean region and other semi-arid areas of the world where climate is characterized by high evaporative demand in late spring when precipitation is low, considerably enhancing the risks of occurrence of severe water deficit during flowering and grain filling of wheat crop leading to strong grain yield limitation (Costa et al., 2013; Páscoa et al., 2017). Moreover, predictions made by IPCC (2014) expect also an augmentation in ambient [CO_2] associated with climate change. Although it is predicted that under optimal growth conditions the atmospheric [CO_2] increases should increase cereal production, grain quality will be negatively affected within a climate change scenario, which might have strong impact on nutritional value of flour and consequently on human health.

1. Effects of water deficit, heat stress, and increasing atmospheric [CO_2] on plant growth and grain yield components

Growth and development of crops are permanently under the influence of various environmental factors, and abiotic stresses are among the main factors reducing crop yields all over the world (Franklin et al., 2010).

Water deficit. One of the most important factors limiting plant growth is the lack of water (Bannayan et al., 2008), being the well-known survival limit factor in arid and semi-arid areas (Chavez et al., 2003). In fact, the projected warming and drying trends over the Mediterranean region represent a substantial threat for wheat production (Yang et al., 2019). In particular, durum wheat (*Triticum durum* Desf.) is usually grown under rainfed conditions where fluctuations in precipitation have caused water scarcity to act as a major limiting factor for crop productivity, especially when drought stress occurs before and after flowering. In general, crops require water in adequate quantities for their optimum growth. For instance, under particular climatic conditions, wheat requires different amounts of water during its stage of growth. Changes in rainfall pattern would lead to imbalances between crop water needs and rainfall during vegetation and have a strong impact on yields and the quality of agricultural products. According to Mulholland et al. (1997), drought stress during the vegetative and reproductive development reduces wheat tillering. Additionally, water scarcity during grain filling affects mainly current assimilation through reduction in both photosynthetic area and activity (Mulholland et al., 1997). It has been reported that drought stress negatively affects the grain-filling period, plant height, peduncle length, number of spikes per plot, number of grains per spike, thousand grains weight, grain yield, biomass, and harvest index of different durum wheat genotypes (Pour-Aboughadareh et al., 2020). Significant decreases in grain yield of durum wheat and bread wheat, due to drought stress, have also been observed by Liu et al. (2015) and Qaseem et al. (2019), respectively. Similarly, Villegas et al. (2010) found that, under Mediterranean drought conditions, tritordeum showed a decrease in grain yield mainly due to low kernel weight. Furthermore, plant growth is also affected by rising temperature since the latter combined with drought stress directly impact photosynthetic rate and plant metabolism (Matthew, 2010). Harvest index may also be reduced if reproductive processes are impaired by heat stress occurring at critical developmental stages (Gordon, 2009; Matthew, 2010).

Heat stress. Heat stress that occurs at late sowing could cause a reduction in the duration of late growth phases. It has been reported that for 1°C temperature rise, wheat growth duration is negatively affected and a wheat yield 10% reduction is recorded (Mulholland et al., 1997; WMO, 2010). According to Shewry (2009), the optimum temperature for wheat anthesis and grain filling ranges from 12 to 22°C. High temperatures reduce grain setting, increase the grain-filling rate and decrease the duration of grain filling, thus resulting in lower yield (Ritchie et al., 1998; WMO, 2010). For wheat, the number of tillers decreases in response to high temperatures, especially high night-time temperatures. Under such conditions, shoot elongation is promoted, but more immature grains are produced and yield decreases because of dark respiration (Masahumi et al., 2011). Under field conditions, when heat and drought stresses were combined, kernels with shrivelled appearance were observed in wheat by Schmidt et al. (2020). This could explain the decrease in wheat grain yield under current and predicted future climatic conditions.

CO_2 concentration ([CO_2]). On the other hand, the rise in atmospheric [CO_2] is predicted to increase crop production and modify, in some way, the adverse effects of global warming. It has been established that rising [CO_2] stimulates plant growth. The effect of [CO_2] on plant growth, and especially on photosynthetic performance, has been extensively studied during recent decades (Ainsworth et al., 2002; Long et al., 2004; Ainsworth and Rogers, 2007; Aranjuelo et al., 2008, 2011; Leakey et al., 2009; Rogers et al., 2009). Although the current [CO_2] in the atmosphere is generally limiting for C_3 photosynthesis, available information suggests that the predicted CO_2 increase will enhance photosynthetic rates in plants (Farquhar et al., 1980; Bowes, 1993; Long et al., 2004). However, the initial stimulation has frequently been described to be partially reversed in a process of acclimation (Ainsworth et al., 2002; Long et al., 2004; Ainsworth and Rogers, 2007; Leakey et al., 2009; Rogers et al., 2009). For wheat and other C_3 crops, reduction in transpiration under enhanced CO_2 due to stomatal closure results in higher water use efficiency and grain yield (Attri and Rathore, 2003). In addition, grain capacity to accumulate assimilates (i.e. sink strength) has been described to be more important in determining wheat yield potential than the supply of assimilates (i.e. source strength) (Borrás et al., 2004). Carbon source-sink relationships are believed to play a major role in determining the ability of a plant to use CO_2 by preventing the downward acclimation of photosynthesis upon prolonged exposure to CO_2 (Kirschbaum, 2010). The downward acclimation determines the maximum carboxylation rate of the key enzyme Rubisco (Leakey et al., 2009) while the activity levels of major carbohydrate metabolism enzymes have shown a different behavior in source and sink organs. All these processes usually result in an increase in the net assimilation so that the wheat grain yield and biomass are enhanced (Li et al., 2019). However, it should be mentioned that plant biomass and grain yield do not increase in the proportion as the net photosynthetic rate (A_n), as a consequence of the acclimation process. The increase in wheat grain yield was also explained by an increase in spike number rather than to an increment in thousand-grain weight, as reported by Tausz-Posch et al. (2012). Similarly, Sabella et al. (2020) found a similar trend for some durum wheat genotypes although no change was detected in one of the cultivars.

Also, Verrillo et al. (2017) found no significant change in thousand grain weight in winter wheat under elevated [CO_2].
These results suggest that this yield component could be controlled in some part genetically, depending on plant response to climatic conditions.

Interacting environmental factors. In addition to C sink strength, several studies remark how environmental parameters interactions have a direct effect on wheat grain yield (Fujimura et al., 2012; Chavan et al., 2019; Yang et al., 2019; Sabella et al., 2020). While the impacts of individual stress factors have been investigated, the interaction among them has received (comparatively) less attention (Lobell, 2012). In fact, when increased atmospheric [CO_2] is studied as a single factor, crop production tends to increase, but under field conditions, various stress factors can occur simultaneously, such as water deficit and high temperature, which could mitigate the positive effect of high [CO_2] on plant yields. For instance, higher temperature during vegetative phase enhances the effect of elevated [CO_2] levels. Total biomass and grain yield increased by 7% per 100 ppm of CO_2 enrichment, compared to yields obtained under ambient atmospheric [CO_2]. The additive effect of elevated [CO_2] and predicted temperature change will result in a 25% to 30% increase of the current potential yield level (Mulholland et al., 1997). However, temperature increase by 1-2°C during the total growth period and 2-3°C during the reproductive phase were sufficient to negate the grain yield increase due to doubling of ambient atmosphere [CO_2] (Mulholland et al., 1997; WMO, 2010). In the same line, Chavan et al. (2019) demonstrated the interactive impacts of elevated [CO_2] and severe heat stress at anthesis on wheat grain yield and found that, despite of biomass and photosynthesis stimulation, a drastic reduction in grain yield was detected due to grain abortion and reduced grain-filling period. Moreover, Yang et al. (2019) assessed winter wheat yield response to potential climate change and found lower yield stimulation under high [CO_2] than that reported by Kimball (2016) under lower CO_2 enrichment. The limited yield response may be explained by the fact that a projected higher temperature above the optimum growth range could partially offset CO_2-induced stimulation of photosynthesis (Yang et al., 2019).

Overall, the response of crop growth to enhanced [CO_2] in the atmosphere is known to depend on climatic conditions and it is difficult to quantify due to complexity of the physiological and environmental processes involved. A common trend towards yield reduction in major cereal crops is being observed despite the existence of breeding programs aimed at developing new genotypes that are more efficient under limiting conditions, and this reflects the combined impact of all environmental factors on global cropproduction. Therefore, in-depth investigations are needed to evaluate the relationship of high [CO_2] with different climatic factors. In addition, targeting genotypes with responsive capacity to increase some yield-related traits under elevated atmospheric [CO_2] could help to mitigate the negative effects of simultaneous stress conditions.

2. Effects of water deficit, heat stress, and increasing atmospheric [CO_2] on nutrients remobilization during grain filling and grain nutritional quality

Another major point of concern is the effect of climate change in grain quality. While cereal breeding is already focusing much more on yield traits, comparatively little attention has been given to grain quality traits. This is a matter of great concern because available studies show that abiotic stress will affect negatively starch (representing 60-70% of grain dry weight), protein, lipid, and mineral composition (Ziska et al., 2012; Loladze, 2014; Goicoechea et al., 2016).

Water availability is an environmental parameter that influences leaf phenology and photosynthetic rate, thus, affecting plant growth, nutritional status and the stoichiometric ratios (Ye et al., 2014). In fact, water deficit affects the uptake and utilization of nutrients and changes the proportion of nutrients in tissues, leading to the imbalance of nutrient elements and affecting other biological activities and metabolic balance (Yan et al., 2020). Adding to that, drought stress accelerates leaf senescence, increases the remobilization of carbon reserves from vegetative tissues to the grains leading to shortening grain-filling period and accelerating grain-filling rate resulting in poor grain setting and grain development (Farooq et al., 2017). Pour-Aboughadareh et al. (2020) indicated that drought stress decreased the grain-filling period of durum wheat by 14% as compared to the control condition. It should be mentioned that grain-filling period is an important phenological trait that is associated with current photosynthesis and relocation of assimilates from reserve pools in vegetative tissues (Pour-Aboughadareh et al., 2020). In the Mediterranean basin, grain filling of cereal crops usually coincides with a progressive increase in drought and temperature during late spring (Acevedo et al., 1999). According to Farooq et al. (2011), grain-filling phase in wheat is very sensitive to high temperature.

Similarly to drought, heat stress generally accelerates the rate of grain filling and shortens the grain-filling duration (Dias and Lidon, 2009). In line with this, Yin et al. (2009) reported that grain-filling duration in wheat may be decreased by 12 days with the increase of 5°C temperature above 20°C. Under such conditions, wheat grain quality is negatively affected essentially because of limitation of assimilates and less remobilization of nutrients.

Grain setting and filling can also be restricted by source and/or sink limitations (Lipiec et al., 2013). When photosynthesis is inhibited by heat stress, stem reserves during pre-anthesis period are the main source of carbon during grain filling (Mohammadi et al., 2009). In fact, high temperature at the pre-anthesis period increased carbohydrate translocation from stem to grain leading to remarkable increase in total soluble sugar (Sumesh et al., 2008; Wang et al., 2012; Asthir and Bhatia, 2014). Grain protein concentration is an important end-use suitability factor in wheat due to its significant effect on cooking quality (Suprayogi et al., 2011), which is affected by crop variety (Baenziger et al., 1985; Rao et al., 1993), air temperature (Bahuguna et al., 2017), water availability (Yadav and Ellis, 2017), nitrogen fertilization (Blacklow and Incoll, 1981), and many other factors. It has been reported that grain nitrogen content varied inversely with the water availability (Clarke et al., 1990). Actually, drought stress had relatively greater impact on grain yield reduction, starch synthesis and grain nitrogen content increase (Farooqet al., 2017; Galindo et al., 2018). Similarly, Triboi et al. (2006) concluded that grain protein concentration increases with drought stress and higher temperatures as a result of reduced starch accumulation. In addition, Castro et al. (2007) found that wheat plants subjected to early heat stress at grain filling had high grain protein content. According to Dias et al. (2008), increased grain protein content is associated with sedimentation index and intensity of essential amino acids.

Among other factors, micro- and macronutrients accumulation in grains is also controlled by the surrounding environment. The effect of drought stress on mineral accumulation was studied by Fischer et al. (2019), comparing food crops in two different regions of East Africa. They found that severe drought caused a decrease in nutrients, while mild drought increased their concentrations. This shows that the nutrient accumulation is dependent not only on the type of abiotic stress but also on its intensity level. The effects of combined water and heat stresses were studied by Velu et al. (2016) in 54 field-grown wheat varieties and found that grain zinc (Zn) concentration was higher under heat and drought stress conditions, whereas a lower increase of grain iron (Fe) was observed under low water availability environments. Moreover, nutrient assimilation, translocation, and remobilization are also affected by increasing [CO_2]. In addition to quantitative changes in crop production and photosynthesis, high[CO_2] leads to modifications in leaf biochemical composition and grain quality. Kernel nitrogen content is largely conditioned by the amount of N remobilized from sowing to late heading reserves accumulated in the shoot, and is also supplemented by the amount of N absorbed by root system from anthesis to kernel maturity (Kichey et al., 2007). In this matter, leaves are classically considered as the main N contributor to kernels due to their large protein content. The fact that Rubisco might represent up to 50% of the total soluble protein and 25% of the nitrogen content in leaves (Parry et al., 2003; Aranjuelo et al., 2013) implies that it can be considered as a major N storage form. It has been reported by Zhou et al. (2016) that the contribution of Rubisco degradation derived N remobilization was different depending on the analysed genotypes and atmospheric [CO_2]. Decreases in Rubisco content have been widely described in plants exposed to elevated [CO_2]. It has been observed that elevated [CO_2] with warming conditions lowered protein concentration in wheat leaves (Jayawardena et al., 2020), which suggests that the low protein levels were due to the inhibition of N and NO_3^- assimilation under such conditions. Different hypothesis have been proposed as responsible of depleted N metabolism in crops exposed to elevated [CO_2]. More specifically, depleted N availability has been associated with processes such as inhibition of photorespiration, transpiration, and the relocation of N within the plant (Rachmilevitch et al., 2004; Bloom et al., 2014; Aranjuelo et al., 2013, 2015; Jauregui et al., 2015).

In addition, under high [CO_2] and as phenology advances, photosynthetic acclimation was explained, in part, by a decrease in Rubisco protein content (Aranjuelo et al., 2015) due to the reallocation of leaf N to ears during grain filling. On the other hand, leaves invest more carbon and are generally less N-rich with higher C/N ratio and lower amount of proteins involved in photosynthesis (Rubisco) (Zhang et al., 2009; Zhu et al., 2009; Aranjuelo et al., 2015; Pandey et al., 2017). When grown under elevated [CO_2], a faster decline in flag leaf nitrogen occurred due to earlier senescence while photosynthesis capacity is increased in ears (Sicher and Bunce, 1998; Zhu et al., 2009). Therefore, the changes in nitrogen and carbon metabolism at the vegetative stage are likely to have a direct impact on determining final wheat grain quality.

It has been reported in many previous studies that wheat grains produced at elevated [CO_2] have generally lower nutritional quality, with lower nitrogen content, less protein, and more starch and fibres (Kimball et al., 2001; Högy et al., 2013; Wroblewitz et al., 2013). It is also worth noting that high [CO_2] causes an increase of free hexoses (fructose, glucose) but less sucrose. Free amino acids concentrations are also reduced, except for valine, leucine, and isoleucine that are significantly more abundant (Wroblewitz et al., 2013; Soba et al., 2019). A decline in the content of several microelements in wheat grains such as iron (Fe) and zinc (Zn) is also observed under atmospheric CO_2 enrichment (Wroblewitz et al., 2013; Pandey et al., 2017; Beleggia et al., 2018). Similarly, Loladze (2014) described a significant reduction in overall mineral concentration by 8% in C_3 plants grown under FACE system, including foliar and edible tissues. Precisely, CO_2 enrichment decreased Fe, Zn, and copper (Cu) concentrations by 6-10%, with manganese (Mn) showing no significant changes. The mechanisms responsible for the overall decline of plant mineral concentrations under elevated [CO_2] conditions are not completely elucidated, but some ideas are suggested by some authors. McGrath and Lobell (2013) claimed that the decrease in transpiration rates reduces mass flow of nutrients, and shifting nutrient allocation driven by altered biochemical processes between tissues can both change nutrient uptake. Adding to that, Poorter et al. (1997) and Loladze (2002) disagreed with the idea that “carbohydrate dilution” could elucidate grain mineral reduction because of the heterogeneous response of each mineral tested for a given crop or for different species.

In general, drought and heat stresses, and increasing [CO_2] have been described to affect negatively flour/dough quality parameters with an impoverishment of grain nutritional value. The interaction effects between these three factors on wheat grain quality are barely reported, but available information foresees major shifts in the grain composition that could have serious impact on human health. In this way, a better understanding of the physiological and molecular processes involved in the response to multifactor growing conditions that are limiting crop performance, will help tothe development of new strategies and tools for enhancing stress tolerance, thus, improving the nutritional performance of grains subjected to climate change. In addition, germplasm screening to identify varieties that have an efficient use of nutrients under changing climate is mandatory to enhance both grain yield and quality.

It is important as well to mention that all the reported changes in grain quality under climate change scenario are not only depending on cultivation conditions, but also on wheat species and cultivars, soil properties, and fertilization management such as the timing and the amount of nitrogen fertilization.

V. Soil nitrogen sources contribution to crop production and quality

Nitrogen (N) has an important role for plant development and its demand is higher than that of any other mineral nutrient (Nunes-Nesi et al., 2010). Since the ‘Green Revolution’, the production increased exponentially due to the combined effect of genetic improvement and new agronomic techniques with a massive use of fertilizers, mainly as nitrogen (Figure 5), which is expected to increase by another 40-50% over the next 40 years (Tamang et al., 2017). However, depending on the crop species and cultivar, only about 30% to 50% of the applied nitrogen fertilizer is absorbed by the growing crop in the year of application (Beckie and Brandt, 1997; Peoples et al., 2004). Therefore, a key challenge is to satisfy crop nitrogen requirements while minimizing nitrogen losses to maintain a sustainable environment and economic benefits to farmers (Lakesh and Sukhwinder, 2018).

1. Application of nitrogen fertilizers and impacts on environment

Since the discovery of nitrogen as an essential nutrient for plants and the capacity of legumes for fixing atmospheric N_2 by Jean-Baptiste Boussingault in 1836, many efforts have been driven to amplify our knowledge of the N cycle (Galloway et al., 2013). Farmers from the 19th century usually employed crop rotation with legumes, organic fertilisation with manures or mineral nitrate deposition to obtain the nitrogen necessary for crop production. However, it was not until 100 years later that the Haber-Bosch process permitted industrial quantities of ammonia production and enabled increasing nitrogen inputs in agriculture

(Galloway et al., 2013). The N input in agricultural soils permits increased crop production, improves soil fertility and maintains sustainable soil productivity (Papastylianou, 2007). Adding to that, climate change is predicted to have an impact on the stability of agricultural productivity and crop yields. Therefore, nitrogen supply is an important driver to meet the demand for agricultural output of an increasing world population and to ensure food security.

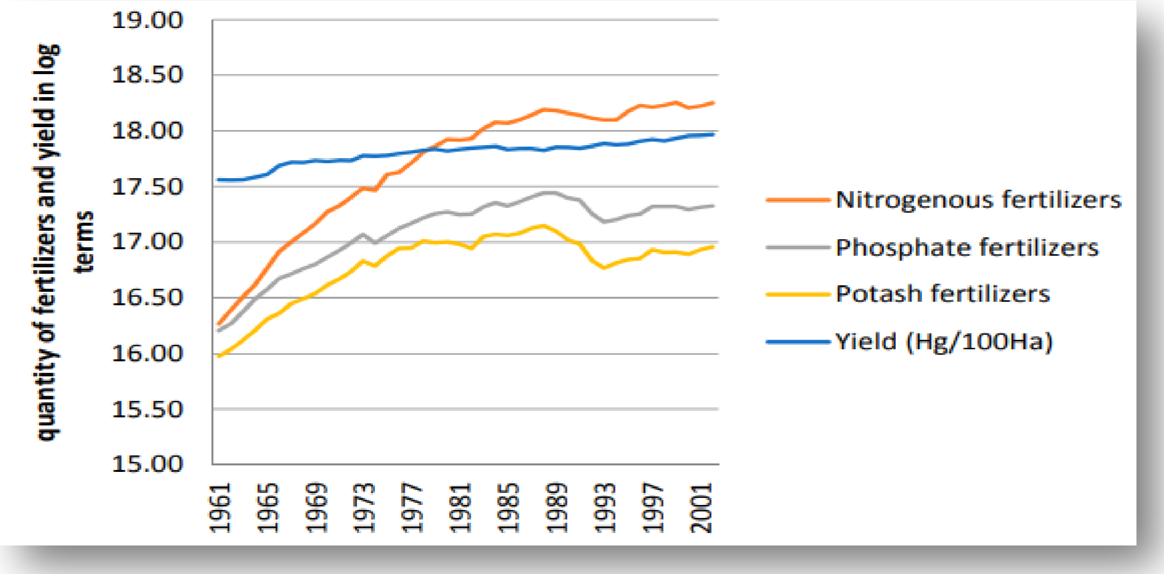

Figure 5: Fertilizer consumption in the world- yield relationship (Erbas and Solakoglu, 2017).

To reduce the likelihood of crop N deficiency and ensure high crop yield and quality, it has been common for farmers all over the world to apply N fertilizer in excess (Peng et al., 2011). Actually, the use of nitrogen fertilizer has increased dramatically worldwide from 112.5 million tons in 2015 to about 118.2 million tons in 2019 (Lakesh and Sukhwinder, 2018). However, the excessive use of nitrogen fertilizers represents a major source of greenhouse gas emission and increases the risk of aquifer pollution. The intensive use of nitrogen fertilizer enhances N losses in the form of NO_3^- leaching, ammonia volatilisation (NH_3) or emission of nitric oxide (NO), nitrous oxide (N_2O) or atmospheric nitrogen (N_2) gas. Besides, with a high amount of fertilizer, the remain quantity of N in the soil at the end of the growing season can be absorbed by the following crops, or lead to negative environmental impacts such as nitrate leaching, greenhouse gas emission, and eutrophication of water bodies (Lyngstad, 2009; Lingan et al., 2014; Kong et al., 2017). In fact, nitrate leaching and water pollution are major environmental problems in Europe, the USA, and China (Yang et al., 2006; Hangs et al., 2013), and according to the European commission (1991), most of European Union countries have restricted the rate of N application to 170 kg N ha^{-1} per year.

It has been reported that an average of 2% of the nitrogen applied to cultivated soils is emitted into the atmosphere as N_2O, thus, inappropriate synchronization between N fertilizer application and crop demand would lead to a further increase in emission rates (Iqbal et al., 2008). While the increase in the amount of nitrogen fertilizer might help farmers to adapt to global warming, this change in and of itself has increased N_2O emissions from agriculture (Erbas and Solakoglu, 2017). Therefore, it is essential to adopt alternative nitrogen fertilizer management (adequate timing of application) to optimize nitrogen use efficiency (NUE) by reducing the input of N fertilizer and increasing the N absorption of crops in order to improve the sustainability of crop production. It is worthy to mention that the efficiency of nitrogen for grain production is higher in low quantities compared to higher quantities (Moll et al., 1982). In fact, NUE is a key element for management of nitrogen application in crop production and has been defined in several ways; the most simple is the ratio of grain yield to nitrogen uptake (Goodroad and Jellum, 1988). The value of NUE in cereals needs to be improved by nitrogen management or breeding methods, maintining or increasing grain yields (Beatty et al., 2010), indeed, coordinated efforts are required to increase N uptake, assimilation and/or remobilization efficiency for improved NUE (Kant et al., 2012). Hence, developing crop varieties with a higher NUE would reduce N loss and decrease input cost (Kant et al., 2011). Together with the genetic variation and agronomic management, environmental conditions are considered as a constituent component that may also affect the efficiency of nitrogen recovery from the available nitrogen, thus, targeting the applied nitrogen fertilizer in response to specific environmental parameter (drought stress, high temperatures or elevated [CO_2]) could also contribute to NUE improvement (Hoseinlou et al., 2013).

2. Effects of nitrogen supply on plant growth and grain yield components

Nitrogen (N) is one of the major nutritional elements of wheat and other crops, and is widely used to increase yield and improve end-use quality (Cao et al., 2008). In fact, wheat grain yield is mainly influenced by nitrogen application rate and timing, the climate, and cultivar (among other factors), as well as by the interactions between these factors (Nakano et al., 2008). Nitrogen fertilization increased wheat biomass (Boukef et al., 2013) since nitrogen is a constitutive component of chlorophyll and proteins affecting photosynthesis process (Tranavicienė et al., 2007), resulting in higher yields. Kursheed and Mahammad (2015) studied the effect of three nitrogen fertilizers on durum wheat and found that all growth parameters and yield components had significantly increased (plant height, number of tillers, flag leaf area, shoot dry weight, leaf chlorophylls, number of spikes, thousand-grain weight, and grain yield). It has been suggested by Ali et al. (2011) that nitrogen increased tryptophan amino acid, which is essential in auxin (Indol- 3-acetic acid) synthesis that is responsible for cells elongation and plant height. The increase in leaf area was due to an increase in length and width of the leaves, which could be related to a nitrogen effect on cell division via increases in auxin (Bojovic and Markovic, 2009). Moreover, it has been reported by Nakano et al. (2008) that nitrogen fertilizer supply at active tillering increased the spikes number of bread wheat increasing thereby grain yield. However, nitrogen application at anthesis and after heading did not increase wheat yield (Takayama et al., 2004; Nakano et al., 2008). On the other hand, increasing the amount of nitrogen fertilizer enhances wheat grain yield, but to a certain extent (Xu et al., 2020). Similarly, Agegnehu et al. (2016) found that N fertilizer application could increase crop yield, whereas excessive N fertilizer application led to yield reduction. In addition, under irrigated conditions, nitrogen fertilization prolonged the vegetative growing period of wheat, delaying consequently the heading date (Shekoofa and Emam, 2008).

Nitrogen supply at different timing of plant growth has also an effect on root system development. According to De Giorgio and Fornaro (2012), high amount of nitrogen fertilization during the first phases of plant development does not stimulate to deepen the root system of durum wheat, and consequently the capacity to take up water and nitrogen from deeper soil layers is reduced, therefore it is more prone to suffer water deficit stress. However, without the addition of fertilizers at first stages of growth, wheat shows stunted growth but pushed its roots down deeper afterwards to recover water resources and the necessary nutrition to satisfy its needs. This allows the plant to overcome water deficit and heat stresses and complete the process of grain ripening with greater uniformity.

3. Effects of nitrogen supply on grain quality

Nitrogen is a crucial mineral element and is involved in several fundamental compounds (amino acids, nucleic acids, chlorophyll, cytokines, polyamines, and secondary metabolites) essential for the biological plant cycle (Yadav et al., 2017). Total nitrogen and protein in durum and bread wheat grains were increased due to an increase in nitrogen uptake under suitable water availability conditions (Weber et al., 2008; Kursheed and Mahammad, 2015; Litke et al., 2018). In addition, changes in grain protein composition may occur under different nitrogen fertilization rates and are also influenced by environment conditions and interactions with nitrogen supply and genotype (Zhu and Khan, 2001; Stepień and Wojtkowiak, 2011). Among grain proteins, gluten content of bread wheat was positively affected by nitrogen fertilization (Litke et al., 2018). Wheat grain quality mainly depends on the content and composition of gluten proteins (gliadins as monomeric proteins and glutenins as polymeric proteins) (He et al., 2005; Anjum et al., 2007). Some studies on bread wheat have suggested that high doses of nitrogen tend to increase the amount of monomer proteins (Zhu and Khan, 2001; Kindred et al., 2008) and to reduce the percentage of polymeric ones causing an increase in the extensibility of the dough (Johansson et al., 2004; Ferrise et al., 2015). In the Mediterranean climate, rainfall variability together with the frequency of high temperature during grain-filling period influence crop responses to nitrogen fertilization, which depend on rainfall amount and distribution during the crop cycle and to the timing and rates of nitrogen applications (Cossani et al., 2012; López-Bellido et al., 2012). In this context, Gagliardi et al. (2020) found that durum wheat genotypes subjected to high temperature during grain filling had low yield and thousand-grain weight together with high protein concentration. Similarly, Triboï et al. (2006) and Ferreira et al. (2012) reported that a moderate high temperature during grain filling (between 25 and 35°C) and short periods of very high temperature (>35°C) at the end of grain-filling phase are frequently associated with a decrease in grain yield and an increase in grain protein concentration. A positive effect of nitrogen fertilization was obvious for grain protein content than for grain yield as reported by Giuliani et al. (2011) and Bouacha et al. (2015) under Mediterranean climate. Oppositely to total protein, grain starch content tended to decrease as a response to increasing nitrogen application as reported by Litke et al. (2018). In addition, wheat grain concentration of P, K, Ca, Mg, Mn, and Zn declined as crop yields increased in response to nitrogen fertilization. Similarly, Dolijanović et al. (2019) claimed that small dose of nitrogen fertilizer had better impact on the concentration of macro- and micro-elements in wheat grain. However other studies showed that larger doses of nitrogen supply increased the micro-element content in bread and durum wheat (Kutman et al., 2011; Klikocka and Marks, 2018).

Overall, it is clear that nitrogen has a crucial role in the vegetative growth and crop quality of wheat and a better understanding of the interactions between nitrogen and other factors is fundamental to improve the efficiency of fertilizer application under challenging climate.

VI. Exploting genetic diversity to increase cereal adaptation to climate change

Rapid human population growth, climate change, and the need to balance increasing agricultural production with increased environmental sustainability make it necessary to optimize the use of available resources (Sansaloni et al., 2020). Actually, since the mid-20th century, genetic improvement has contributed markedly to sustaining growth in crop yields, but, in spite of this achievement, genetic gains still fall short of meeting the predicted demands by 2050 (Rosegrant and Agcaoili, 2010) and the current genotypes are unlikely to be the best varieties in the future which represents a serious challenge for food security especially in the light of the challenges associated with climate change. Therefore, exploring genetic resources such as landraces or in wild relatives of domestic crops may be a useful tool in breeding to find traits that confer better adaptation to changing climate and could be incorporated into new varieties with better tolerance to increased stresses (Heisey and Rubenstein, 2015).

It should be noticed that the polyploidization events, domestication of landraces, and recent selective breeding have all introduced restrictions in genetic diversity and many advantageous alleles or even genes relating to valuable traits, including NUE, may have been lost in modern commercial lines and thus, it is highly desirable to evaluate germplasm diversity in as wide a selection of germplasm as possible (Hawkesford, 2017). The existence of variability for NUE among genotypes has been indicated in different genetic studies in wheat (Brancourt-Hulmel et al., 2003; Guarda et al., 2004; Cormier et al., 2013) which shows the importance of screening wheat germplasm for genetic markers associated with nitrogen response to develop lines with high yield potential coupled with high NUE. Adding to that, genetic improvement of NUE in wheat has been previously reported under a range of N conditions and the selection under low as well as high N environments is of paramount importance to identify high nitrogen use efficient wheat genotypes, with the potential to perform well under low N conditions (Tyagi et al., 2020), ensuring environmentally sustainable increases in wheat production.

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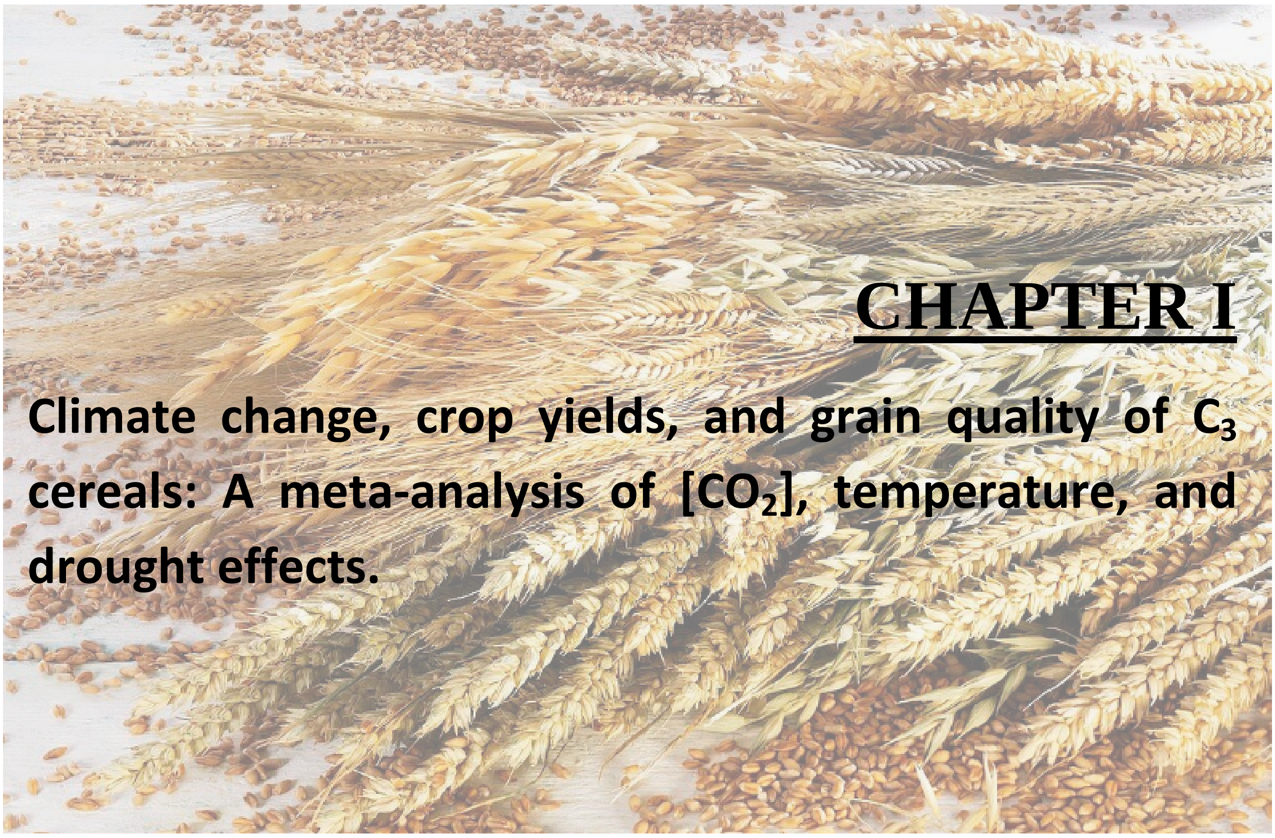
OBJECTIVES

The main objective of this thesis is the use of new wheat selection criteria to identify better adapted genotypes and crop management practices that allow high nitrogen use efficiency (NUE) to achieve higher yields and better grain quality under climate change conditions.

This global objective is divided into the following specific objectives:

1. Summarize the current knowledge related to the effect of increasing atmospheric CO_2 concentration ([CO_2]), elevated temperature, and drought stress on cereal crops development.
2. Assessing the changes in wheat yield and grain nutritional quality of archived grain samples collected from different locations and grown under field conditions over long-term period.
3. Identify genotypes with higher/lower nitrogen use efficiency (NUE), as well as yield and grain quality traits, by screening and testing of durum wheat and tritordeum germplasm collections exposed to different nitrogen fertilizer levels.
4. Identification of metabolic mechanisms linked with NUE and grain quality parameters, and involved in the response of durum wheat and tritordeum genotypes (with contrasting NUE selected in objective 3) exposed to high [CO_2] and drought stress conditions.



CHAPTER I

Climate change, crop yields, and grain quality of C_3 cereals: A meta-analysis of $[CO_2]$, temperature, and drought effects.

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INTRODUCTION

Food security is threatened by the impacts of climate change on agriculture and by increasing world population (FAO, 2018a; UNPD, 2018). Actually, climate change has already slowed global agricultural productivity growth, and in a recent study, Ortiz-Bobea et al. (2021) found that anthropogenic climate change (ACC) has reduced global agricultural total factor productivity since 1961 by about 21%, with a greater impact for warm regions such as Africa (-34%) than for cooler regions such as Europe and Central Asia (-7.1%). Over the next few decades, climate change is expected to affect the world's supply of cereal grains, impacting their quantity and quality due to the complex effects of elevated atmospheric [CO_2] and changing temperature and rainfall patterns on crops (Nuttal et al., 2017). Cereals contribute to a substantial part of world's plant-derived food production and comprise a majority of the crops harvested. In fact, FAO statistics show that in 2016, sugar cane had the highest production globally, followed by corn, wheat, and rice (FAO, 2018b). Adding to that, according to the Foreign Agricultural Service/USDA, preliminary world production in 2018 of maize, wheat and rice was estimated at around 1076, 763, and 495 million tons, respectively (FAS/USDA, 2019). Further, their nutritional quality has a significant impact on human well-being and health, especially in the developing world (Singh et al., 2012). Thus, one of the major challenges that plant breeders are facing currently is to increase cereal grain production while taking into consideration an adequate grain nutrient content.

Numerous effects of elevated atmospheric [CO_2] on plants have been documented through a photosynthesis-mediated CO_2 fertilization effect, including increased carbon (C) assimilation, growth, yield, and C content (Ainsworth and Long, 2005; Loladze, 2014). Thus, elevated [CO_2] could enhance the concentration of photosynthesis-derived carbohydrates in grains, starch being the major component (Zhang et al., 2013a, 2013b; Broberg et al., 2017). Since grains are predominantly composed of carbohydrates (mostly in the form of starch), it has been suggested that increases in starch concentration can cause a dilution effect on other nutrients, including proteins, lipids, vitamins, and minerals. In addition, adjustments in the photosynthetic apparatus and later on the redistribution from senescing leaves to grains must be considered as the key mechanisms. Due to the different biochemistry of C_3 and C_4 photosynthesis, the positive effect of elevated [CO_2] on photosynthesis is more pronounced in C_3 crops such as wheat and rice but less notable in C_4 crops such as maize (Ghannoum et al., 2000). Rising [CO_2] is likely to lead to “globally imbalanced plant stoichiometry (relative to pre-industrial times)” (Loladze, 2002), which in turn would “intensify the already acute problem of micronutrient malnutrition” (Loladze, 2002), particularly regarding minerals, such as Fe, Zn, and I, as well as protein (or N) (Taub et al., 2008; Broberg et al., 2017; Medek et al., 2017).

Elevated [CO_2] has been reported to decrease mineral concentrations in barley grains (-6.9%), rice grains (-7.2%), and wheat grains (-7.6%), and increasing the ratio of non-structural carbohydrates (TNC) to protein by 6-47% in grains and tubers (Loladze, 2014). For the grain crops barley, rice, and wheat, the reduction in protein mediated by elevated [CO_2] was reported to be 15%, 10%, and 10%, respectively (Taub et al., 2008). In their meta-analysis of the impact of elevated [CO_2] on wheat grains, Broberg et al. (2017) found a significant reduction in the concentration of the majority of minerals (Ca, Cd, Cu, Fe, Mg, Mn, P, S, and Zn), while B and Na were not significantly affected, and K significantly increased (<2%). These meta-analytic results are in line with those from individual wheat FACE experiments (Högy et al., 2009; Fernando et al., 2012a, 2012b, 2014a, 2014b). Two minerals, Fe and Zn, are already deficient in the diets of hundreds of millions of people, and CO_2-induced reductions in Fe and Zn have been reported in the edible parts of major crops (Loladze, 2014; Myers et al., 2014; Dietterich et al., 2015) and are projected to have negative effects on human nutrition (Weyant et al., 2018; Beach et al., 2019). Furthermore, emerging evidence points to elevated [CO_2] affecting nutrients beyond protein and minerals that are essential to human, such as vitamins and carotenoids (Zhu et al., 2018; Loladze et al., 2019). The decrease in mineral concentrations is notable in C_3 plants but less so in C_4 plants (Loladze, 2014; Myers et al., 2014) and is consistent with differences in physiology; the simulation of carbohydrate production by elevated [CO_2] is stronger in C_3 plants, while reduced transpiration is present in both C_3 and C_4 plants.

During the last two decades, air temperature has increased by 0.85°C (IPCC, 2014). In fact, annual average minimum temperatures in Spain have increased over the last century by 1.5°C and by 0.6°C during the last 25 years (Lopez-Bustins et al., 2014). The most probable outcome of climate ensemble model projections foresees increases of 1.8°C to 4.0°C by the end of the 21st century (2090-2099) relative to the period 1980-1999. These numbers originate from the best estimate of greenhouse gas time series deduced from the six marker scenarios alone (Meehl et al., 2007). Heat stress is a major constraint to sustainable cereal production, with reductions in grain yield being associated with high temperatures during the reproductive or grain-filling stages in wheat (Castro et al., 2007; Lizana and Calderini, 2013) and rice (Usui et al., 2014; Bahuguna et al., 2015; Chaturvedi et al., 2017).

High-temperature impacts on grain filling can vary enormously, depending on timing (days after anthesis) and duration. Both chronic moderately high temperature (25-35°C) and heat shocks (>35°C) during the grain-filling phase are frequently associated with an increase in grain protein concentration in wheat (Ferreira et al., 2012; Lizana and Calderini, 2013; Guzmán et al., 2016) and rice (Jing et al., 2016; Chaturvedi et al., 2017). Indeed, high temperature primarily impacts the accumulation of starch in wheat grain, with accumulation beginning earlier than under cooler temperatures, the duration of its accumulation also being reduced, and the result is a greater concentration of protein in the grain. Further, the duration of protein accumulation is reduced, while the rate of protein accumulation is substantially increased. In addition, leaves senesce before the heads mature, suggesting that high temperatures might enhance N remobilization from leaves and stems (Dupont et al., 2006; Savill et al., 2018). Moreover, the timing and duration of heat stress during grain filling have been shown to be important sources of variation in dough properties in wheat (Wardlaw, 2002). Grain protein and mineral composition are quality characteristics that can change due to high temperature, and they respond to changes in enzymes involved in starch and protein synthesis. Yang et al. (2018) observed that the activity of glutamate synthase was enhanced by heat stress, while sucrose phosphate synthase, sucrose synthase, and soluble starch synthase were significantly decreased during grain filling. However, Monjardino et al. (2005) found that protein concentration was negatively affected by heat stress during the early stage of endosperm development. They found that among the protein fractions, zeins are the most affected by heat stress. In fact, zein accumulation was repressed under high temperature rather than being degraded in the early developmental stages. In rice, elevated temperature also alters grain protein and mineral nutrient composition (Ziska et al., 1997; Chaturvedi et al., 2017). Ferreira et al. (2012) showed that the total quantity of N per grain in wheat is generally little affected by the growing temperature but, due to the above-mentioned lower grain yield, the percentage of N on a dry weight basis rises under higher temperatures. Similar increases in the percentage of dry weight have been reported for wheat (Randall and Moss, 1990; Gooding et al., 2003).

Increasing greenhouse gas emissions may also lead to rainfall reductions in the coming decades, which will increase the frequency and intensity of drought in the Mediterranean basin (Habash et al., 2009; IPCC, 2013; McKersie, 2015). Climate change projections for the Mediterranean region indicate a precipitation decrease of 25-30% for the last decades of the 21st century (Giorgi and Lionello, 2008). Adding to that, the seasonality of rainfall is much more important. In fact, the expected shortage in Mediterranean rainfall should impact summer precipitation much more than winter precipitation. Mediterranean crop growth, however, is mainly driven by winter rain. Moreover, drought is considered one of the most important factors limiting crop yields around the world. Wheat crop responses to water scarcity depend on several factors, including plant development status, duration, and intensity of the stress and genetic variables (Altenbach et al., 2003). Although rainfall during winter has been traditionally abundant and coincides with the lowest evapotranspiration rates, the occurrence of drought in winter during the early stages of the crop cycle has been recently reported (Russo et al., 2015). This can further constrain wheat growth and thus final grain yield, mostly through a decrease in ear density and the number of kernels per unit crop area (Araus et al., 2008; Rebolledo et al., 2013). Grain yield reductions mediated by drought have been widely reported in wheat (Kiliç and Yagbasanlar, 2010; Balla et al., 2011), and depending on the genotype, the reductions may reach up to 50%. The thousand-grain weight (TGW) is also reduced significantly, above 30% in droughted wheat (Altenbach et al., 2003; Balla et al., 2011; Houshmand et al., 2014). Drought stress leads to reduced photosynthetic area and acceleration of leaf senescence during late grain filling in cereals, resulting in a shorter grain-filling period. In wheat, this smaller photosynthetic area and accelerated leaf senescence limit the amount of assimilates translocated to the grain, which implies reductions in grain yield (Altenbach et al., 2003). Grain composition is also affected. Drought stress affects starch accumulation (Altenbach et al., 2003; Sharma and Carena, 2016) more severely than N accumulation during grain filling, putatively influencing the conversion of sucrose into starch (Panozzo and Eagles, 1998). This tends to increase the grain protein concentration (expressed as % protein) in wheat (Kiliç and Yagbasanlar, 2010;

Houshmand et al., 2014; Chaturvedi et al., 2017) and rice (Crusciol et al., 2008). In some cases, the opposite effect has been observed in wheat (Singh et al., 2010; Abd El-Kareem and El-Saidy, 2011), possibly related to differences in stress levels and plant development status (Flagella et al., 2010). Knowledge of the effects of drought stress on grain mineral composition is scarce (Peleg et al., 2008; Singh et al., 2012). Crusciol et al. (2008) explained the increase in rice grain N, Ca, Mg, Fe, and Zn concentration under rainfed conditions being due to a dilution effect because productivity was higher in irrigated than rainfed systems.

All the above-mentioned changes in grain composition linked to the changing environmental conditions are expected to have important implications for the nutritional quality of foods. During the last decade, different meta-analyses have characterized elevated [CO_2] effects on crop yield and quality traits. However, comparatively little attention has been given to how other target environmental parameters such as temperature and drought will affect crop yield, and especially grain nutritional characteristics. Considering the economic and social importance of cereal crops and the impact of climate change not only on grain production but also on the nutritional value, this meta-analysis aims to provide an overview of the effects and interactions of multiple climate stressors, specifically high [CO_2], drought, and elevated temperatures, on the productivity and grain quality of C_3 cereals.

MATERIAL AND METHODS

Data search and selection criteria

To find relevant studies related to the issue of the current meta-analysis, literature searches of primary research in published peer-reviewed journal sources were conducted from Google, Web of Science, and Scopus in June 2017. To search the literature, the following keywords were used: grain yield, cereal, high [CO_2], elevated temperature, drought stress, climate change, and C_3 grain quality. More than 150 papers were found, but 78 articles were selected according to the following criteria: (i) the article studies the effect of at least one climate parameter, including [CO_2], temperature, and drought, (ii) the article contains at least one response variable from the following list: grain yield, thousand-grain weight (TGW), starch, total protein, gluten, glutenins, gliadins, and a set of minerals (Al, N, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mo, Mg, Mn, Na, Ni, P, Pb, S, Se, Si, and Zn). The most abundant C_3 species that are reported in the literature are wheat, rice, and barley. All papers included in this meta-analysis were published between 1990 and 2019 (Table S1). The study is based on comparing plants grown at elevated [CO_2] (550-900 ppm) using Open Top Chamber (OTC) facilities or in the field using Free-Air-CO_2-Enrichment (FACE) systems with those grown at ambient [CO_2] (currently at ca. 400 ppm). Studies comparing different ranges of temperature, from ambient (10-25°C) to elevated temperature (28-37°C), and two levels of irrigation (limited irrigation or well-watered) are also included in the current report. Response means of plants grown under the different environmental conditions stated previously were taken from tables. The time of occurrence of stress during the crop cycle and the duration of stress applied differ among the studies. Most studies reported that treatments were maintained until the end of the experiments, when the plants reached maturity.

Data analysis

All the data described above were organised in an Excel datasheet pairwise (control and experimental value) for each experimental factor ([CO_2], temperature, water). The datasheet was loaded into and analysed in RStudio v1.1.456. For the effect size metric, we used the natural log of the response ratio, lnR = ln(HF/LF), where LF and HF are reported mean nutrient concentrations at low and high treatment, respectively, with the treatment being any of the three climate factors considered in this study (CO_2, temperature, or water). The log response ratio eliminates asymmetry between percentage decreases limited to 100% and unlimited percentage increases; it is a standard approach for analyzing elevated [CO_2] and other ecological studies (Hedges et al., 1999). After performing statistical analyses, all the results were back-transformed to regular percentage changes using the formula: (exp(lnR) − 1)*100%. For estimating the 95% confidence intervals for the mean effect size, a non-parametric test, namely bootstrapping with 999 replacements, was used for sample sizes of seven or more (i.e., when seven or more independent studies reported any given nutrient concentration at low and high treatments) (Loladze et al., 2019). The advantage of this approach is that it does not require the distribution of effect sizes to be normal. However, for the confidence intervals to be accurate, they can be applied only for sufficiently large sample sizes (>7). For sample sizes <7, we had a choice of discarding the data completely, which would result in the loss of potentially valuable information, or making a normality assumption and applying a parametric method.

We chose the latter for sample sizes of 3 to 7. No confidence intervals were derived for sample sizes of two or less. In all cases, unweighted methods were used, with each study having equal weight.

RESULTS

[CO$_2$], temperature, and drought stress effects on grain yield components

The overall effect of elevated [CO$_2$] on C$_3$ crops resulted in significant increases in grain yield and thousand-grain weight (TGW) of 30.10% and 7.41%, respectively (Figure 1). Nevertheless, a contrasting drastic loss in grain yield and TGW was observed under high temperatures and drought stress. Results presented in Figure 1 indicate that the heat and drought stress effects were similar for TGW and recorded -20.17% and -20.29% reductions, respectively, but the negative effect of drought on cereal grain yield was larger than the effect of elevated temperatures (-70.53% vs. -24.85%).

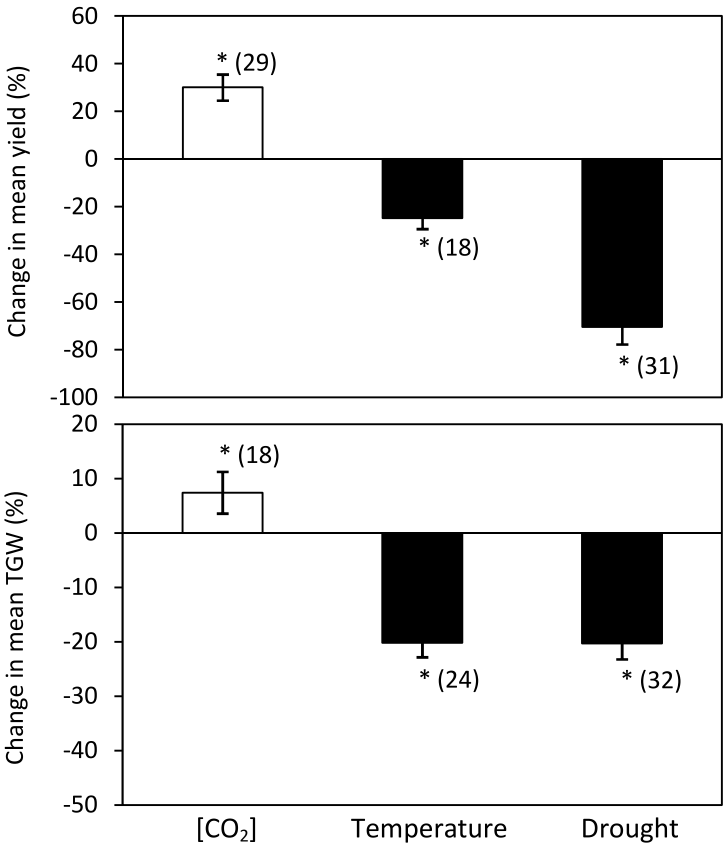

Figure 1: Change (%) in the mean of **yield** and **TGW** of plants grown under elevated [CO$_2$], high temperature, and drought stress relative to the control. Data within parenthesis indicate the number of observations. Error bars indicate the standard error of the mean. * indicates a statistically significant difference at $p<0.05$.

$[CO_2]$, temperature, and drought stress effects on grain quality

Starch

In cereals grown under high $[CO_2]$, there was a significant increase in grain starch concentration (5.65%), whereas there was a significant decrease (-9.91%) under elevated temperature (Figure 2). Regarding water availability, there was no significant change as responses were sprayed in a broad range of both positive and negative changes (Figure 2).

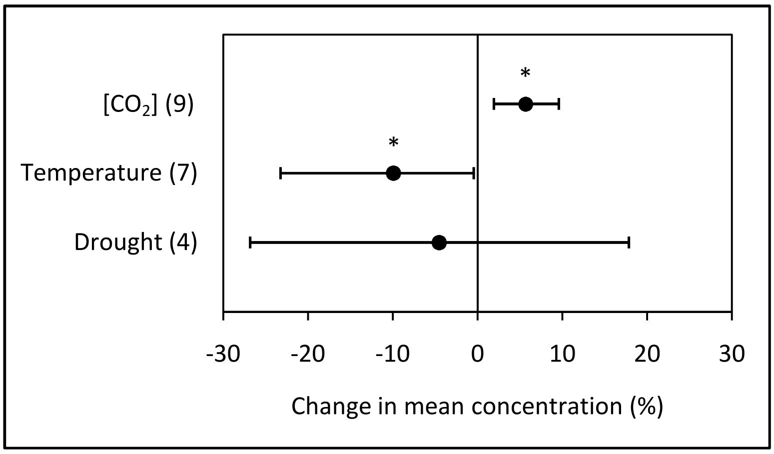

Figure 2: Change (%) in the mean concentration of **grain starch** of plants grown under elevated $[CO_2]$, high temperature, and drought stress relative to the control. Data within parenthesis indicate the number of observations. Error bars indicate 95% CI. * indicates a statistically significant difference at $p<0.05$.

Total protein

Grain total protein concentration was negatively affected (-8.90%) by high $[CO_2]$. However, it was significantly increased by temperature and drought (10.40% and 12.44%, respectively) as shown in Figure 3. Among the proteins that were studied, the gluten, gliadin, and glutenin concentrations were analysed under elevated $[CO_2]$. The grain gluten, gliadin, and glutenin concentrations presented in Figure 4 reveal a significant decrease in the gluten and gliadin concentrations (−11.54% and −7.41%, respectively). In contrast, rising $[CO_2]$ decreased the glutenin concentration, but it was not significant. Regarding the effects of drought and heat stress on these proteins, it was not possible to generate statistically powerful results due to the low amount of data (less than three repetitions).

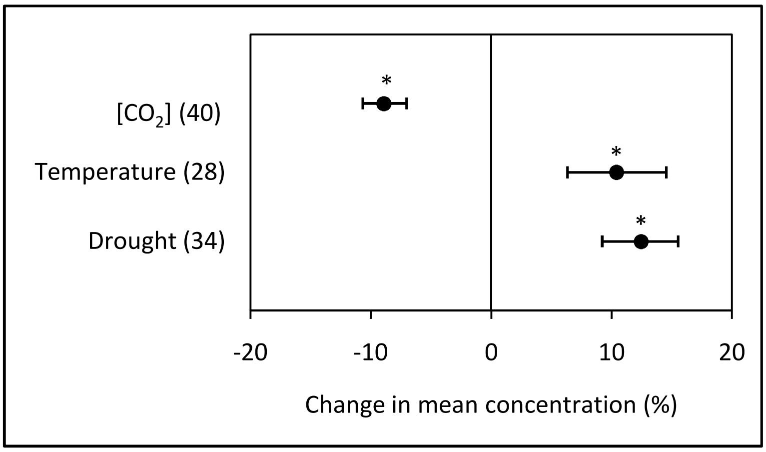

Figure 3: Change (%) in the mean concentration of **grain total protein** of plants grown under elevated [CO_2], high temperature, and drought stress relative to the control. Data within parenthesis indicate the number of observations. Error bars indicate 95% CI. * indicates statistically significant difference at $p<0.05$.

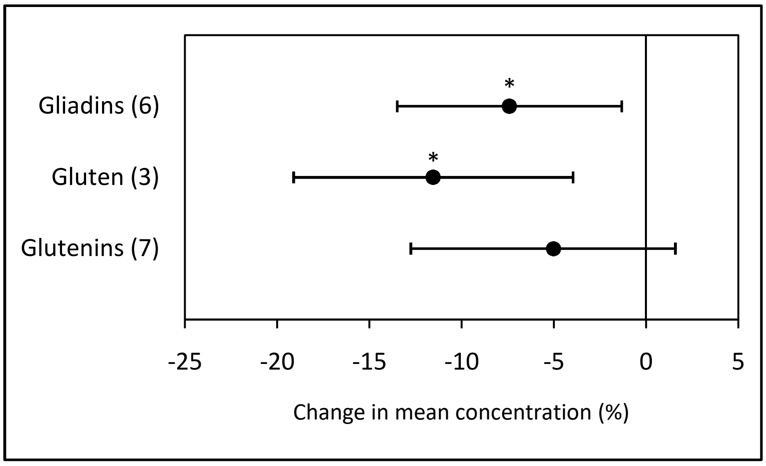

Figure 4: Change (%) in the mean concentration of **grain gliadins, gluten, and glutenins** of plants grown under elevated [CO_2] relative to ambient level. Data within parenthesis indicate the number of observations. Error bars indicate 95% CI. * indicates a statistically significant difference at $p<0.05$.

Mineral composition

The results presented in Figure 5.A show an overall decrease in micro/macronutrients in C_3 grains under elevated [CO_2]. Across all the data, the mean change ranged between -4.70% (recoded for P) and -39.41% (recorded for Mo). The changes in B and Se were not significant. Among all the measured elements, only Na concentration increased significantly (52.05%) under high [CO_2]. Heat stress had no significant effect on any of the grain mineral concentrations (Figure 5.B), and this could be due to data scarcity and small sample sizes leading to high data variability. Slight increases in Mg and N of 1.91% and 6.31%, respectively, were recorded, whereas the Ca, Fe, Mn, and Zn concentrations were reduced.

Regarding water scarcity, the data analysis showed distinct effects between minerals (Figure 5.C). In fact, drought stress induced an accumulation of Ca and Na in grains and recorded a significant increase by 19.92% and 9.56%, respectively. However, no significant increase was obtained regarding Fe, Mg, P, and Zn concentrations. Under low water availability, S and K concentrations declined, but not significantly, by -10.43% and -7.59%, respectively.

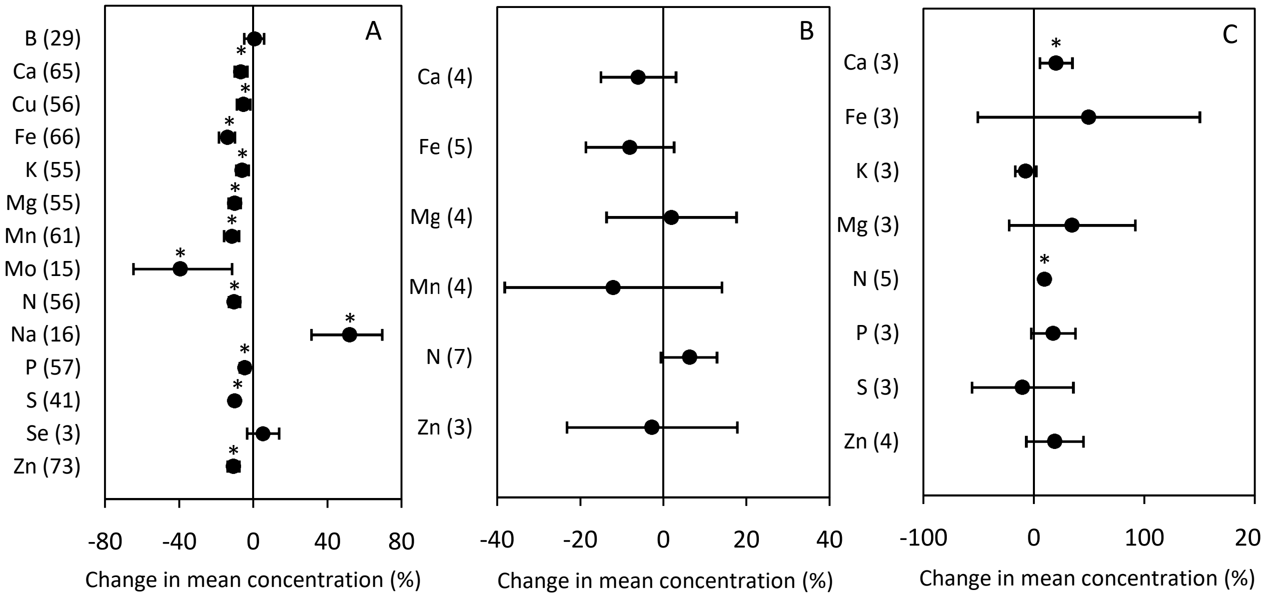

Figure 5: Change (%) in the mean concentration of **grain minerals** of plants grown under (A) elevated [CO_2], (B) high temperature, and (C) drought stress relative to the control. Data within parenthesis indicate the number of observations. Error bars indicate 95% CI. * indicates a statistically significant difference at p<0.05.

DISCUSSION

[CO_2], temperature, and drought stress effects on grain yield components

Current scientific knowledge indicates that grain yield and quality will face serious challenges under the projected future climate. In line with previous papers (Wilcox and Makowski, 2014; Knox et al., 2016; Dixit et al., 2018), our meta-analysis shows that the predicted elevated [CO_2] will increase crop grain production (Kimball et al., 2001; Ainsworth and Long, 2005; Högy et al., 2009). However, as noted by studies conducted over recent decades, it is essential to consider that the [CO_2]-derived “fertilization” effect might decline or be eliminated when combined with stressful growth conditions, such as drought and temperature stress (Lobell et al., 2011; Aranjuelo et al., 2011, 2013). Moreover, in cereals such as wheat, increased grain yields have been associated with increases in the numbers of tillers and grains per spike rather than spike number or grain size (Bourgault et al., 2013; Pleijel and Högy, 2015). The results of the current study have also revealed an association with an increase in the number of grains rather than their weight (larger increase in grain yield than TGW).

Both high temperature and drought negatively affected crop yield. Data analysis showed that yield was more markedly affected under drought than under heat stress conditions. Lower yields in stressed plants can be associated with (i) a shortened duration of the grain-filling period and/or (ii) a lowered photosynthetic rate during grain filling. Dixit et al. (2018) applied a crop simulation model to assess the impact of climate change on wheat production and found a loss of 15% in wheat grain yield in stressed plants, which was associated with a reduction in the number of days to reach grain maturity. Indeed, Mitchell et al. (1995) attributed the direct negative effect of rising temperature on wheat yield to the temperature-dependent shortening of the phenological stages. Such decreases in the duration of grain filling would imply a shorter time available for accumulating resources for grain formation (Gooding et al., 2003; Lizana and Calderini, 2013). The time and duration of heat stress could cause different physiological responses in the plant, therefore, affect crop production. Many studies have reported that heat stress applied prior to anthesis negatively affects the grain yield of wheat due to many reasons (Lizana and Calderini, 2013; Talukder et al., 2014). High temperature accelerates leaf senescence and reduces post heading duration (Talukder et al., 2014). Adding to that, heat stress significantly reduces seed germination and negatively affects microspores and pollen cell, leading to non-functional florets or abortion of fertile florets and resulting in male sterility (Akter and Islam, 2017). In fact, the decline in grain yields under high day temperatures was primarily caused by a reduction in the seed set percentage. Meanwhile, under high night temperature, the combination of decreased spikelet number per panicle, grain weight, and biomass production in addition to decreased seed set percentage contributed to the grain yield loss (Xiong et al., 2017). Altenbach et al. (2003) reported that high temperature during anthesis promoted both grain shrinkage and a decrease in weight. Additionally, under heat stress conditions, plants tend to have a shorter grain-filling period, which reduces grain size and thousand-kernel weight, while under drought conditions, plants tend to produce fewer grains per spikelet (and/or fewer tillers) (Li et al., 2013a). This finding matches the TGW analyses stated above.

In fact, heat stress and water scarcity showed similar effects on TGW in the current data analysis, suggesting that under drought conditions, the drastic decline in C_3 cereal yields is instead linked to a decrease in grain number produced per plant.

[CO_2], temperature, and drought stress effects on grain quality

Another major consideration is the effect of climate change on grain quality. While crop breeding is already much more focused on yield traits, comparatively little attention has been given to grain quality traits. This is a matter of great concern because, as described in more detail below, environmental stress will affect the relative abundance of starch, protein, and minerals (Ziska et al., 2012; Loladze, 2014; Goicoechea et al., 2016).

Starch

Starch is the most abundant end-product of cereal growth and development, representing around 70% of the dry weight (w/w) of grains (Jung et al., 2008). Rising [CO_2] increases photosynthetic rates in C_3 plants; increased carbohydrate translocation from the source (leaves and stems) to the sink (grains) is expected to increase the starch content in grains (Thitisaksakul et al., 2012). Indeed, the current data analysis has shown that growth under elevated [CO_2] has a significant positive effect on the grain starch concentration, which contrasts with the non-significant results reported by Högy and Fangmeier (2008) and Broberg et al. (2017). Fangmeier et al. (1999) reported that elevated [CO_2] significantly increased starch only for plants under high levels of N fertilizer.

Despite no significant effect due to drought, we revealed an overall decrease in grain starch concentration under drought stress. Worch et al. (2011) observed that changes in endosperm starch content positively correlated with grain yield and concluded that grain starch content is one of the leading causes of reduced yield in crops subjected to drought conditions. This can be due to water deficit compromising both production of photoassimilates (source of carbon skeletons for the synthesis of starch) and the activity of enzymes involved in starch biosynthesis in the endosperm. Thus, the lower starch content observed in grains of genotypes subjected to water deficit could be correlated with the availability of reducing sugars (Avila et al., 2017).

Elevated temperature also negatively affected the starch concentration in grains. It has been reported that the reduction in starch concentration under high-temperature conditions is due to two factors; (i) shortening of the grain-filling period, which may reduce the duration of starch accumulation (Altenbach et al., 2003), and (ii) impairment of starch metabolism. While data for grains of plants exposed to high temperatures are scarce, Hawker and Jenner (1993) and Keeling et al. (1993) reported the inhibition of starch metabolism by high temperature (generally around 30°C), possibly due to thermal denaturation negatively affecting the activity of starch synthase.

Total protein

Elevated [CO_2] has been documented to reduce grain protein (or N) content in edible parts of crops (Loladze, 2002; Taub et al., 2008; Medek et al., 2017). In line with these earlier studies, the current meta-analysis showed that elevated [CO_2] significantly decreased grain protein concentrations. This reduction has been associated with increased photosynthesis and accumulation of grain carbohydrates, leading to reductions in the amount of grain protein (due to a dilution effect) (Högy et al., 2009; Chaturvedi et al., 2017). However, Goufo et al. (2014) reported decreases in protein without associated increases in starch in grains of rice exposed to elevated [CO_2]. Decreased protein concentrations in cereal grains under elevated [CO_2] might be a consequence of reduced leaf protein concentrations in photosynthetic tissues, leading to decreased seed protein (Fangmeier et al., 1999, 2000). The suppression of nitrate assimilation by elevated [CO_2] could be another contributor (Bloom et al., 2014). Our study also showed that there was a change in protein composition in grains of plants grown at elevated [CO_2]. In line with the results of Wieser et al. (2008) and Högy et al. (2009), gluten, gliadins, and glutenins concentrations decreased under increasing [CO_2]. Differences in the amounts and proportions of gluten protein fractions and types have significant effects on dough mixing and rheological characteristics. One of the most important characteristics for baking quality is bread volume, which has been strongly correlated with crude protein, total gluten proteins, and glutenin macropolymers (Weegels and Hamer, 1996; Nuttall et al., 2017). Consequently, a reduction in bread quality can be expected due to the higher sensitivity of gluten fractions to elevated [CO_2].

Grain protein content is sensitive to environmental conditions and controlled by a number of factors, particularly the duration and rate of grain filling and the availability of assimilates, which are negatively affected in crops subjected to stressful growth conditions (Yang and Zhang, 2006; Brdar et al., 2008). In contrast to elevated [CO_2], we found that high temperatures increased the grain protein concentration by 10.4%, which could be attributed to a greater remobilization of shoot-derived protein. The grain protein concentration is expressed as a percentage of grain dry mass, which alongside the lower size and weight of the affected grains (also detected in our meta-analysis), would contribute to them having lower carbohydrate levels and consequently higher grain protein (Barnabás et al., 2008). We note that the increase in grain protein concentration (10.4%) is almost the same as the decrease in grain starch concentration (-9.9%), suggesting that starch depletion increases the relative content of total protein.

Drought affects plant phenology and physiology. Water scarcity has been previously described as reducing photosynthetic rates, shortening the grain-filling period (Gallé et al., 2009; Zhang et al., 2013b), and accelerating leaf senescence after anthesis. We detected significant increases in grain total protein associated with low water availability. Bhullar & Jenner (1986) reported that during the grain-filling period, drought stress hinders the conversion of sucrose into starch but has a milder effect on protein biosynthesis. Our findings did not corroborate Bhullar and co-workers' conclusions. As mentioned before, the fact that the grain starch concentration was not significantly affected by drought would discard the lower carbohydrate level as a factor that induces increased grain protein content. Singh et al. (2012) observed that together with lower rates of carbohydrate accumulation in the grain of plants subjected to drought, the increase in flour protein was mainly due to higher rates of grain N accumulation. The present meta-analysis supports this assertion because grain N concentration was affected by drought. Adding to that, the increased grain protein concentration under drought could be explained by the shortened maturation time common to stress conditions, which tends to favor protein over starch accumulation in cereal grains (Wang and Frei, 2011). Drought, among other stresses, accelerates the translocation of senescence-inducing resources (including amino acids) from leaves to seeds during grain filling. Several studies have demonstrated that the contribution of reserve mobilization to the final grain yield is higher under stressful conditions than relatively well-irrigated conditions (Blum, 1998; Yang et al., 2003; Srivastava et al., 2017).

Mineral composition

The present study showed that elevated [CO_2] leads to an impoverishment of macro/micro-elements in grains. Moreover, there is a variation among minerals in the magnitude of the reductions, and this supports previous results (Högy et al., 2009; Fernando et al., 2012a, 2014b; Houshmandfar et al., 2015; Zhu et al., 2018). In fact, only the Na concentration was significantly increased, with surprisingly few studies having investigated this element in relation to the effect of [CO_2], and so there is little background information to explain this trend. Basically, most studies have focused on the main minerals that affect human health, such as Fe, Zn, P, K, and Ca, and have underlined a common decline in these minerals under raising [CO_2]. With respect to our results, the concentrations of Zn, Fe, S, Ca, Mg, P, Mn, K, and Mo were significantly decreased. Such reductions have been associated with increased production of spikes and grains that translates into a grain nutrient-dilution effect, diminishing the nutritional value. Furthermore, by reducing transpiration (linked to stomatal closure due to long-term exposure to elevated [CO_2]), high [CO_2] can reduce the mass flow in the soil toward roots, which diminishes the availability of mobile minerals in the rhizosphere (Loladze, 2002). While carbohydrate dilution should lower all other nutrients in plant tissues evenly (Gifford et al., 2000), other effects of elevated [CO_2] on plant physiology are not evenly distributed among the minerals. For example, reduction in transpiration and elevated biosynthesis affect some minerals more than others. This means that the stoichiometry of plants exposed to elevated [CO_2] should “differ not only in C:(other elements) ratios but also in the ratios among other elements (e.g. C:N, N:P, and P:Zn should be different)” (Loladze, 2002). Indeed, Loladze’s meta-analysis of over 7500 pairs of observations from studies of elevated [CO_2] published over 30 years (1984-2014) showed a significant reduction in foliar Mg (and N, P, K, Ca, S, Fe, Zn, and Cu) but not the Mn content in C_3 plants, and underlying biochemical mechanisms responsible for the increased Mn:Mg ratio have been proposed (Bloom and Lancaster, 2018).

Changes in the elemental composition in grains are also detected under heat and drought stress. Previous studies suggested that both stress factors tend to increase mineral concentrations (including Fe, N, S, Zn, K, and P). However, the low number of reports means that there is relatively large uncertainty about the magnitude of the increase. The observed increase in grain protein and N concentrations (and the concomitant decrease in starch) under elevated temperature means that there is more N per unit of starch (Stone et al., 1997). In addition, Fe and Zn tend to increase under drought. Although water plays a significant role in mineral uptake and later mobilization within the plant, with these processes decreasing during water stress, our meta-analysis agrees with Ge et al. (2010) reporting that soil drought stress improved transport mechanisms and/or routes for some minerals, such as Fe and Zn, leading to increased grain concentrations of these elements. Moreover, according to other studies (Farahani et al., 2010; Ge et al., 2010), the increase in the levels of Fe and Zn may be related to the more efficient remobilization of these nutrients from leaves to grains. However, according to other authors (Miller et al., 1994), the increase in Fe and Zn concentrations is linked to sink strength at the single grain level. More specifically, Miller et al. (1994) observed in maize how the mineral content in drought-sensitive genotypes (which produced lower numbers of grains than the tolerant ones) was higher than in fully watered plants. According to this explanation, the increase in nutrients in the grains may be related to the number of grains formed, with each grain being a specific sink (Avila et al., 2017). Furthermore, as we mentioned above, heat and drought cause a decrease in the number and size of cereal grains, which suggests that there might be a concentration effect due to the smaller grains (Velu et al., 2016).

CONCLUSIONS AND PERSPECTIVES

This study highlights that while current and near-future environmental conditions will severely affect cereal yield, the nutritional value of cereal grain will also be affected. It seems that within the three factors related to climate change investigated, the rise in atmospheric [CO_2] is possibly the one more detrimental and difficult to face because elevated [CO_2] will impact grain quality traits all over the world while the impacts of the increase in temperature and the decrease in water availability will be localized or easy to counterbalance. In fact, although the increase in [CO_2] might promote yield enhancement and starch accumulation through higher rates of photosynthesis, the grains of these plants will have lower concentrations of total protein and minerals, leading to reduced baking quality and deficient nutritional value. On the other hand, even if both high temperate and drought severely decrease crop yields, the available data shows that grain quality will be differentially affected. Heat stress will negatively affect grain starch concentration due to depleted starch biosynthesis metabolism and shortening of the grain-filling period, but it might increase total protein and N concentration. Regarding water availability effects, grain yield could be conditioned by the final starch concentration of affected plants. Adding to the increase in the Fe and Zn concentrations, we found that total protein concentration is significantly increased, which is probably due to a dilution effect on starch and the accelerated reserve remobilization from source to sink to compensate for the nutrient uptake deficit that results from low soil water content. According to numerous climatic models, precipitation patterns are expected to change in the future with more frequent drought events in semiarid and arid regions but, it is also predicted that in other regions, precipitation will likely increase. Therefore, while drought and elevated temperature can be potentially mitigated (by increasing irrigation, planting crops at higher altitudes within a given latitude or displaced to cooler and wet latitudes within a country), the effect of rising [CO_2] is present at all latitudes and will act independently of where crops will be established. Hence, [CO_2]-induced reductions in grain quality would be much more challenging to mitigate.

Our study highlights the fact that within the context of the present and near-future environments, it is crucial to increase crop yield through the development of stress-adapted cultivars. While the current breeding programs and agricultural incentives are almost exclusively yield-based, breeding for improved cereal quality can meaningfully improve the nutritional status of humanity. For this purpose, a better understanding of how environmental growth conditions (such as elevated temperature, drought, etc.) affect grain yield and nutritional parameters of cereals will help developing more nutrient-dense crops. Adding to that, exploring genetic diversity and variability of major crops in needed to discover genotypes more resilient to ongoing climate change.

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SUPPLEMENTARY MATERIAL

Table S1: List of papers and species used for the data analysis of each environmental factor.

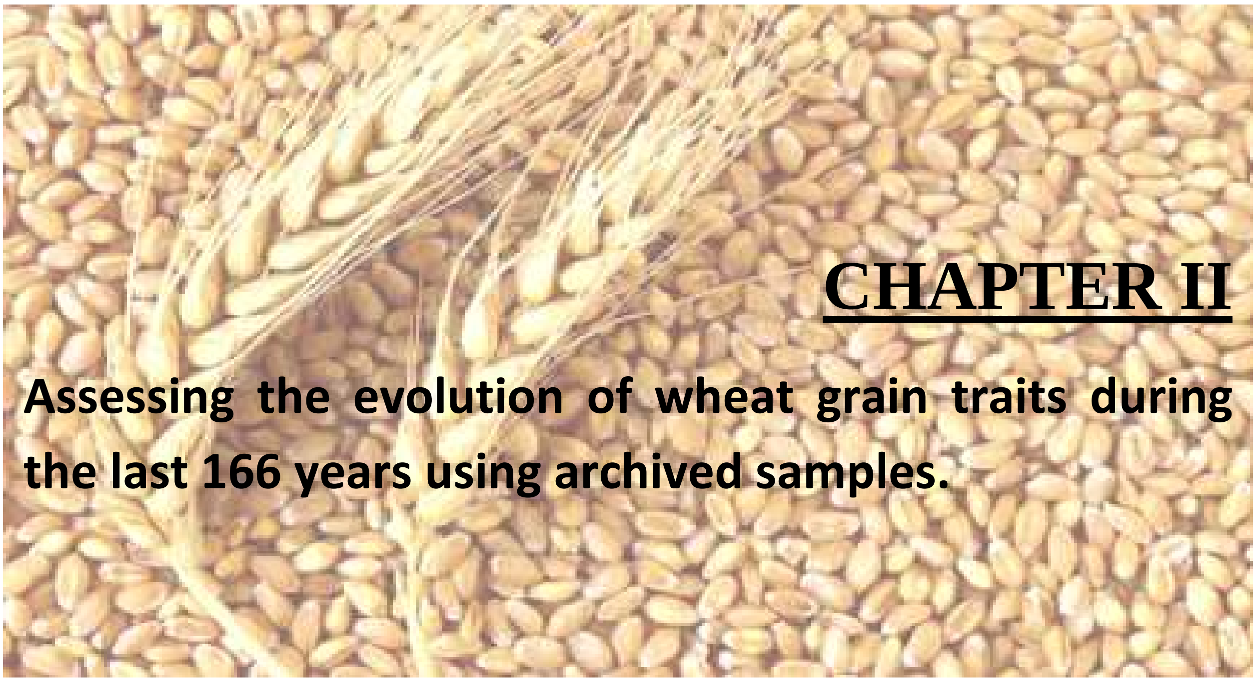
Papers	Species
High [CO_2]	
(Wieser et al., 2008)	*Triticum durum* L.
(Högy et al., 2010)	*Triticum aestivum* L.
(Högy et al., 2009)	*Triticum aestivum* L.
(Högy et al., 2013)	*Triticum aestivum* L.
(Erbs et al., 2010)	*Triticum aestivum* L./*Hordeum vulgare*
(Zhu et al., 2018)	*Oryza sativa* L.
(Usui et al., 2016)	*Oryza sativa* L.
(Pleijel and Högy, 2015)	*Triticum aestivum* L.
(Dietterich et al., 2015)	*Triticum durum* L./*Oryza sativa* L.
(Panozzo et al., 2014)	*Triticum aestivum* L.
(Fernando et al., 2014b)	*Triticum aestivum* L.
(Bencze et al., 2004)	*Triticum aestivum* L.
(Blumenthal et al., 1996)	*Triticum aestivum* L.
(Conroy et al., 1994)	*Triticum aestivum* L./*Oryza sativa* L.
(De la Puente et al., 2000)	*Triticum aestivum* L.
(Fangmeier et al., 1997)	*Triticum aestivum* L.
(Fangmeier et al., 1999)	*Triticum aestivum* L.
(Fangmeier et al., 1996)	*Triticum aestivum* L./*Hordeum vulgare*
(Wroblewitz et al., 2014)	*Triticum aestivum* L.
(Weigel and Manderscheid, 2005)	*Triticum aestivum* L.
(Wu et al., 2004)	*Triticum aestivum* L.
(Rogers et al., 1998)	*Triticum aestivum* L.
(Mitchell et al., 1995)	*Triticum aestivum* L.
(Manderscheid et al., 1995)	*Triticum aestivum* L./*Hordeum vulgare*
(Erice et al., 2019)	*Triticum durum* L.
(Jing et al., 2016)	*Oryza sativa* L.
(Goicoechea et al., 2016)	*Triticum durum*L.
(Fernando et al., 2012a)	*Triticum aestivum* L.
(Fernando et al., 2014a)	*Triticum aestivum* L.

Table S1: (Continued).

(Carlisle et al., 2012)	*Triticum aestivum* L.
(Guo et al., 2011)	*Triticum aestivum* L./*Oryza sativa* L.
(Ma et al., 2007)	*Triticum aestivum* L. /*Oryza sativa* L.
(Pleijel and Danielsson, 2009)	*Triticum aestivum* L.
(Beleggia et al., 2018)	*Triticum durum* L.
(Chaturvedi et al., 2017)	*Oryza sativa* L.
(Sakai et al., 2019)	*Oryza sativa* L.
Drought stress	
(Chang-Xing et al., 2009)	*Triticum aestivum* L.
(Houshmand et al., 2014)	*Triticum durum* L.
(Eivazi and Habibi, 2012)	*Triticum aestivum* L.
(Baric et al., 2005)	*Triticum aestivum* L.
(Houshmand et al., 2004)	*Triticum durum* L.
(Arzani, 2002)	*Triticum durum* L.
(Saint Pierre et al., 2008)	*Triticum aestivum* L.
(Kiliç and Yagbasanlar, 2010)	*Triticum durum* L.
(Abd El-Kareem and El-Saidy, 2011)	*Triticum aestivum* L.
(Goicoechea et al., 2016)	*Triticum durum* L.
(Crusciol et al., 2008)	*Oryza sativa* L.
(Singh et al., 2010)	*Triticum aestivum* L.
(Erice et al., 2019)	*Triticum durum* L.
(Wu et al., 2004)	*Triticum aestivum* L.
(Balla et al., 2011)	*Triticum aestivum* L.
(Souza et al., 2004)	*Triticum aestivum* L.
(Guzmán et al., 2016)	*Triticum aestivum* L./*Triticum durum* L.
(Mkhabela et al., 2010)	*Triticum aestivum* L.
(Guttieri et al., 2001)	*Triticum aestivum* L.
(Li et al., 2013b)	*Triticum aestivum* L.
(Altenbach et al., 2003)	*Triticum aestivum* L.
Heat stress	
(Ferreira et al., 2012)	*Triticum durum* L.
(Altenbach et al., 2003)	*Triticum aestivum* L.
(Castro et al., 2007)	*Triticum aestivum* L.
(Dias et al., 2008)	*Triticum aestivum* L./*Triticum durum* L.

Table S1: (Continued).

(Panozzo and Eagles, 1998, 2000)	*Triticum aestivum* L.
(Randall and Moss, 1990)	*Triticum aestivum* L.
(Corbellini et al., 1997)	*Triticum aestivum* L./*Triticum durum* L.
(Lizana and Calderini, 2013)	*Triticum aestivum* L.
(Li et al., 2013b)	*Triticum aestivum* L.
(Dupont et al., 2006)	*Triticum aestivum* L.
(Mitchell et al., 1995)	*Triticum aestivum* L.
(Daniel and Triboi, 2000)	*Triticum aestivum* L.
(Gooding et al., 2003)	*Triticum aestivum* L.
(Labuschagne et al., 2009)	*Triticum aestivum* L./*Triticum durum* L.
(Spiertz et al., 2006)	*Triticum aestivum* L.
(Guzmán et al., 2016)	*Triticum aestivum* L./*Triticum durum* L.
(Viswanathan and Khanna-Chopra, 2001)	*Triticum aestivum* L.
(Wrigley et al., 1994)	*Triticum aestivum* L.
(Wardlaw, 2002)	*Triticum aestivum* L.
(De Leonardis et al., 2015)	*Triticum durum* L.



CHAPTER II

Assessing the evolution of wheat grain traits during the last 166 years using archived samples.

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INTRODUCTION

Environmental changes, including climate change, land degradation, and biodiversity loss, have been particularly apparent in recent decades and are predicted to become even more extreme in the 21st century (Scheelbeek etal.,2018). The environmental conditions in which plants have been growing during the last century have changed considerably since the Industrial Revolution (IPCC,2014). The atmospheric carbon dioxide concentration [CO_2] has increased from about 280 ppm in pre-industrial times to 406 ppm reached in 2017 (EEA, 2019). A direct consequence of the elevated atmospheric [CO_2] (and other greenhouse gas concentrations) is the increase in global temperature and evaporative water demand, paralleled by a reduction in water availability in many regions. It is reported that the average global and ocean surface temperature has increased by approximately 0.85°C [0.65 to 1.06°C] over the period 1880 to 2012 (IPCC, 2014). These changes have affected agriculture globally and will create significant challenges for food security and nutrition in the future. Indeed, as observed by Oury et al. (2012), the beneficial effects expected from the increase in [CO_2] in European crop production during recent decades have been constrained by the effects of temperature increases and extended drought periods. This is a major issue, because cereal grains are a key source of carbohydrates, proteins, amino acids, lipids, vitamins, and minerals, all of which determine the nutritional value and quality of wheat products (Erice et al., 2019). More specifically, those crops provide 44% of the daily dietary intake of Fe, 27% of Mg, 25% of Zn, and 31% of Cu (Fan et al., 2008). Further, wheat is the second most important food crop after rice, and provides 20% of the daily protein and food calories worldwide (Nirgude and Sonawane, 2017).

Environmental conditions have a significant impact on crop production. Since the current atmospheric [CO_2] is generally limiting for plants with photosynthetic C_3 metabolism, the available information suggests that increases in [CO_2] should contribute to increased crop growth and yield (Kant et al., 2012; Degener, 2015). Alongside changes in crop yield, other studies carried out during the last few decades (Taub et al.,2008; DaMatta et al., 2010) have revealed that exposure to increasing [CO_2] causes alterations to the mineral content of plant tissues, total protein concentration, and lipid composition. Within this context, it has been suggested that because grains are predominantly composed of carbohydrates (mostly in the form of starch), the expected increases in starch concentrations due to the high [CO_2] could dilute other nutrients, including proteins, lipids, vitamins, and minerals (Högy and Fangmeier, 2008; Loladze, 2014).

Most plant studies are based on experiments in controlled environment (such as growth chambers) or field conditions, in which [CO_2] concentrations were fixed at 550-700 ppm. According to the different scenarios proposed by the IPCC (2014), these may be the ambient [CO_2] values that will be reached by 2050 and 2100, respectively. Nevertheless, new approaches to field studies have been developed. These include free air systems, such as the free air [CO_2] enrichment (FACE), controlled [CO_2] greenhouses (CGH) or open-top chambers (OTC) (Körner, 2006; Morales et al., 2014). It should be noted that, according to Högy et al. (2009a), the high [CO_2] stimulation effect might be larger in growth chambers and glasshouses compared to field exposure. While FACE overcomes many of the disadvantages associated with chamber and glasshouse experiments, some potential limitations have been attributed to these facilities when simulating natural growth conditions. A number of challenges exist when conducting FACE experiments, including the difficulty of managing large numbers of sites, replication within sites, physiological impact of a relatively abrupt increase in [CO_2], effect of CO_2 influx on breaking up an inversion layer, and consequent impact on temperature fluctuations within the open-top chamber (Pinter et al., 2000; Pinkard et al.,2010). Consequently, while FACE experiments approach natural conditions more closely than open-top chambers or other means of exposing plants to elevated [CO_2], they may still represent an approximation of natural growing conditions, albeit a method that is close to natural growth conditions. This may be a major issue to consider because the more realistic the experimental conditions, the more likely it is that the resultant predictions will reflect the reality of the future. However, few studies have addressed the effect of lower-than-present [CO_2] on grain quality and to the best of our knowledge, all of them have been performed in growth chambers simulating past conditions. As an alternative, the analyses of old samples from herbaria and other repositories should allow direct assessment of the effect of past growing conditions on contemporary samples from crops that were grown at that time. Within this context, in the last two decades, several studies have highlighted the use of archived material to analyse the changes in plant mineral composition over recent decades (Zhao et al., 2001; Fan et al., 2008).

However, herbarium material has often insufficient information about the location and environmental conditions where the plants were grown or the cultivars used.

The data presented in this paper aims to evaluate the impact of long-term changes in atmospheric [CO_2], temperature, and rainfall conditions on wheat grain quality traits (carbohydrates, protein, and mineral concentration) in archived samples during the last 166 years and the association between such quality traits and increased yield.

MATERIAL AND METHODS

Grain material collection

In this study, bread and durum wheat grains were collected from archives of 16 countries around the world (Table 1), from 1850 to 2016. All grain samples selected were intact, without any visible signs of degradation. One part of the archived grain samples originated from the Broadbalk Wheat experiment (Rothamsted, UK). They were taken from plots given annual applications of Farmyard Manure (35 tha^{-1} fresh material) since 1843. Another part of the samples (called the 'global samples') originated from 16 countries, and were removed from herbarium specimens stored at the Universitat Politecnica de Catalunya (Castel ldefels, Spain), Muséum National d'Histoire Naturelle (Paris, France), Real Jardín Botánico (Madrid, Spain), and Royal Botanic Gardens (Kew Richmond, United Kingdom).

The Broadbalk experiment is the oldest continuous agronomic field experiment in the world, which started in 1843. This 176 year-old experiment provides a large number of archived crop and soil samples from a wide range of agricultural, environmental, and ecological conditions. Grain yield data (1850-2016) were available for the Broadbalk experiment, but not from the other sites used in this study. Wheat varieties analyzed in the current study are shown in table S1. In addition, thousand-kernel weight (TKW) were available for Broadbalk from 1974 until 2016. In the early years (1844-1901), the crop from each plot was cut by hand with scythes, bound into sheaves and carted into the barns to await threshing. Broadbalk is now harvested by a small plot combine harvester with a 2m cut width. Yields of grain and straw are recorded, and samples stored for chemical analyses.

Table 1: Geographic origin and **sampling year** of archived wheat grain samples.

Continent	Country	Location	Sampling year	Number of replicates per year
Eurasia	Spain	Albacete	1900	3
		Lleida	1900	3
		Logroño	1850	3
		Madrid	1920	3
		Lugo	1953	3
		Segovia	1985	3
		Valladolid	1985	3
		Serrania de Ronda	1905	3
		Aranjuez	2015	3
		Cordoba	2015	3
		Zamora	2015	3
	United Kingdom	Rothamsted	[1850-2016]	3
		Regent Road, Leicester	1944	3
		City of London, Middlesex	1945	3
	New Caledonia	No data	1937	3
		Pouembout	1965	3
	Italy	Tuscany	1883	3
		Fiorenzuola d'Arda	2016	14
	Serbia/UK	Suva plains but cultivated at Royal Botanic Gardens Kew	1923	3
	Germany	Hohenheim	2016	3
	Russia	No data	1900	3
	No data (Asia)	No data	1910	3
Africa	Algeria	Oran	1856	3
	Nigeria	No data	1921	3
South America	Argentina	No data	1900	3
North America	USA	No data	1900	3
		Beltsville	2016	3
Asia (Near East)	Iraq	Zakh-Mosul	1947	3
		Mariye 4 km NW of Rawa	1947	3
	Iran	locality illegible	1885	3
	Yemen	Wadi Hadhramant	1946	6
Asia	China	No data	2016	6
Oceania	Australia	No data	2016	8

Environmental [CO_2], temperature, and precipitation data between 1850 and 2016

Global atmospheric [CO_2] values (Table 2) for the period from 1850 to 2016 were obtained from the European Environment Agency web page (EAA, 2019). Data corresponding to the evolution of ambient temperature were extracted from the Intergovernmental Panel on Climate Change (IPCC, 2007). Average temperature and precipitation recorded at Rothamsted (Table 2) were provided by the Department of Computational and Analytical Sciences.

Table 2: Average **environmental data** recorded from 1850 to 2016.

Area	**[CO_2] (ppm)**		**Temperature (°C)**		**Precipitation (mm)**	
	Years	values	Years	values	Years	values
Global	1850	286	1850	13.7	No data	
	1856	286	1860	13.6		
	1883	293	1870	13.8		
	1885	293	1880	13.9		
	1900	297	1890	13.7		
	1905	299	1900	13.8		
	1910	299	1910	13.5		
	1921	303	1920	13.7		
	1923	305	1930	13.9		
	1937	308	1940	14.0		
	1947	310	1950	13.8		
	1953	312	1960	13.9		
	1955	314	1970	13.9		
	1965	318	1980	14.0		
	1975	331	1990	14.2		
	1985	345	2000	14.4		
	1990	352	2010	14.7		
	2000	378	2016	14.9		
	2010	390				
	2016	400				
Rothamsted	1850	286	1878	9.28	1878	814.2
	1900	297	1900	9.37	1900	715.8
	1955	314	1955	8.99	1955	592.1
	1975	331	1975	9.60	1975	612.3
	1990	352	1990	10.25	1990	597.4
	2000	378	2000	10.20	2000	973.5
	2010	390	2010	9.01	2010	644.2
	2016	405	2016	10.35	2016	679.3

Grain quality parameters

Carbon isotope discrimination ($\Delta^{13}C$)

Carbon isotope composition was determined in milled grain samples. For each sample, 15 mg of finely milled material was weighed and analysed at the research support service of the Universidade da Coruña (Spain) using an elemental analyzer (EA1108; Carlo Erba Strumentazione, Milan, Italia) coupled to an isotope ratio mass spectrometer (Delta C; Finnigan, Mat., Bremen, Germany) operating in continuous flow mode. Values were expressed in composition units as $\delta^{13}C$ (‰) = $[(R_{sample}/R_{standard}) - 1] \times 1000$ (1), where the $^{13}C/^{12}C$ ratio of the sample is noted as $\delta^{13}C$ and expressed in ‰, whereas $R_{standard}$ is the molar abundance ratio of the secondary standard calibrated against the primary standard Pee Dee Belemnite ($\delta^{13}C$). The $\delta^{13}C$ values were later transformed into carbon isotopic discrimination values ($\Delta^{13}C$) according to Farquhar et al. (1989) as follows: $\Delta^{13}C$ (‰)= ($\delta^{13}C_{air}$ (‰)-$\delta^{13}C_{VPDV}$ (‰))/(1+($\delta^{13}C_{VPDV}$ (‰)/1000)) (2) where $\delta^{13}C_{air}$ (‰) is the ratio of the isotopes of ^{13}C and ^{12}C in the air, which varies through time, and $\delta^{13}C_{VPDV}$ (‰) refers to carbon isotope discrimination of grain sample. Air $\delta^{13}C$ values were obtained from Zhao et al. (2001).

Starch and soluble sugar concentrations

Milled grain samples were extracted by the addition of 0.5mL of 100% ethanol then another 0.5mL of 80% ethanol to approximately 25mg of sample and heated in a thermomixer (70°C, 90 min, 1100 rpm). The mixture was centrifuged (22°C, 10 min, 14000 rpm) and the supernatant was used for the determination of soluble sugars (glucose, fructose and sucrose). The samples were diluted with water (300µl sample + 700µl H_2O Mili-Q) and measured using an ionic chromatograph (ICS-3000, ThermoScientific^{TM}Dionex^{TM}, USA). Reference was made to sugar standards of known concentrations (50mM). The pellet was used to determine the starch content. Starch was solubilized by adding KOH (0.2N) to the pellet, and the pH was adjusted to 4.8 with acetic acid (0.1N). The extraction was performed with the kit containing the enzyme amyloglucosidase (R-Biopharm, AG; Darmstadt, Germany) and the absorbance was measured with a spectrophotometer at 340nm.

Protein content

Grain protein content (%) was determined according to Suchy et al. (2007).

Mineral composition

In each case, 100mg of pulverized dry grain samples were analysed (Gàmez et al., 2019). C and N concentrations (%) were determined using an elemental analyzer (EA1108; Carlo Erba Strumentazione, Milan, Italia). In addition, micro- and macronutrients (Cu, Zn, Fe, Mn, K, P, Mg, Ca, and Na) were quantified using ICP/OES (inductively coupled plasma/optical emission spectrometry, iCAP 6500 Duo, Thermo Fisher Scientific, Waltham, USA).

Data analyses

Given the long time-series considered, wheat samples received from herbaria were harvested from different locations and periods. Distinct genotypes were represented within each sampling year, with different number of repetitions. In view of the available data, the statistical analyses aimed to evaluate the trend of grain quality parameters over 166 years by calculating the average per year, without considering the genotype effect. To study the 'year' effect on the different parameters measured for global and Rothamsted grain samples, a simple analysis of variance (ANOVA) was performed by using STATGRAPHICS Centurion version 17.1.02 program (Bitstream, Cambridge, MN). For analytical variables, a multiple-range test (Fisher's least significant differences, LSD) was applied to test for statistical differences between years. Multifactor analyses of variance and Pearson correlation analyses were performed between the different parameters and the environmental factors ([CO_2], temperature, and precipitation) with the R software (RStudio v.3.4.2, 2017; Boston-Seattle, USA). The correlations between variables were considered significant when $p<0.05$.

RESULTS

Global samples

Environmental conditions

Atmospheric [CO_2] has been rising since 1850, and results in Table 2 showed that two periods can be distinguished: a first period (1850-1965), during which the average [CO_2] slowly rose by 31 ppm in 115 years; and a second period (1965-2016) during which a swift increase of [CO_2] of 82 ppm was recorded over 51 years. Therefore, the analysis of the effect of climate change on global wheat grain quality was based on the comparison among years, and also between these two periods (Table 3). The global mean annual temperature presented in Table 2 showed that it has increased by 1.2°C from 1850 to 2016, with fluctuations recorded in-between.

Table 3: Global averages of **wheat grain quality traits** in [1850-1955] and [1965-2016]. Mean ±standard error (SE) (n=3-18). The calculation of p-values is based on one-way ANOVA. Values in bold indicate statistical significance ($p<0.05$).

Grain quality traits	[1850-1955]	[1965-2016)	*p-value*
$\Delta^{13}C$ (‰)	16.53 ±0.23	16.50 ±0.36	0.940
Starch (µmol/g DW)	2713±117.48	2900 ±33.11	0.344
Sucrose (µmol/g DW)	13.83 ±2.93	15.19 ±3.33	0.794
Glucose (µmol/g DW)	1.01 ±0.19	1.89 ±0.51	**0.027**
Fructose (µmol/g DW)	3.14 ±0.59	2.92 ±0.41	0.821
Protein content (%)	16.96 ±1.28	13.07 ±0.8	**0.049**
Carbon content (%)	40.81 ±0.33	41.53 ±0.4	0.187
C/N (%)	17.16 ±0.56	20.6 ±0.84	**0.039**

Carbon isotope discrimination ($\Delta^{13}C$)

The results of carbon isotope discrimination calculated from 1850 to 2016 did not show a clear trend during this period (Figure 2.A). While the global carbon isotope discrimination ($\Delta^{13}C$) showed significant differences among years between 1850 and 2016 ($p<0.001$; Figure 2.A), no significant difference was observed between 1850-1955 and 1965-2016 (16.53‰ *vs.* 16.50‰ respectively; Table 3). Pearson analyses did not show any significant correlation, neither between $\Delta^{13}C$ and [CO_2] nor between $\Delta^{13}C$ and temperature (Table 5). Similarly, the multifactor analysis of variance (Table 6) indicated that there are no significant effects of [CO_2] and temperature on carbon isotope discrimination, and only the interaction between these two environmental factors was statistically significant ($p= 0.004$).

Non-structural carbohydrates

Starch concentration

The comparison of grain starch content among years (Figure 3.A) showed significant differences between years until 1946, but since 1953, the results showed higher values compared to the previous period and stability in starch content was detected. Further, the comparison between the two periods (Table 3) showed a non-significant increase of grain starch content by 7%. Results presented in Table 5 showed non-significant negative correlations between starch content and high temperature ($r= -0.175$) whereas significant positive correlation was detected with [CO_2] ($r= 0.247$). The multifactor analysis of variance presented in Table 6 showed that [CO_2] and temperature have significant effects on grain starch content, while the interaction [CO_2] x temperature has no significant effect (p= 0.525).

Soluble sugar concentrations

Sucrose, glucose, and fructose concentrations of global samples showed an increasing trend since 1975 (Figure 4.A). The comparison between the two periods (1850-1955 *vs.* 1965-2016; Table 3) showed non-significant differences for sucrose and fructose, whereas a significant increase was found for glucose (p= 0.027). Pearson analyses showed significant positive correlations between [CO_2] and glucose and sucrose concentrations, and between temperature and glucose and fructose concentrations (Table 5). Table 5 shows that [CO_2] had a significant effect on increasing glucose and sucrose concentrations, but no effect was recorded on fructose concentration. The interaction [CO_2] x temperature only significantly affects sucrose concentration.

Protein content

Protein content has varied among years since 1850 (Figure 5.A), and the comparison between 1850-1955 and 1965-2016 revealed a significant decrease of 23% (Table 3). Significant correlations were detected between protein content and [CO_2] and temperature (r= -0.265 and r= 0.269, respectively; Table 5). We also detected a significant effect of [CO_2] and temperature on protein content in the ANOVA, but no effect of the interaction effect (Table 6).

Mineral composition

At the global level, no significant difference was detected in C content during the period of study (Figure 6.A). However, given the decrease in protein content (that is tightly linked with the N content), the C/N ratio showed a significant increase of 20% between 1850-1955 and 1965-2016 (Figure 7.A, Table 3). A significant positive correlation was found between [CO_2] and C/N ratio, as well as a significant effect of [CO_2] on this ratio in the ANOVA (Tables 5 and 6). On the contrary, a significant negative correlation was found between the temperature and the C/N ratio (and a significant effect of the temperature in the ANOVA; Tables 5 and 6).

The global analyses of macro/micro-elements showed that their concentrations tend to decrease as ambient [CO_2] increased (Figure 8.A). In fact, negative correlations were detected between all macro/micro-elements and [CO_2], and these correlations were significant (and the effect of [CO_2] significant in the ANOVA) for K, Mg, Zn, Fe, and Mn (Table 5). The magnitude of the reduction differed between minerals, and the most notable reductions were observed for Mn, Fe, Zn, and Mg (Figure 8.A; Table 6). Temperature has also significantly affected Mg, Fe, and Mn concentrations, but no interaction with [CO_2] was detected (except for Cu; Table 6).

Table 4: Pearson correlation analyses (r) between environmental parameter (precipitation), and grain yield and quality traits of the Rothamsted Broadbalk experiment. A statistically significant effect is indicated with *** for $p<0.001$. ' - ' indicates unavailable coefficient of correlation (r).

Parameters	Yield	Starch	Sucrose	Glucose	Fructose	Protein
Precipitation	0.662***	-0.198	0.207	0.126	-0.310	0.004
TKW	0.193	-	-	-	-	-

Table 5: Pearson correlation analyses (r) between grain yield and quality traits, and environmental factors ([CO_2] and temperature). Statistically significant effects are indicated with *** for $p<0.001$, ** for $p<0.01$, and * for $p<0.05$. ' - ' indicates unavailable coefficient of correlation (r).

Grain quality traits	**Global**		**Rothamsted**	
	[CO_2]	Temperature	[CO_2]	Temperature
Yield	-	-	0.631**	0.688***
$\Delta^{13}C$	0.048	0.003	-0.688***	0.143
Starch	0.247*	-0.175	0.458*	0.435*
Glucose	0.379**	0.238*	0.591**	0.755***
Sucrose	0.676***	-0.03	0.209	-0.424
Fructose	0.131	0.257*	-0.224	0.293
Protein content	-0.265**	0.269**	-0.771***	-0.474*
C/N ratio	0.229**	-0.396***	0.691***	0.614**
C	-0.120	0.107	0.482*	0.315
K	-0.452***	-0.151	0.486*	0.425*
P	-0.124	0.036	-0.860***	-0.514*
Ca	-0.037	0.202*	-0.391	-0.626**
Mg	-0.560***	0.169	-0.852***	-0.698***
Cu	-0.165	-0.116	-0.437*	-0.370
Na	0.054	0.080	-0.324	-0.557**
Zn	-0.285*	0.097	-0.717***	-0.346
Fe	-0.188*	0.212*	-0.314	-0.300
Mn	-0.348***	0.364***	-0.942***	-0.444*

Broadbalk Wheat experiment (Rothamsted, UK)

Environmental conditions

Since the beginning of the Broadbalk wheat experiment in Rothamsted, the [CO_2] concentration increased by 45 ppm from 1850 to 1975 (*i.e.*, in 125 years), and by as much as 47 ppm from 1975 to 2016 (*i.e.*, in 41 years; Table 2). Annual temperatures were recorded from 1878 to 2016. Available data showed that, while mean temperature during 1878 was of 9.3°C, during, 2016, this value reached 10.3°C. Precipitation data recorded between 1878 and 2016 in Rothamsted did not show a clear pattern over time and rather revealed random fluctuations among years (Table 2).

Grain yield and thousand-kernel weight (TKW)

The results provided by the Broadbalk experiment about grain yield and thousand-kernel weight (TKW) are presented in Figure 1. Grain yield was more or less constant from 1850 until 1960, but has been subsequently increasing. Correlation analyses revealed significant positive correlations between grain yield and precipitation, [CO_2] and temperature (Tables 4 and 5). Highly significant effects of [CO_2] and temperature on wheat yield were also observed (Table 6). Further, available data showed that TKW has been decreasing since 1974 (Figure 1). Pearson analysis revealed non-significant correlation between TKW and grain yield (Table 4), and the multifactor ANOVA showed highly significant effects of [CO_2] and temperature on TKW (Table 6).

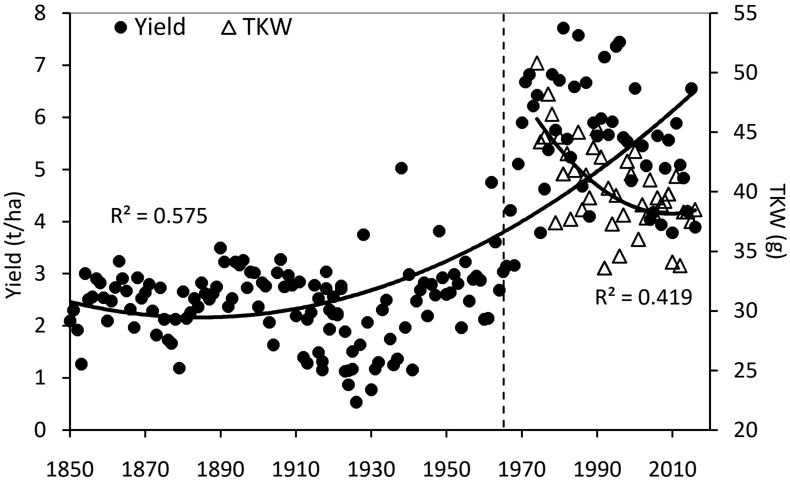

Figure 1: Trends **in wheat yield** and **thousand-kernel weight (TKW)** of **Broadbalk experiment** from 1850 to 2016. Data are means. The dashed line represents the introduction of dwarf cultivars in 1968.

Carbon isotope discrimination ($\Delta^{13}C$)

We observed a clear decrease of $\Delta^{13}C$ during the last decades (Figure 2.B). The [CO_2] concentration was highly significantly and negatively correlated with $\Delta^{13}C$ (Table 5), and had a significant effect on this variable (Table 6). The temperature has also significantly affected $\Delta^{13}C$, but there was no significant effect of the [CO_2] x temperature interaction.

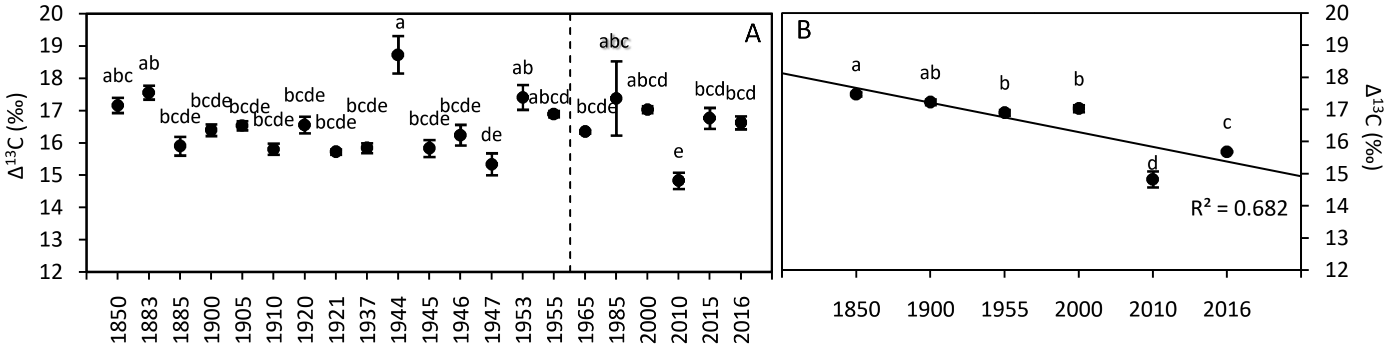

Figure 2: Wheat grain carbon isotope discrimination ($\Delta^{13}C$) of **global** (A) and **Broadbalk experiment** (B) samples. The dashed line corresponds to the separation between the [1850-1955] and [1965-2016] periods, based on the increase of [CO_2]. Data are means ±standard errors (n=3-14). The same letters indicate no statistically significant differences among years (Fisher's LSD, $p \geq 0.05$).

Non-structural carbohydrates

Starch concentration

Grain starch content varied significantly among years since 1850 and reached the highest value in 2016 (Figure 3.B). It should also be noted that at Rothamsted, the increasing temperature might have contribute to favor starch accumulation (P= 0.034) by 43% (Table 5), but there was no correlation between precipitation and starch content (Table 4). Results presented in Table 4 showed insignificant negative correlations between starch content and precipitations (r= -0.198) whereas significant correlations have been recorded with [CO_2] and temperature (Table 5). According to the results presented in Table 6, only raising [CO_2] has a significant effect on grain starch content since 1850 to 2016.

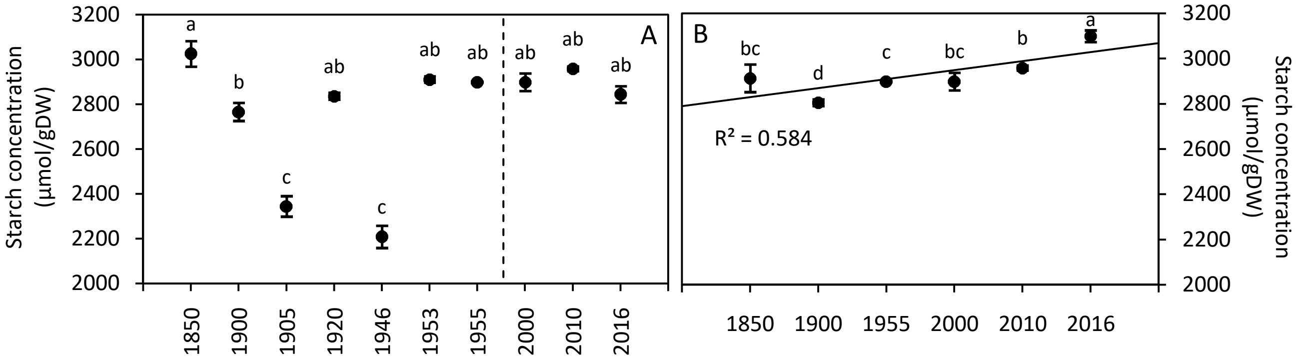

Figure 3: Wheat grain starch concentration of **global** (A) and **Broadbalk experiment** (B) samples. The dashed line corresponds to the separation between the [1850-1955] and [1965-2016] periods, based on the increase of $[CO_2]$. Data are means ±standard errors (n=3-14). The same letters indicate no statistically significant differences among years (Fisher's LSD, $p \geq 0.05$).

Table 6: Multifactor analysis of variance (ANOVA). The calculation of p-values in based on multifactor analysis of variance ANOVA. Values in bold indicate significance ($p<0.05$). ' - ' indicates unavailable p-value.

Traits	**Global**			**Rothamsted**		
	$[CO_2]$	Temperature	$[CO_2]$ X Temperature	$[CO_2]$	Temperature	$[CO_2]$ X Temperature
	p-value			*p-value*		
Yield	**-**	**-**	-	**<0.001**	**0.003**	**0.015**
TKW	**-**	**-**	-	**<0.001**	**<0.001**	0.164
$\Delta^{13}C$	0.612	0.906	**0.004**	**<0.001**	**<0.001**	0.837
Starch	**0.009**	**0.008**	0.525	**0.026**	0.247	0.700
Glucose	**0.001**	0.079	0.249	**<0.001**	**<0.001**	**<0.001**
Sucrose	**<0.001**	0.215	**0.002**	0.147	<0.001	0.806
Fructose	0.273	**0.023**	0.962	0.233	**0.002**	0.434
Protein content	**<0.001**	**<0.001**	0.901	**<0.001**	0.737	**0.015**
C/N ratio	**0.001**	**<0.001**	0.064	**<0.001**	0.060	0.794
C	0.137	0.093	0.158	**0.022**	0.717	0.852
K	**<0.001**	0.446	**0.001**	**0.016**	0.296	0.318
P	0.174	0.488	0.248	**<0.001**	0.538	0.700
Ca	0.682	0.018	0.552	**0.035**	**0.010**	0.857
Mg	**<0.001**	**<0.001**	0.264	**<0.001**	**0.004**	0.151
Cu	0.065	0.345	**0.027**	**0.038**	0.416	0.849
Na	0.559	0.444	0.755	0.084	**0.019**	0.218
Zn	**0.013**	0.498	0.528	**<0.001**	0.809	0.845
Fe	**0.035**	**0.047**	0.273	0.141	0.448	0.340
Mn	**<0.001**	**<0.001**	0.586	**<0.001**	0.298	**0.002**

Soluble sugar concentrations

The concentrations of sucrose, glucose, and fructose in wheat grains showed significant variations among years, as presented in Figure 4.B. Pearson analyses showed non-significant correlations between these concentrations and precipitation (Table 4). [CO_2] and temperature were significantly related to glucose concentration, as shown by both the positive correlations (r= 0.591 and r= 0.755, respectively; Table 5) and the ANOVA (Table 6).

Protein content

Climate change has negatively impacted total protein content: A 26% reduction in protein content was recorded between 1850 and 2016 (Figure 5.B). Pearson analyses revealed that water availability was not correlated with protein content (Table 4), whereas temperature and (more significantly) [CO_2] were both negatively correlated with such content (Table 5). Based on the ANOVA, only [CO_2] but not temperature had a significant effect on protein content (Table 6).

Mineral composition

Carbon content (Figure 6.B) showed a significant 3% increase since 1850. Similarly, C/N ratio (Figure 7.B) showed a highly significant 40% increase, mainly caused by the significant decrease of protein content (and therefore N content) reported above. Only [CO_2], but not the temperature, was significantly correlated with and had a significant effect on C content (and consequently on C/N ratio; Tables 5 and 6).

Macro/micro-elements concentrations have also been modified since 1850 (Figure 8.B). Statistical analyses showed that both [CO_2] and temperature (but most importantly [CO_2]) had significant negative effects on mineral compositions (except for the K concentration that increased; Tables 5 and 6).

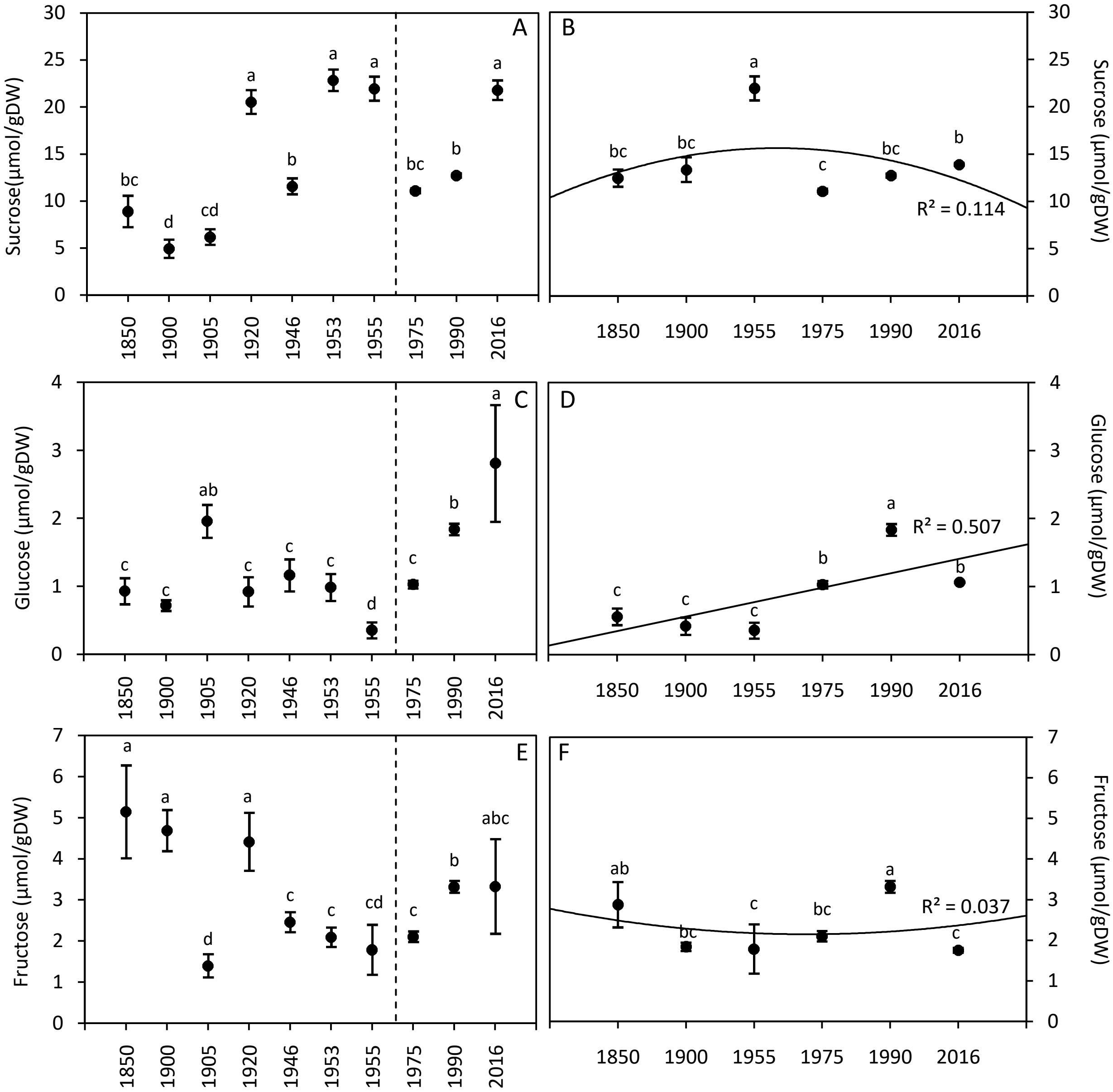

Figure 4: Wheat grain soluble sugar (sucrose, glucose, and fructose) concentrations of **global** (A, C, E) and **Broadbalk experiment** (B, D, F) samples. The dashed line corresponds to the separation between the [1850-1955] and [1975-2016] periods, based on the increase of [CO_2]. Data are means ±standard errors (n=3-14). The same letters indicate no statistically significant differences among years (Fisher's LSD, $p \geq 0.05$).

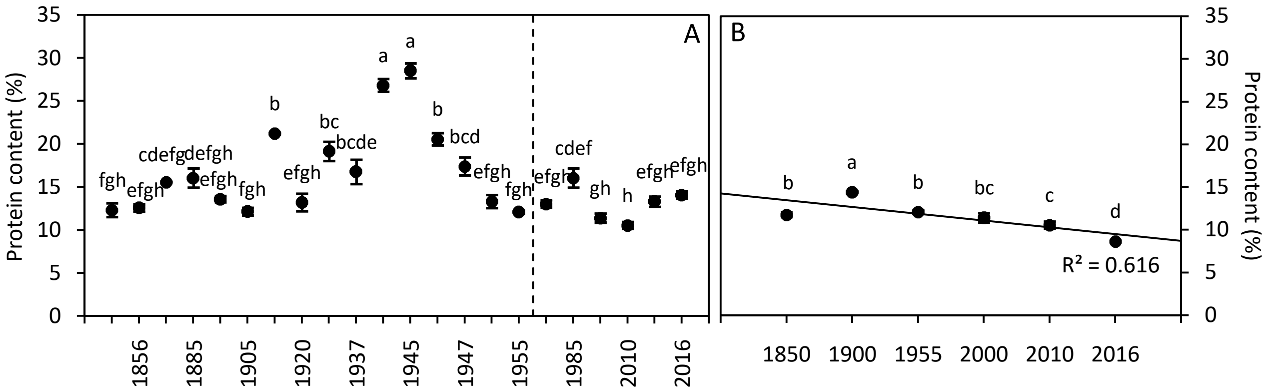

Figure 5: Wheat grain protein content of **global** (A) and **Broadbalk experiment** (B) samples. The dashed line corresponds to the separation between the [1850-1955] and [1965-2016] periods, based on the increase of [CO_2]. Data are mean ±standard errors (n=3-14). The same letters indicate no statistically significant differences among years (Fisher's LSD, p ≥ 0.05).

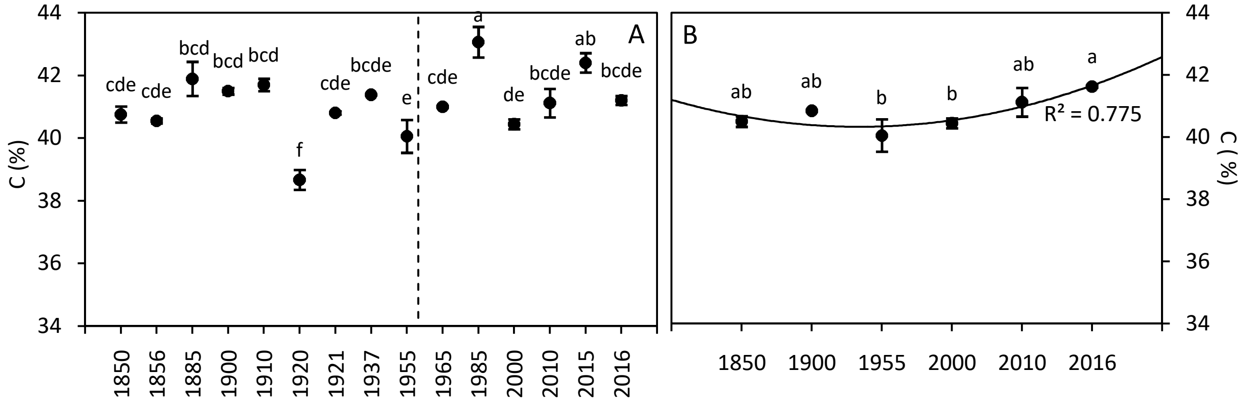

Figure 6: Wheat grain carbon content of **global** (A) and **Broadbalk experiment** (B) samples. The dashed line corresponds to the separation between the [1850-1955] and [1965-2016] periods, based on the increase of [CO_2]. Data are means ±standard errors (n=3-14). The same letters indicate no statistically significant differences among years (Fisher's LSD, p ≥ 0.05).

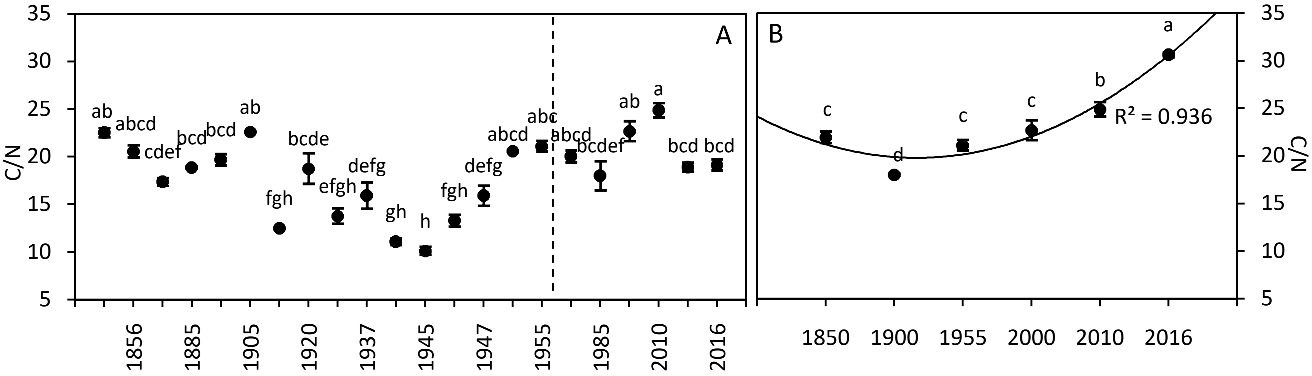

Figure 7: Wheat grain C/N ratio of **global** (A) and **Broadbalk experiment** (B) samples. The dashed line corresponds to the separation between the [1850-1955] and [1965-2016] periods, based on the increase of [CO_2]. Data are means ±standard errors (n=3-14). The same letters indicate no statistically significant differences among years (Fisher's LSD, p ≥ 0.05).

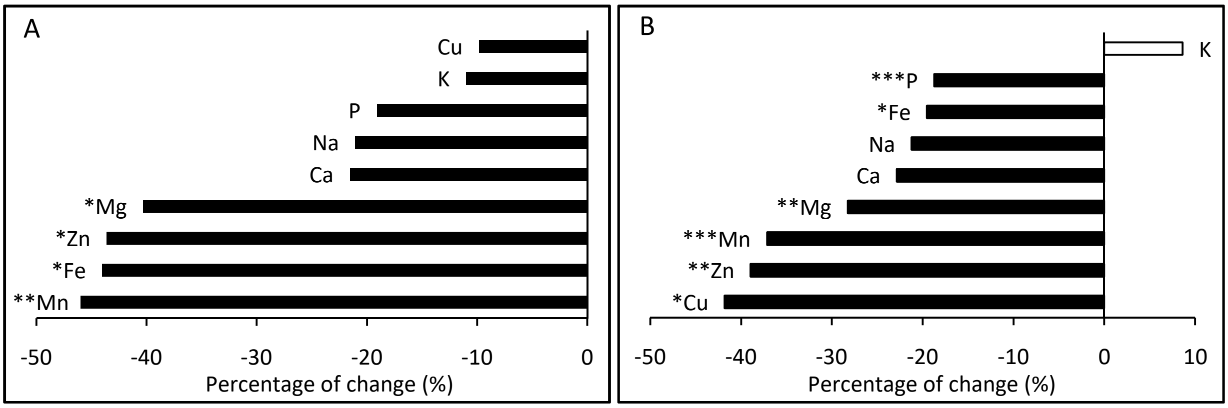

Figure 8: Change in **grain mineral concentrations** relative to [1850-1955] period of **global** (A) and **Broadbalk experiment** (B) samples. Data are means ±standard errors (n=3-14). Statistically significant effects are indicated with *** for p<0.001, ** for p<0.01, and * for p<0.05 (Fisher's LSD).

DISCUSSION

The current study, based on historical wheat samples collected over a 166-year period, has confirmed that grain quality and yield have been affected by raising atmospheric [CO_2] and temperature. The increase of [CO_2] and temperature, together with the introduction of dwarfing genotypes (see below), have increased harvest index, leading to rising wheat yield. Furthermore, carbon isotope discrimination has decreased over 166 years in the Broadbalk experiment (but not on worldwide samples collected across the same time period), which indicates that plants have been subjected to stressful conditions causing stomatal closure. With respect to grain quality parameters, our findings showed that non-structural carbohydrate concentrations have increased, while total protein content and mineral compositions have decreased.

The decreasing trend in TKW indicated that the increase in grain yield was not linked to heavier grains, but to a larger number of grains. While the effect of introducing semi-dwarf varieties should also be considered, our study showed that increasing [CO_2] favored crop production. Previous experiments, carried out with wheat plants grown in environments where atmospheric [CO_2] was increased by 150-300 ppm, showed similar increases in grain yield values (Högy et al.,2009b; Erice et al., 2019) As has been widely described in these previous experiments, the higher yield records would be associated with the stimulation of photosynthetic rates. According to this study, alongside increasing radiation levels, the temperature rise was also an important factor explaining yield increases. More specifically, the enhancement of ambient temperatures (below heat stress values) might have contributed to extend the grain-filling period, which subsequently resulted in a higher biomass production and a higher yield. Finally, when analyzing changes in crop yield during the last decades, together with increasing [CO_2] and temperature effects, changes in crop varieties could have an important effect. After 1968, high-yielding semi-dwarf cultivars (with increasing harvest index) were cultivated in the Rothamsted field trials and these cultivars have been reported to distribute a greater proportion of photosynthates to the grains than other cultivars (Flintham et al.,1997).

The use of stable isotope variation has grown steadily in plant research during the past two decades. Stable isotopes are time-integrated indicators of how plants interact and respond to their abiotic and biotic environment (Yousfi et al., 2013). Consequently, alterations in $\Delta^{13}C$ have been used as a selection indicator of stomatal opening, water transpiration and water use efficiency (WUE) under different water availability and [CO_2] (Yousfi et al., 2013; Erice et al., 2019). In the case of Rothamsted, the negative correlation between [CO_2] and $\Delta^{13}C$ detected after the 1960's revealed that following the increase in [CO_2] and temperature, and the decrease of precipitation, plants tended to decrease stomatal opening and reduce water loss via canopy transpiration (Zhao et al., 2001). As described by Aranjuelo et al. (2005), exposure to elevated [CO_2] might increase WUE by decreasing water consumption (due to a decline in stomatal opening and transpiration), by enhancing CO_2 fixation or by the interaction of both mechanisms. However, large genetic variation in carbon isotope discrimination exists among and between species. This variation could be widely explained by genotypic differences in stomatal conductance and photosynthetic capacity (Dixon et al., 2019). Hence, the absence of a temporal trend at the global (worldwide) level, between 1850 and 2016, is probably mainly due to genotypic variability of cultivars grown at different locations.

About carbohydrate concentrations, studies conducted in controlled and FACE facilities showed that rising [CO_2] contributes to increases in starch and soluble sugar concentrations in wheat grains (Sinha et al., 2011; Pandey et al., 2017; Yadav et al., 2019). The larger photosynthetic rates of C_3 plants such as wheat under increasing [CO_2] may contribute to increased carbohydrate translocation from the source (leaves and stems) to the sink (grains), where the C is mainly stored in the form of starch. Furthermore, the positive correlation between yield and temperature at Rothamsted contributed to increased starch content. Considering that photosynthesis increases as leaf temperature rises (peaking at an optimum temperature and then declining), and that average temperature values in this area are below the optimum (15-25°C), such an increase in ambient temperature might have contributed to increase photosynthesis and grain development (Nuttall et al., 2017; Posh et al., 2019). Increases of ambient temperatures have been previously described and often results in an environmental temperature closer to the optimal, which results in increased photosynthesis (Sage and Kubien, 2007; Albert et al.,2011).

Alongside the increases in grain carbohydrates mentioned above, decreases in total protein and nutrient concentrations have been extensively described in plants exposed to elevated [CO_2](Loladze, 2014;Zhu et al., 2018). Different explanations have been proposed. According to some studies (Kimball et al., 2001; Högy et al., 2009a), decreased protein and mineral concentrations could be a consequence of the larger carbohydrate content in grains. Our study showed a clear correlation between [CO_2] and C/N ratio in all cases. Furthermore, we found that the starch concentration globally increased by 7% while grain protein content decreased by 23% between 1850-1955 and 1965-2016. This may support the fact that grain protein decrease should also be associated with other mechanisms such as decreased transpiration-driven mass flow of nitrogen (Myres et al., 2014; Udling et al., 2018). Furthermore, other factors such as the crop breeding approaches aimed at increasing crop yields (and declining response to N fertilizer) and limitations to N assimilation (Vicente et al., 2015) may have been involved. The positive correlation found between temperature and starch content in the Broadbalk experiment, as well as changes to the C/N ratio, would also highlight how the temperature-associated increases in grain C content are involved in the lower grain protein contents observed.

The current study showed an overall decrease in all micro- and macronutrient concentrations in wheat grain over 166 years. Similarly, other historical studies have shown that mineral composition of the dry matter of wheat grains, vegetables and some fruits have decreased over time (White and Broadley, 2005; Garvin et al., 2006; Fan et al., 2008; Morgounov at al., 2013). Such impoverishment may in part be associated with changes in atmospheric [CO_2] and temperature. The fact that the decreases were more evident in the Broadbalk experiment could be associated with different factors such as the strong increases in crop yield and lower C^{13} discrimination (Δ) values detected in those plants during the recent decades. The potential impact of other factors such as alterations on nutrient uptake, and remobilization from leaves to grain and a greater transport of carbohydrates to grain (dilution) should also be considered. In addition, lower stomatal opening and a decrease of crop transpiration may have altered the mass flow of minerals from the soil to aboveground plant parts (Wang and Frei, 2011; Pilbeam, 2015). As observed by Fan et al. (2008), this explanation is especially likely since the concentrations of soil nutrients have not decreased at Rothamsted since the Green Revolution.

CONCLUSIONS AND PERSPECTIVES

Overall, this study highlighted that there has been a global trend of altered wheat grain quality characterized by an increase in non-structural carbohydrates and an impoverishment in total protein and mineral nutrients concentrations during the last 166 years. This trend has been especially prominent since the 1960s and linked to the introduction of higher yielding short-strawed varieties, together with an increase in air [CO_2] and temperature. It seems likely that during this period enhanced photosynthetic rates linked to the increase in [CO_2] may have favored carbohydrate synthesis and carbon accumulation in grains and that this has negatively affected mineral composition. Along with a potential C-derived dilution effect, the current paper also implicates other factors such as depleted transpiration (affecting mineral transport) and the lower responsiveness of modern cultivars to current fertilization strategies. In view of the findings stated above, breeding strategies should develop new genotypes better adapted to changing environmental conditions with greater resource use efficiency and combine high grain nutritional values with high-yielding traits by exploring genetic variation in proteins and nutrients concentrations in wheat germplasm, since these traits are not only affected by environmental factors, but also are controlled genetically.

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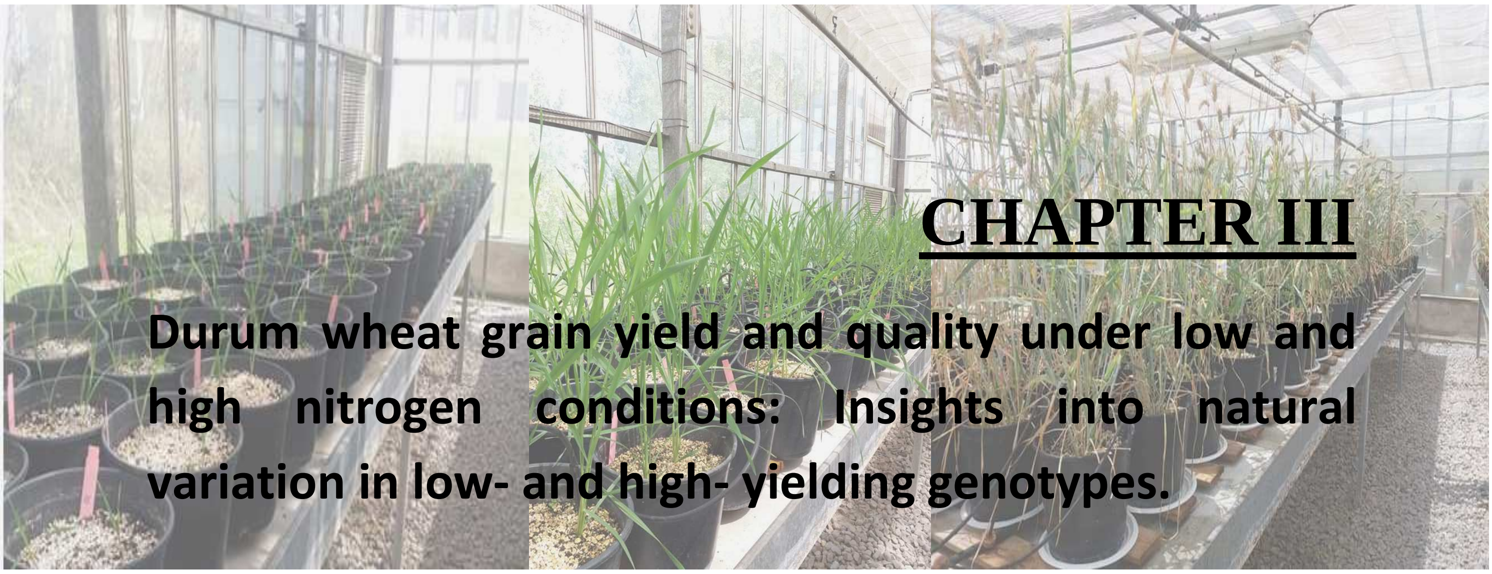
SUPPLEMENTARY MATERIAL

Table S1: Winter wheat varieties grown on Broadbalkduring 1849-2018.

Years	Variety
1849-1852	Old Red Cluster
1853-1881	Red Rostock
1882-1899	Red Club
1900-1904	Squarehead's Master
1905	Giant Red
1906-1909	Squarehead's Master
1910	Browick Red
1911-1912	Little Joss
1913-1916	Squarehead's Master
1917-1928	Red Standard
1929	Squarehead's Master
1930-1939	Red Standard
1940-1941	Squarehead's Master
1942	Stand up
1943	Squarehead's Master
1944-1945	Red Standard
1946-1967	Squarehead's Master
1968-1978	Cappelle Desprez
1979-1984	Flanders
1985-1990	Brimstone
1991-1995	Apollo
1996-2012	Hereward
2013-2014	Crusoe
2015	Mulika (Spring wheat)
2016-2018	Crusoe



CHAPTER III

Durum wheat grain yield and quality under low and high nitrogen conditions: Insights into natural variation in low- and high- yielding genotypes.

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INTRODUCTION

Cereals are the most abundant field crops globally and considered as staple foods for humanity. Wheat is one of the most important cereal crops with an outstanding role in worldwide population nutrition (Ciudad-Mulero et al., 2020). Currently, Triticum grains contribute largely to human diet by providing carbohydrates, protein, dietary fiber, minerals, vitamins, and also phenolic acids that complement a balanced diet by their anti-oxidative potential (Engert et al., 2011). Durum wheat (*Triticum turgidum* L. var. *durum*) represents 8% of the whole area cultivated with wheat and about 5% of world wheat production (Colasuonno et al., 2019). In spite of being relatively less important than bread wheat (*Triticum aestivum* L.), durum wheat is cultivated in many areas of the Mediterranean basin as a main cereal crop widely used for making pasta (Kabbaj et al., 2017). In fact, considering its cooking quality, durum wheat flour is technologically a preferred raw material for this purpose (Sissons et al., 2005).

Wheat grain yield and quality is determined by genotype, environment and the interaction between them (Asthir et al., 2017). Among the environmental factors that affect strongly crop productivity and nutritional quality of cereal crops, fertilizers management is an important factor to obtain high yield and high quality harvests (Dolijanovic et al., 2019). Nitrogen is one of the major nutritional elements required for adequate plant growth, and hence fertilization with N increases grain yields and improves end-use quality (Blandino et al., 2016; Litke et al., 2018; Zörb et al., 2018). As a consequence, N fertilizers application has been dramatically increased (Sharma and Bali, 2018). However, it has been estimated that only 30% to 40% of the applied N is absorbed by the crop and harvested in the grain (Lyngstad, 1975), thus, the excessive application leads to a huge loss of N contributing thereby to environment pollution. Therefore, an optimized N fertilization, i.e., a rational use of N fertilizers, is an important task for agronomists to improve crop N uptake, increase nitrogen use efficiency (NUE) and yields (Fageria, 2014; Zavalin et al., 2018).

Increasing wheat yield has often been associated with grain quality losses. Research has been focused to investigate the relationship between N and grain protein. In this regard, different observations have been reported regarding grain protein content and composition depending on the rate of N and the timing of application. Nitrogen supply increased N accumulation in bread wheat grains, which enhanced protein content (Rodriguez-Felix et al., 2014), resulting in an increase in both gliadins and glutenins (González-Torralba et al., 2011). Similar results were found by Abedi et al. (2011) but, an over-dose N application decreased wheat seed protein content. Late application of N resulted in gluten enhancement (Abedi et al., 2011). Makowska et al. (2008) found a positive correlation between N dose and protein content as well as glutenin in durum wheat grains. They claimed also that fertilization level influenced gluten proteins properties.

Conversely, less attention has been devoted in the literature to assess the effect of N fertilization on wheat grain carbohydrates. Regulation of metabolic processes by sugars depends on N supply, suggesting that N and sugar signaling pathways interact (Wingler et al., 2004). In fact, N metabolism requires C sources and energy from C metabolism, whereas C metabolism requires N metabolism to provide N-containing compounds, such as photosynthetic pigments and enzymes. Thus, the application of N fertilizer has indeed a significant effect on crop non-structural carbohydrate content (Wu et al., 2019). Furthermore, C assimilation, allocation and partitioning are strongly influenced by N supply affecting as a consequence carbohydrate distribution within the plant (Bala et al., 2016). In the same way, Pan (2010) found that, under low N conditions, the concentration and apparent transferred mass of non-structural carbohydrates were higher than those under high N conditions.

Mineral composition and content in wheat grain are also impacted by N fertilization. Numerous studies have shown that N application can promote the accumulation of some macro- and micro-elements in wheat grain (Svecnjak et al., 2013; Ma et al., 2018; Singh et al., 2018), whereas they declined in other studies (Smith et al., 2018), showing that grain nutrient composition is controlled in part by genotype and in part by environmental factors. Adding to that, Dolijanovic et al. (2019) found that an appropriate rate of N fertilizer has better impact on the concentration of macro- and micronutrients in wheat grain than using over-doses.

Phenolic compounds are secondary plant metabolites with strong antioxidant activity (Shahidi and Ambigaipalan, 2015). Ma et al. (2015) indicated that N fertilization and irrigation have positive effects on wheat grain phenolic content and antioxidant activity.

Other studies showed that wheat grain antioxidant properties are influenced by genotype, environment and genotype-environment interaction (Zhou et al., 2004; Okarter et al., 2010). A crucial first step in any genetic mapping and breeding approach is to identify the existing variability in available germplasm. Genetic differences in NUE have been reported in the past in target crops such as wheat. However, the molecular knowledge governing genetic variation among varieties in changing environmental conditions is still incomplete. In this work, a collection of 20 durum wheat varieties were provided by the International Maize and Wheat Improvement Center (CIMMYT, Mexico), which were selected in field conditions from a set of 120 genotypes as the ones having higher grain yield, protein, and starch concentrations under stressful growth conditions.

Taking into account the above-mentioned background, the aim of this work was to elucidate the response of durum wheat yield and grain quality to N fertilization. Towards this aim, we investigated the effects of two N levels in the fertilizer composition on grain yield and quality in different genotypes of durum wheat.

MATERIAL AND METHODS

Plant material and experimental design

The experiment was carried out from March to July 2017 with 20 durum wheat (*Triticum turgidum* L. var. *durum*) genotypes. Seeds were obtained from CIMMYT (International Maize and Wheat Improvement Centre, Mexico) (Table S1). Those genotypes were selected from a set of 120 genotypes as the ones having highest grain protein and/or starch content. Seedlings were vernalized for one week in a cold room at 4°C and then transplanted to 6L pots containing a peat/perlite/vermiculite 2:2:1 (v/v/v) substrate mixture. After sowing, the plants were transferred to a greenhouse located at the Institute of Agrobiotechnology (IdAB), Pamplona (Spain). Plants were grown under natural sunlight (with no supplemental lighting) and day-length at 15-17/19-23°C from March to June and 17-20/19-26°C in July night/day. Plants were irrigated with water until they reached anthesis stage (nutrients were released from peat). Then, half of the plants were watered by ½ Hoagland nutrient solution (100% N: high N treatment), whereas the other half were watered with modified ½ Hoagland solution with 1/3 N (low N treatment). For each treatment, 4 pots were used per genotype with 2 plants per pot and the experiment was conducted according to randomized complete block design. Plants were irrigated 3 times per week (0.5L each per pot), two times with Hoagland solution and once with water. At maturity, plants were harvested and grains were collected to determine yield and quality traits of the different genotypes.

Grain yield and thousand-grain weight

Grain yield and thousand-grain weight (TGW) were determined for each plant. TGW was determined by calculating the weight of 20 grains and then converted to the weight of 1000 grains as follows: TGW(g)= (weight of 20 grains (g) x 1000)/20. These parameters were determined for the 20 genotypes, as a prerequisite to select 6 genotypes (3 as high-yielding genotypes and 3 as low-yielding ones) in order to analyze grain quality under the two above-mentioned N levels.

Grain carbon and nitrogen concentrations and nitrogen use efficiency (NUE)

C and N concentration (% of DW) analyses were determined using an elemental analyzer (FlashEA1112, ThermoFinnigan) equipped with a MAS200R autosampler. Grains were ground to a fine powder and ≈1mg samples were weighed and stored in tin capsules for elemental analyses (MX5 microbalance, Mettler-Toledo) and introduced into a quartz reactor filled with WO_3 and copper and heated at 1020°C. The combustion gas mixture was carried by a helium flow to a WO_3 layer to achieve a complete quantitative oxidation, followed by a reduction step in a copper layer to reduce nitrogen oxides and SO_3 to N_2 and SO_2. The resulting components, N_2, CO_2, H_2O and SO_2 were separated in a chromatographic column (Porapak 2m, Santa Clara, CA, USA) and detected with a thermal conductivity detector. Nitrogen use efficiency (NUE) of production was determined as the ratio of grain yield to the total N concentration in grains, which must be differentiated from other ways of measuring the efficiency of N use by plants (Hawkesford and Riche, 2020).

Grain mineral composition

Micro- and macronutrients concentrations were determined by inductively coupled plasma/optical emission spectrometry (ICP/OES, iCAP 6500 Duo, Thermo Fisher Scientific, Waltham, USA).

Grain carbohydrates composition

Wheat grains were milled and 25mg of each sample were added to 0.5mL of 100% ethanol then another 0.5mL of 80% ethanol was added and heated in a thermomixer (70°C, 90 min, 1100 rpm). The mixture was centrifuged (22°C, 10 min, 20800g) and the supernatant was used for the determination of soluble sugars (glucose, fructose and sucrose) concentration, using an ionic chromatographer (ICS-3000, Thermo ScientificTM, Waltham, MA, USA). Reference was made to sugar standards of known concentrations (50mM). The pellet was used to determine starch content. Starch was solubilized by adding KOH (0.2N) to the pellet, and the pH was adjusted to 4.8 with acetic acid (0.1N). Quantification was performed with the kit containing the enzyme amyloglucosidase (R-Biopharm, AG; Darmstadt, Germany) measuring the absorbance at 340nm with a spectrophotometer.

Grain gliadin and glutenin concentrations

To determine grain gliadin concentration, 167mg of milled samples were placed in 2mL test tube. First, albumins and globulins were extracted with 1mL of buffer A (0.05M sodium phosphate pH 7.8 and 0.05M NaCl) for 1 h at 4°C. Extraction was followed by centrifugation at 20800g for 5 min at 4°C. Amphyphilic proteins were then extracted from the pellet resuspended in 1mL of 2% (w/v) Triton X-114 in buffer A for 1 h at 4°C. After centrifugation at 20800g for 5 min at 4°C, gliadins were separated from the residue with 1mL of 70% (v/v) aqueous ethanol for 1h at 20°C and centrifuged at 20800g for 5 min at 20°C. Gliadins were located in the supernatant. For glutenin extraction, only 50mg of white flour were used to avoid a too-viscous supernatant after glutenin extraction. After gliadin extraction, glutenins were extracted overnight with 1.5mL of buffer B (0.05M disodium tetraborate pH 8.5, 2% (v/v) β-mercaptoethanol, 8M urea, and 1g L^{-1} glycine) at 20°C. Samples were centrifuged at 20800g for 5 min at 20°C, and an aliquot of 0.5mL was alkylated with 15µL of 4-vinylpyridine for 45 min at 60°C. Afterward, 1 volume of 2-propanol was added to precipitate polysaccharides, and samples were centrifuged at 20800g for 1 min at 20°C. Supernatants containing gliadins and glutenins were filtered with a 0.45µm polyvinylidene fluoride filter before quantification by reverse phase high performance liquid chromatography (RP-HPLC) in a Waters 2695 Separations Module (Waters Corporation, Milford, USA) using a Europa Protein 300 C18 column (300 Å, 5µm, and 250 x 4.6mm) at 50°C with a Guard Column Protein 300 C18 (10 x 3.2mm) (Teknokroma, SantCugat del Vallès, Spain). Eluents and gradient conditions for RP-HPLC were as described in Triboi et al. (2000). Amounts of gliadins and glutenins were calculated by integration of the areas under the curve of the ultraviolet signal (220nm) and expressed as the chromatogram area per milligram of whole-meal flour on a dry matter basis (mV min mg^{-1}).

Grain polyphenol fractions and anti-radical activity

Soluble polyphenols were determined in 500mg of freeze ground grain samples by extraction with 4mL of acidified methanol (HCl/methanol/water, 1:80:10, v/v/v) at room temperature for 2h (Gao et al., 2002). Hydrolysable polyphenols were determined in sample residues remained after extraction of soluble phenolics, by mixing with 5mL of methanol and concentrated sulfuric acid (10:1, v/v) at 85°C for 20 h in a shaking water bath (Hartzfeld et al., 2002). Bio-accessible polyphenols were assessed by conducting an “in vitro” digestive enzymatic mild extraction that mimics the conditions in the gastrointestinal tract, according to the procedure of Glahn et al. (1998) and adapted for flours and breads by Angioloni and Collar (2011). Supernatants obtained after extractions were combined and used for respective determination of soluble, hydrolysable and bio-accessible polyphenols using the Folin-Ciocalteu spectrophotometric method. A calibration curve was made using gallic acid, and therefore, obtained amounts of phenolics were expressed as gallic acid equivalents. The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to measure the radical scavenging capacity of milled samples according to the DPPH method modified by Sánchez-Moreno et al. (1998) and adapted by Collar et al. (2014). Plots of micromoles of DPPH vs. time (min) were drawn and calculations were made to determine the anti-radical activity (AR) as follows: ((DPPH INITIAL – DPPH PLATEAU) × 100) / DPPH INITIAL.

Statistical analysis

To explore the N fertilization effect on yield components and used to select 6 out of the 20 genotypes tested, one-way analysis of variance (ANOVA) (STATGRAPHICS, Centurion XV, Version 15.1.02) was conducted using ‘genotypes’ as factor. Two-way analysis of variance has been also conducted to analyze the interaction between genotypes and N treatments. Regarding to grain quality parameters, univariate statistic analyses were performed by two-way analysis of variance using the factor ‘N treatments’ with two values (high and low) and the factor ‘genotypes’ having 6 values (6 different genotypes).

One-way ANOVA was also performed to evaluate differences among genotypes. Results were considered to be significant when p<0.05. When the main factors 'genotypes' or 'N treatments' gave statistically significant differences, Least Significant Difference (LSD) was used to determine statistical differences among genotypes and treatments.

RESULTS

Wheat yield components: grain yield and thousand-grain weight

Significant differences were detected regarding grain yield and TGW among the 20 wheat genotypes fertilized by the two N levels (Table 1). This comparison allowed selecting 6 genotypes for grain quality analysis. Genotypes 18, 6, and 10 were selected as high-yielding genotypes since they recorded the highest values of grain yield and TGW under the two N levels, while genotypes 3, 9, and 16 showed the lowest values. It was surprising results of genotypes 20 and especially 17, which had higher grain yield under low than under high N. We are not sure if it could be related with pot placement in the greenhouse, because pots were placed according to a randomized complete block design. Causes for that behavior in genotypes 20 and 17 were not further explored. Overall, high N fertilization treatment had significant positive effect on mean grain yield per plant while no effect was detected on TGW (Table 1). Therefore, these data suggest that the increase of wheat grain yield observed under the high N treatment can be due to the increase in grain number produced per plant. The interaction genotype-treatment was not significant (Table 1).

Wheat grain nitrogen and carbon concentrations

Results presented in Table 2 show that the high N treatment significantly increased grain N and C concentrations by 29.24% and 2.43% respectively. These changes led to a significant decrease in the C/N ratio (Table 2). Regarding to genotype effect, the comparison among genotypes revealed that, generally, averages of grain N and C concentration were higher in the low-yielding genotypes (3, 9, and 16) (Table 2). Adding to that, NUE was significantly increased for plants grown under low N treatment and, as predicted, high-yielding genotypes (18, 6, and 10) had on average the highest values (Table 2).

Table 1: Gain yield and thousand-grain weight (TGW) of 20 wheat genotypes fertilized by two nitrogen levels (High and Low). Means are followed by standard error (n=8). Grey shading indicates the genotypes that were selected for grain quality analyses. Statistical analysis was made by one-factor ANOVA. Within each treatment, letters indicate significance differences between genotypes at p<0.5 as determined by LSD test. Values in bold indicate significance (p<0.05). G: Genotype, T: Treatment.

Genotypes	**Grain yield (g/plant)**		**TGW (g)**	
	High N	**Low N**	**High N**	**Low N**
18	9.43^{a} ±0.58	8.22^{ab} ±0.28	58.63^{a} ±1.88	56.50^{a} ±2.49
4	9.09^{ab} ±0.57	6.57^{defg} ±0.49	50.75^{cd} ±1.69	47.14^{e} ±0.61
6	8.68^{abc} ±0.46	7.85^{abc} ±0.54	57.25^{ab} ±0.54	51.38^{cd} ±2.43
5	8.40^{abcd} ±0.45	6.94^{cdef} ±0.52	48.75^{de} ±0.53	47.69^{de} ±1.23
8	8.33^{abcd} ±0.48	7.72^{abcd} ±0.47	51.06^{cd} ±1.02	52.50^{bc} ±1.05
15	7.98^{abcdef} ±0.82	8.27^{a} ±0.50	51.00^{cd} ±1.67	51.81^{bc} ±0.91
14	7.96^{bcde} ±0.42	6.92^{cdef} ±0.34	46.56^{efg} ±1.34	48.94^{cde} ±1.52
10	7.41^{cdefg} ±0.31	7.36^{abcdef} ±0.39	52.88^{c} ±1.00	52.13^{bc} ±1.42
1	7.31^{cdefg} ±0.34	6.37^{efgh} ±0.55	46.38^{efg} ±1.41	42.88^{fg} ±1.83
13	7.17^{defg} ±0.36	7.55^{abcde} ±0.45	53.50^{bc} ±0.73	50.00^{cde} ±1.33
2	7.16^{defg} ±0.39	6.90^{cdef} ±0.40	43.56^{g} ±1.13	42.44^{g} ±1.45
12	7.06^{defgh} ±0.45	6.23^{fgh} ±0.45	54.25^{bc} ±1.88	50.38^{cde} ±1.22
19	6.86^{efgh} ±0.52	7.05^{bcdef} ±0.38	43.56^{g} ±1.02	50.63^{cde} ±0.69
7	6.83^{efgh} ±0.75	5.19^{h} ±0.35	43.94^{fg} ±1.52	52.06^{bc} ±0.65
11	6.78^{efgh} ±0.64	6.52^{efg} ±0.44	58.56^{a} ±1.16	55.69^{ab} ±1.57
20	6.70^{efgh} ±0.30	7.77^{abcd} ±0.33	47.56^{def} ±1.48	50.38^{cde} ±0.91
3	6.53^{efgh} ±0.37	6.28^{fgh} ±0.49	44.06^{fg} ±1.59	42.13^{g} ±1.26
16	6.43^{fgh} ±0.53	6.17^{fgh} ±0.43	50.69^{cd} ±0.87	46.69^{ef} ±0.97
9	6.27^{gh} ±0.53	5.40^{gh} ±0.34	48.81^{de} ±1.98	49.19^{cde} ±0.64
17	5.74^{h} ±0.26	7.37^{abcdef} ±0.38	54.19^{bc} ±2.22	52.88^{abc} ±1.97
Two-way ANOVA				
p-value (G effect)	**<0.001**		**<0.001**	
p-value (T effect)	**0.03**		0.34	
p-value (G x T)	0.75		0.40	

Table 2: Grain nitrogen concentration (N), carbon concentration (C), C/N, ratio, and nitrogen use efficiency (NUE) of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Means are followed by standard error (n=8). Statistical analysis was made by one-factor ANOVA. Within each treatment, letters indicate significant differences between genotypes at p<0.5 as determined by LSD test. *** indicates significance at p<0.001. Values in bold indicate significance (p<0.05). HYG: High-yielding genotypes (grey shadowed), LYG: Low-yielding genotypes**,** G: Genotype, T: Treatment.

Genotypes	**N (%)**		**C (%)**		**C/N**		**NUE**	
	High N	**Low N**	**High N**	**Low N**	**High N**	**Low N**	**High N**	**Low N**
Genotype 18 (HYG)	2.8^{d}±0.07	2.2^{b} ±0.08	41.0^{cd} ±0.11	39.7^{c} ±0.09	14.6^{a} ±0.34	18.1^{a} ±0.64	35.70^{a} ±0.92	45.55^{a} ±1.56
Genotype 6 (HYG)	2.9^{cd} ±0.04	2.1^{b} ±0.06	40.9^{d} ±0.08	40.5^{b} ±0.06	13.9^{ab} ±0.17	19.4^{a} ±0.51	34.03^{ab} ±0.45	47.98^{a} ±1.27
Genotype 10 (HYG)	3.1^{bc} ±0.09	2.1^{b} ±0.04	41.6^{b} ±0.06	41.2^{a} ±0.07	13.6^{b} ±0.38	19.5^{a} ±0.37	32.69^{bc}±0.88	47.23^{a} ±0.88
Genotype 3 (LYG)	3.2^{ab}±0.12	2.6^{a} ±0.13	41.3^{c} ±0.11	40.7^{b} ±0.14	13.1^{bc} ±0.48	15.9^{b} ±0.71	31.77^{bcd} ±1.20	39.19^{b} ±1.84
Genotype 9 (LYG)	3.4^{a} ±0.10	2.7^{a} ±0.10	41.2^{c} ±0.06	40.3^{b} ±0.15	12.2^{c}±0.36	14.8^{b} ±0.55	29.52^{d}±0.87	36.78^{b} ±1.44
Genotype 16 (LYG)	3.2^{ab}±0.06	2.7^{a} ±0.14	42.6^{a} ±0.09	40.3^{b} ±0.30	13.2^{b} ±0.24	15.5^{b} ±0.80	31.05^{cd} ±0.57	38.40^{b} ±2.00
N effect (%)	+29.24***		+2.43***		-21.89***		-23.66***	
Two-way ANOVA								
p-value (G effect)	**<0.001**		**<0.001**		**<0.001**		**<0.001**	
p-value (T effect)	**<0.001**		**<0.001**		**<0.001**		**<0.001**	
p-value (G x T)	0.194		**<0.001**		**<0.001**		**0.005**	

Wheat grain carbohydrates composition

High N fertilization affected positively soluble sugars concentration in grain (Figure 1). Aside from fructose, the other sugars (glucose, sucrose, and maltose) increased significantly (p<0.001) (Figure 1). Maltose recorded the largest increase (+117.5%) followed by glucose (+60.24%), while fructose and sucrose concentrations also rose but much less (+6.65% and +4.6%, respectively) (Figure 1). Genotypes showed also significant differences for all soluble sugars (p<0.001) except for maltose (p= 0.35) (Figure 1). It should be noted that monosaccharide concentrations (glucose and fructose) of high-yielding genotypes (18, 6, and 10) were higher when compared to those of low-yielding ones (3, 9, and 16) (Figure 1). Nevertheless, grain starch concentration declined significantly under the high N treatment for all genotypes except genotype 16 that showed similar data under the two treatments (Figure 2). The mean change in starch concentration recorded in the high N treatment was -16.03% relative to the low N one. As occurred for the monosaccharide concentrations, grain starch was higher in the high-yielding genotypes (Figure 2).

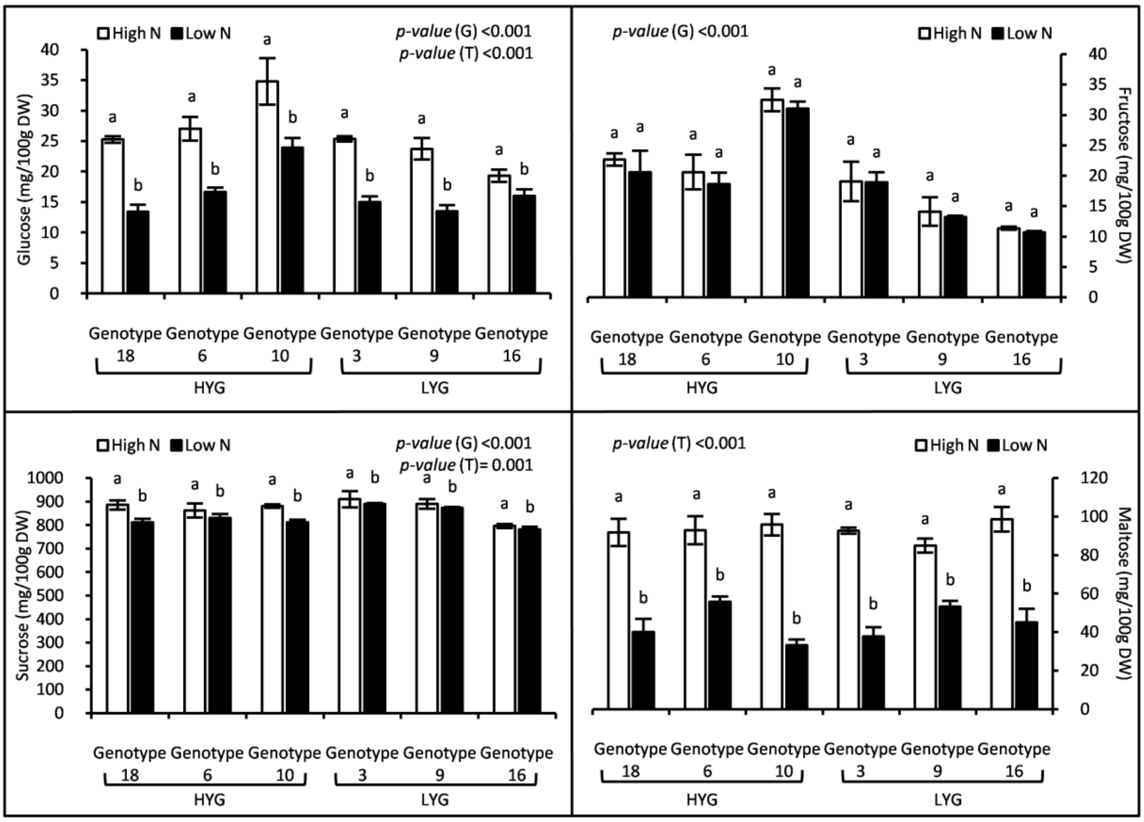

Figure 1: Grain soluble sugar concentrations of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Data correspond to the means ±standard error (n=4). Statistical analysis was made by one-factor ANOVA. For each genotype, letters indicates significant differences between the two treatments at p<0.5 as determined by LSD test. *p-values* corresponds to two-way ANOVA analysis. HYG: High-yielding genotypes, LYG: Low-yielding genotypes, G: Genotype, T: Treatment.

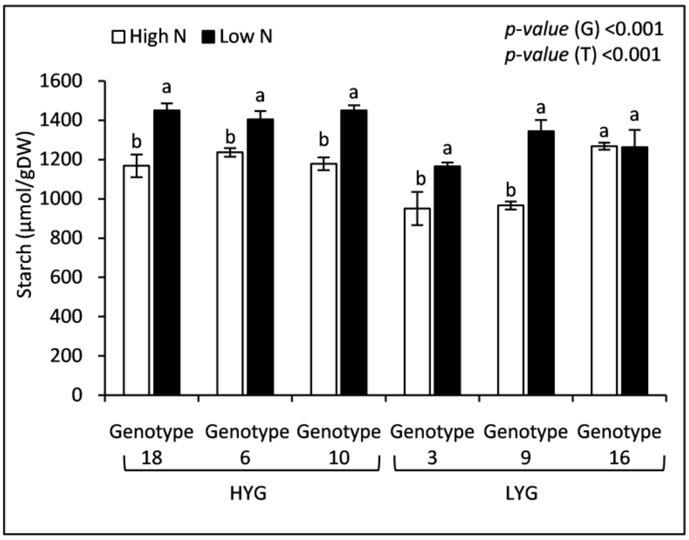

Figure 2: Grain starch concentration of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Data correspond to the means ±standard error (n=4). Statistical analysis was made by one-factor ANOVA. For each genotype, letters indicates significant differences between the two treatments at p<0.5 as determined by LSD test. *p-values* corresponds to two-way ANOVA analysis. HYG: High-yielding genotypes, LYG: Low-yielding genotypes, G: Genotype, T: Treatment.

Wheat grain gliadin and glutenin concentrations

As expected, N supply stimulated the synthesis of gliadins and glutenins in wheat grains but the increases were genotype-dependent (Figure 3). Results presented in Figure 3 indicated that high-yielding genotypes increased significantly total gliadin and glutenin concentrations, while it remained more or less constant for low-yielding genotypes. Thus, with respect to the change in mean concentration due to the high N treatment, total gliadins and glutenins were increased by 39.73% and 46.05% respectively for the high-yielding genotypes, while the increases in low-yielding genotypes were insignificant (+6.07% and +2.44%). Significant differences were detected among genotypes with the highest mean values recorded for the low-yielding wheat genotypes (Figure 3). Gliadin to glutenin (Gli/Glu) ratio ranged from 0.77 to 1.01 under low N and from 0.79 to 0.95 under high N (Figure 4). A high N fertilization did not affect Gli/Glu ratio, whereas genotype did (Figure 4). Genotypes 6 and 9 showed a significant decrease in their Gli/Glu ratios under the high N treatment, however, this trait remained fairly constant in the other genotypes. The analysis of the gliadins fractions showed that the α/β fraction was quantitatively predominant, increasing by 18.97% under high N (Table 3). ω and γ fractions also increased significantly in response to a higher N supply, by 35.48% and 11.19%, respectively (Table 3). The differences of high- and low-yielding genotypes with respect to N fertilization described for total gliadins (Figure 3) were also observed for their fractions (Table 3). Glutenin fractions were also significantly affected when N increased (Table 3). High (HMW) and low molecular weight (LMW) subunits of glutenins significantly increased in the high N treatment when compared with the low one, being the largest increase found (23.25%) in those of HMW (Table 3).

Wheat grain mineral composition

Grain micro- and macronutrients concentration was significantly increased in wheat grown under high N (Table 4). Only Zn showed a slight, non-significant increase of 1.81%. Across the 10 nutrients that increased, the mean change ranged between 3.69% and 84.77% ($p = 0.023$, $p < 0.001$). The lowest increases were recorded for Cu and K (3.69% and 4.85%, $p = 0.033$ and $p = 0.008$ respectively), whereas Ca and Al increased the most (31.44% and 84.77% respectively, $p < 0.001$). In general, whether in high or low N level, the mean concentration of most nutrients recorded for the low-yielding genotypes (3, 9, and 16) was larger than that recorded for the high-yielding ones (18, 6, and 10) (Table 4).

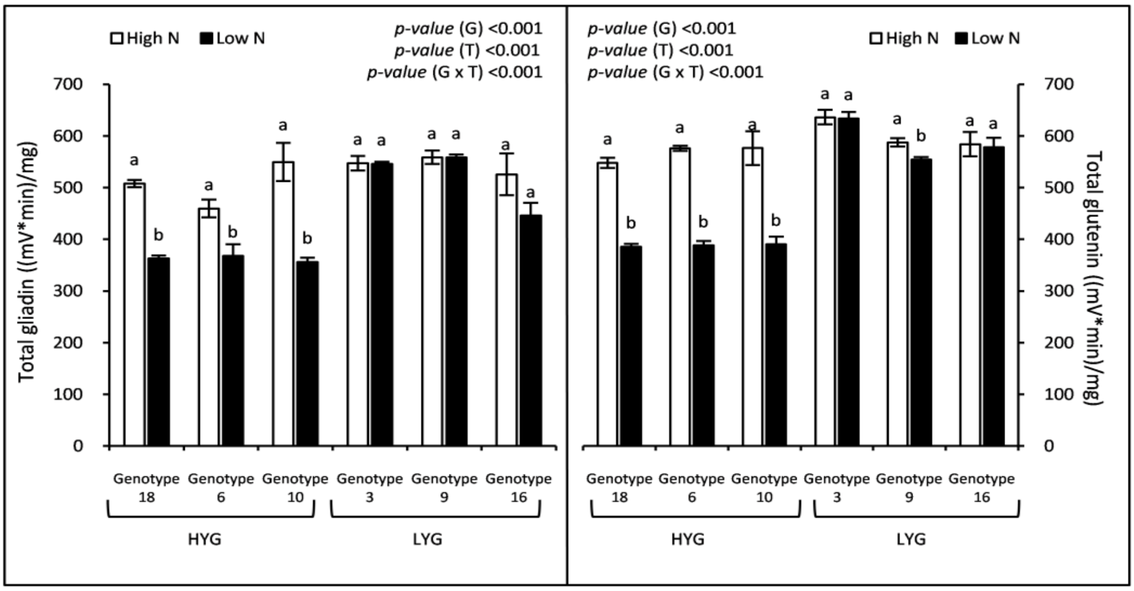

Figure 3: Grain gliadin and glutenin concentrations of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Data correspond to the means ±standard error (n=4). Statistical analysis was made by one-factor ANOVA. For each genotype, letters indicates significant differences between the two treatments at p<0.5 as determined by LSD test. *p-values* corresponds to two-way ANOVA analysis. HYG: High-yielding genotypes, LYG: Low-yielding genotypes, G: Genotype, T: Treatment.

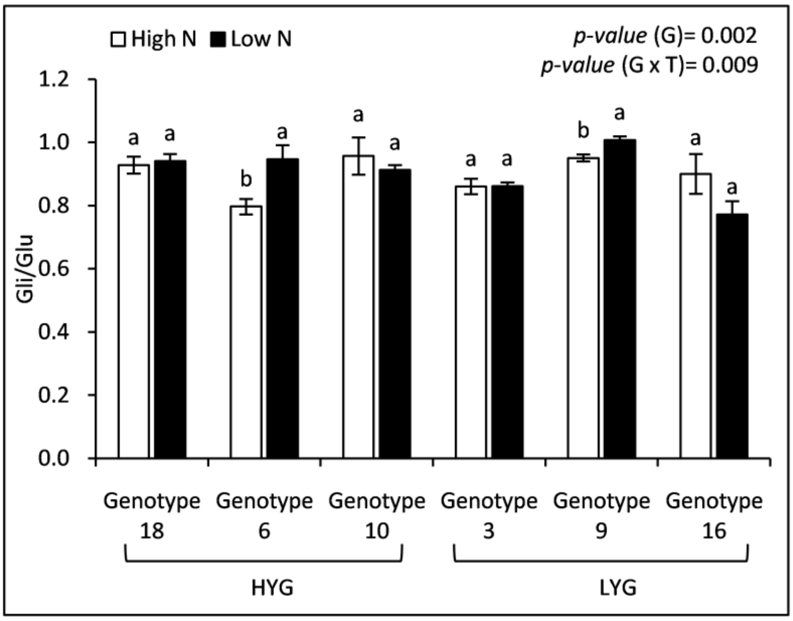

Figure 4: Grain gliadin to glutenin ratio (Gli/Glu) of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Data correspond to the means ±standard error (n=4). Statistical analysis was made by one-factor ANOVA. For each genotype, letters indicates significant differences between the two treatments at p<0.5 as determined by LSD test. *p-values* corresponds to two-way ANOVA analysis. HYG: High-yielding genotypes, LYG: Low-yielding genotypes, G: Genotype, T: Treatment.

Among the genotypes, specifically, genotype 3 had generally the highest nutrient values both under low N as well as under high N supply (Table 4).

Table 3: Total gliadin and glutenin fractions of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Means are followed by standard error (n=4). Statistical analysis was made by one-factor ANOVA. Within each treatment, letters indicate significant differences between genotypes at p<0.5 as determined by LSD test. *** indicates significance at p<0.001. Values in bold indicate significance (p<0.05). HYG: High-yielding genotypes (grey shadowed), LYG: Low-yielding genotypes. HMW: High molecular weight, LMW: Low molecular weight, G: Genotype, T: Treatment.

N level	**Genotypes**	**Total gliadin ((mv*min)/mg)**			**Total glutenin ((mv*min)/mg)**	
		ω	**α-β**	**γ**	**HMW**	**LMW**
High N	Genotype 18 (HYG)	25.32^{ab} ±2.14	302.07^{ab} ±8.59	180.31^{cd} ±5.71	89.42^{b} ±3.1	458.55^{c} ±8.59
	Genotype 6 (HYG)	19.00^{b} ±1.17	248.07^{c} ±12.63	192.42^{c} ±3.49	101.72^{ab} ±2.98	474.39^{bc} ±2.45
	Genotype 10 (HYG)	26.44^{a} ±2.28	294.67^{ab} ±24.52	222.74^{ab} ±10.31	110.82^{a} ±7.96	465.72^{bc} ±13.57
	Genotype 3 (LYG)	25.57^{a} ±1.65	321.29^{a} ±11.17	200.51^{bc} ±5.9	105.30^{ab} ±2.94	522.25^{a} ±12.28
	Genotype 9 (LYG)	19.54^{b} ±2.22	261.51^{bc} ±15.67	246.03^{a} ±9.43	89.37^{b} ±7.03	498.18^{ab} ±1.50
	Genotype 16 (LYG)	24.80^{ab} ±2.67	310.00^{a} ±14.58	165.66^{d} ±10.92	106.50^{a} ±6.22	475.62^{bc} ±20.17
Low N	Genotype 18 (HYG)	11.36^{c} ±0.23	198.23^{c} ±3.06	153.13^{cd} ±2.36	51.46^{c} ±2.18	334.15^{c} ±3.47
	Genotype 6 (HYG)	12.36^{c} ±1	204.05^{c} ±14.89	151.81^{d} ±7.13	51.53^{c} ±1.94	336.48^{c} ±6.84
	Genotype 10 (HYG)	13.11^{c} ±0.56	180.28^{c} ±4.64	162.68^{cd} ±3.64	55.14^{c} ±3.19	335.60^{c} ±10.96
	Genotype 3 (LYG)	25.77^{a} ±0.26	316.52^{a} ±1.73	215.58^{b} ±0.62	113.02^{a} ±0.65	520.89^{a} ±11.73
	Genotype 9 (LYG)	24.13^{a} ±0.73	303.38^{a} ±6.13	237.23^{a} ±2.21	101.13^{b} ±2.08	453.27^{b} ±3.41
	Genotype 16 (LYG)	17.12^{b} ±2.82	258.01^{b} ±14.87	165.69^{c} ±6.99	117.08^{a} ±7.23	462.10^{b} ±12.67
N effect (%)		+35.48***	+18.97***	+11.19***	+23.25***	+18.51***
Two-way ANOVA						
p-value (G effect)		**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
p-value (T effect)		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
p-value (G x T)		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Wheat grain polyphenols and anti-radical activity

Soluble and hydrolysable polyphenols (in mg gallic acid 100g^{-1} flour) constitute minor (range between 172 and 297) and major (range between 525 and 928) sub-fractions, respectively, in all wheat genotypes under both N treatments (Table 5). In response to an increase in N supply, a significant decrease was observed in soluble polyphenols (-19.36%), meanwhile hydrolysable polyphenols increased 47%. A significant difference was also detected among genotypes under both N treatments (Table 5). In fact, under high N, soluble and hydrolysable polyphenols concentrations were higher in low-yielding genotypes but, under low N, soluble polyphenols concentration was higher in high-yielding genotypes and no significant difference was detected for hydrolysable polyphenols. Bio-accessible polyphenols accounted for 44-66% and 59-74% of the total polyphenols under high and low N (Table 5), in line with a significant decrease (-4.62%) in the mean concentration of grain bio-accessible polyphenols in response to an increased N supply (Table 5). As a consequence of the already mentioned changes in soluble and hydrolysable polyphenols, total polyphenols concentration significantly increased (25.21%), the highest accumulation being observed in low-yielding genotypes (Table 5). Additionally, anti-radical activity was determined by the extent of the reduction of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Results correspond to the remaining unreacted DPPH amount when 0.494µmol of the free radical is initially available to react with 2.4-2.6mg of flour extracted with methanol/acetone/water (Table 5). The comparison between the two N treatments gave similar anti-radical activities (a mean of 53%, p= 0.91). However, significant differences were detected among genotypes (Table 5). Aside from genotype 3, results indicate that, under high N supply, anti-radical activity of the high-yielding genotypes was higher than that of the low-yielding genotypes (genotypes 9 and 16), while an opposite trend was observed under the low N treatment.

Table 4: Grain micro- and macronutrients of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Means are followed by standard error (n=4). Statistical analysis was made by one-factor ANOVA. Within each treatment, letters indicate significant differences between genotypes at $p<0.5$ as determined by LSD test. *, ** and *** are significant at $p<0.05$, $p<0.01$, and $p<0.001$, respectively. 'ns' means insignificant at $p<0.05$. Values in bold indicate significance ($p<0.05$). HYG: High-yielding genotypes (grey shadowed), LYG: Low-yielding genotypes**,** G: Genotype, T: Treatment.

N level	Genotypes	K (mg/100g)	P (mg/100g)	Mg (mg/100g)	S (mg/100g)	Na (mg/100g)	Ca (mg/100g)	Mn (mg/100g)	Fe (mg/100g)	Zn (mg/100g)	Cu (mg/100g)	Al (mg/100g)
High N	Genotype 18 (HYG)	513.82^{bc} ±30.39	367.42^{bc} ±21.03	113.76^{b} ±6.85	99.25^{b} ±5.45	40.04^{b} ±1.71	20.45^{d} ±0.94	4.91^{c} ±0.05	2.24^{c} ±0.11	1.56^{e} ±0.02	0.44^{d} ±0.03	0.40^{b} ±0.07
	Genotype 6 (HYG)	504.41^{c} ±19.93	353.91^{c} ±13.44	112.85^{b} ±4.40	110.96^{ab} ±6.96	39.23^{b} ±2.24	25.07^{c} ±0.86	5.26^{bc} ±0.22	2.65^{b} ±0.07	1.79^{d} ±0.03	0.53^{b} ±0.02	1.18^{a} ±0.09
	Genotype 10 (HYG)	554.13^{ab} ±5.96	404.93^{b} ±4.18	128.86^{a} ±1.24	109.13^{ab} ±0.89	51.62^{a} ±2.74	24.76^{c} ±1.18	5.44^{ab} ±0.04	2.43^{bc} ±0.05	1.61^{e} ±0.06	0.46^{cd} ±0.00	0.55^{b} ±0.13
	Genotype 3 (LYG)	595.62^{a} ±9.73	442.43^{a} ±8.02	136.54^{a} ±2.06	114.96^{a} ±1.90	51.38^{a} ±1.90	30.25^{b} ±0.59	5.76^{a} ±0.08	3.35^{a} ±0.20	2.55^{a} ±0.05	0.61^{a} ±0.01	0.58^{b} ±0.04
	Genotype 9 (LYG)	553.28^{ab} ±9.67	402.62^{b} ±6.81	139.21^{a} ±2.51	116.79^{a} ±1.64	53.55^{a} ±2.90	34.82^{a} ±0.95	5.42^{b} ±0.07	3.26^{a} ±0.09	2.24^{b} ±0.02	0.54^{b} ±0.01	0.99^{a} ±0.06
	Genotype 16 (LYG)	552.34^{ab} ±5.97	405.25^{b} ±4.21	129.55^{a} ±1.12	100.62^{b} ±1.43	43.32^{b} ±0.73	30.94^{b} ±0.73	5.53^{ab} ±0.07	3.15^{a} ±0.18	2.11^{c} ±0.04	0.50^{bc} ±0.01	0.32^{b} ±0.08
Low N	Genotype 18 (HYG)	549.15^{a} ±4.64	354.80^{bc} ±2.17	102.10^{b} ±0.51	85.46^{b} ±0.76	36.63^{b} ±2.35	19.12^{cd} ±0.47	4.81^{bc} ±0.04	2.78^{ab} ±0.24	1.91^{bc} ±0.03	0.46^{b} ±0.00	0.40^{b} ±0.05
	Genotype 6 (HYG)	563.80^{a} ±24.31	345.24^{c} ±14.11	105.69^{b} ±4.45	88.59^{b} ±3.80	38.44^{b} ±0.82	19.06^{cd} ±1.25	4.81^{bc} ±0.20	2.32^{b} ±0.08	1.85^{c} ±0.10	0.53^{a} ±0.02	0.73^{a} ±0.09
	Genotype 10 (HYG)	503.84^{b} ±3.03	356.51^{bc} ±1.98	103.05^{b} ±0.91	88.43^{b} ±0.56	40.83^{ab} ±0.28	16.16^{d} ±0.18	4.56^{c} ±0.05	2.64^{ab} ±0.23	1.55^{d} ±0.02	0.45^{bc} ±0.01	0.39^{bc} ±0.00
	Genotype 3 (LYG)	567.74^{a} ±6.06	403.63^{a} ±3.66	123.32^{a} ±1.49	95.35^{b} ±0.82	43.07^{a} ±2.58	26.74^{a} ±2.74	5.09^{b} ±0.04	2.76^{ab} ±0.18	2.06^{b} ±0.07	0.56^{a} ±0.01	0.16^{d} ±0.02
	Genotype 9 (LYG)	481.11^{bc} ±6.70	373.31^{b} ±5.47	121.81^{a} ±1.64	110.78^{a} ±6.38	42.97^{a} ±0.74	23.90^{ab} ±0.66	5.78^{a} ±0.07	2.96^{a} ±0.01	2.63^{a} ±0.04	0.54^{a} ±0.00	0.23^{d} ±0.02
	Genotype 16 (LYG)	456.41^{c} ±20.07	337.84^{c} ±13.54	107.96^{b} ±4.89	81.16^{b} ±3.31	41.08^{ab} ±0.78	21.54^{bc} ±0.98	4.48^{c} ±0.19	2.36^{b} ±0.14	1.63^{d} ±0.06	0.42^{c} ±0.02	0.26^{cd} ±0.05
N effect (%)		+4.85**	+9.45***	+14.59***	+18.55***	+14.86***	+31.44***	+9.39***	+7.98*	(ns)	+3.69*	+84.77***
Two-way ANOVA												
p-value (G effect)		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
p-value (T effect)		**0.008**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.023**	0.25	**0.033**	**<0.001**
p-value (G x T)		**<0.001**	0.107	0.08	0.51	**0.046**	**0.001**	**<0.001**	**0.001**	**<0.001**	**0.008**	**<0.001**

Table 5: Polyphenol fractions and anti-radical activity of 6 wheat genotypes fertilized by two nitrogen levels (High and Low). Means are followed by standard error (n=4). Statistical analysis was made by one-factor ANOVA. Within each treatment, letters indicate significant differences between genotypes at $p<0.5$ as determined by LSD test.* and *** are significant at $p<0.05$ and $p<0.001$, respectively. 'ns' means insignificant difference at $p<0.05$. Values in bold indicate significance ($p<0.05$). b corresponding to 2.4mg flour or 2.4-2.6mg bread at flour basis that consumed DPPH when 0.494µmol of the free radical are initially available to react. The plateau was decided at 120 min of reaction. HYG: High-yielding genotypes (grey shadowed), LYG: Low-yielding genotypes**,** G: Genotype, T: Treatment.

		Soluble polyphenols		Hydrolysable polyphenols		Total polyphenols	Bio-accessible polyphenols		Anti-radical activity^{b}	
N level	Genotypes	mg gallic acid/100g flour	% of total polyphenols	mg gallic acid/100g flour	% of total polyphenols	mg gallic acid/100g flour	mg gallic acid/100g flour	% of total polyphenols	Remaining µmol DPPH at steady state	%
High N	Genotype 18 (HYG)	224^{b} ±6.77	19	928^{a} ±33.66	81	1152^{a} ±31.00	540^{abc} ±13.13	47	0.220^{c} ±0.004	55
	Genotype 6 (HYG)	172^{c} ±6.12	19	719^{b} ±27.31	81	892^{b} ±26.86	586^{a} ±21.91	66	0.212^{c} ±0.006	57
	Genotype 10 (HYG)	200^{bc} ±8.25	21	745^{b} ±30.83	79	949^{b} ±41.17	552^{ab} ±20.74	58	0.215^{c} ±0.003	56
	Genotype 3 (LYG)	294^{a} ±9.68	24	924^{a} ±35.81	76	1218^{a} ±36.94	575^{ab} ±14.59	47	0.206^{c} ±0.009	58
	Genotype 9 (LYG)	265^{a} ±17.90	23	905^{a} ±85.29	78	1159^{a} ±80.31	531^{bc} ±23.40	46	0.257^{b} ±0.006	48
	Genotype 16 (LYG)	205^{bc} ±17.08	18	924^{a} ±52.25	82	1129^{a} ±45.49	494^{c} ±13.87	44	0.278^{a} ±0.009	44
Low N	Genotype 18 (HYG)	297^{ab} ±19.29	32	619^{a} ±40.72	68	916^{a} ±20.35	543^{cd} ±9.33	59	0.248^{a} ±0.002	50
	Genotype 6 (HYG)	297^{a} ±14.89	34	573^{a} ±25.42	66	873^{ab} ±17.10	517^{d} ±21.13	59	0.243^{ab} ±0.005	51
	Genotype 10 (HYG)	293^{a} ±12.58	34	559^{a} ±36.03	66	851^{ab} ±51.15	541^{cd} ±14.89	64	0.253^{a} ±0.011	49
	Genotype 3 (LYG)	284^{ab} ±12.52	32	602^{a} ±50.13	68	886^{ab} ±45.07	601^{ab} ±14.89	68	0.206^{c} ±0.009	58
	Genotype 9 (LYG)	255^{b} ±5.55	29	622^{a} ±51.08	71	876^{ab} ±52.72	647^{a} ±18.43	74	0.219^{c} ±0.006	56
	Genotype 16 (LYG)	263^{ab} ±5.48	33	525^{a} ±23.87	67	788^{b} ±23.38	587^{bc} ±21.87	74	0.223^{bc} ±0.010	55
N effect (%)		-19.36***		+47.03***		+25.21***	-4.62*		ns	
Two-way ANOVA										
p-value (G effect)		**<0.001**		**0.01**		**<0.001**	**0.02**		**<0.001**	
p-value (T effect)		**<0.001**		**<0.001**		**<0.001**	**0.01**		0.91	
p-value (G x T)		**<0.001**		0.06		**0.002**	**<0.001**		**<0.001**	

DISCUSSION

Grain and thousand-grain weight traits were used to differentiate high- and low-yielding durum wheat genotypes

In the current study, 20 genotypes from CIMMYT were chosen from a set of 120 genotypes as the ones having highest grain protein and/or starch content. Grain yield per plant and TGW traits were used to identify genotypes of contrasting grain production among 20 durum wheat genotypes tested. Genotypes 18, 6, and 10 were selected as high-yielding genotypes since they recorded the highest values of grain yield and TGW under the two N levels tested, while genotypes 3, 9, and 16 showed the lowest values and were tagged as low-yielding genotypes. These genotypes were used to investigate the effects of N fertilization in grain yield and quality.

A supplementary nitrogen addition post-anthesis slightly improves durum wheat grain yield

Nitrogen fertilization generally (Klikocha et al., 2016; Litke et al., 2018; Bielski et al., 2020; Xu et al., 2020) but not always (Nakano et al., 2008) stimulates grain yield in triticale and wheat. Our results with durum wheat are in line with the former rather than the latter since grain yield significantly increased (7%) in the high N treatment with respect to the low N supply when applied post-anthesis with a similar behavior in all genotypes tested. The observed increase in grain yield can be attributed to many components such as ears number, number of grains produced per ear, and TGW that may respond positively to N fertilization. In the current study, we suggest that the increase of wheat grain yield observed under the high N treatment might be due to the increase in grain number produced per plant because (i) TGW increased, but not significantly (1.3%), under the high N treatment with respect to the low one, and (ii) no significant differences between treatments were found in a fast screening made for number of tillers per plant (not shown). These results agree with those reported by Li et al. (2001) and Abedi et al. (2011) in bread wheat.

A high nitrogen supply increases durum wheat grain nitrogen but decreases nitrogen use efficiency

We fertilized plants from anthesis to maturity. The recommendation of applying N several times to the durum wheat crop, in order to achieve greater efficiency, is commonly accepted. However, the number of applications may vary. Thus, in some cases, the proposed fertilization includes application during sowing and vegetative growth up to flowering phase (Garrido-Lestache et al., 2005; Visioli et al., 2018). However, it has been shown that a late supply of N increases the protein content of the grain and improves the quality of durum wheat (Blandino et al., 2015). In the experimental design of our experiment, special emphasis was made on studying the ability of the different genotypes to use this late application of N, considering that the N present in the initial substrate covered the needs of the crop up to anthesis in an analogous way to the applications that farmers usually carry out during sowing and pre-anthesis. Our approach of using pots and greenhouse, however, simplify the logistics (field surface to be cultivated, number of analyses, etc.) and can be used as a pre-selection trial for data based genotype choice to be planted in a field test.

Using the above-mentioned approach, N concentration was, as expected, increased in durum wheat grains regardless of yields when the N dose was increased. This could be due to a large N availability post-anthesis coming from root N uptake and N remobilization to the grain during grain filling (Belete et al., 2018). Belete et al. (2018) and López-Bellido et al. (2004) found genotypic variability in grain N content at different N rates between genotypes of high and low yield. In line to that, the highest amount of grain N was recorded in the low-yielding genotypes irrespective of N supply. The low N concentration in grains of the high-yielding genotypes when grown at low N could be explained by dilution of N due to higher yields comparing them to those of the low-yielding genotypes. On the other hand, C grain concentration also significantly increased under the high N treatment but the extent of the increase was small, much lower than that of the grain N increase (+2.43% vs. +29.24%), which explains the decrease in C/N ratio. In rice panicle at filling and maturity stages Ye et al. (2014) and mature wheat grains Yan et al. (2015) found similar results. In this line, it has been reported that tissue C concentration is relatively unaffected by N fertilization due to the stable plant C structural basis that accounts almost for 50% of plant dry mass (Ye et al., 2014; Yan et al., 2015). Nitrogen use efficiency (NUE) was highly influenced by the applied N dose and genotype. An increased N fertilization rate led to a decrease in NUE. This is coherent because a large part of the additional N is driven to grain protein synthesis whereas yield increases were small (i.e., the concentration of N in the grain increases more than the yield). This result confirms previous reports made working with bread (Haile et al., 2012; Belete et al., 2018; Xu et al., 2020) and durum (Ierna et al., 2016) wheat. Under both N treatments, high-yielding genotypes showed higher efficiency in the use of the applied N. This finding led us to hypothesize that NUE can be a trait under genetic control. Having highest or lowest NUE between genotypes was independent of N dose, which is an advantage when using this parameter in genetic improvement programs, although it is true that under conditions of low N is where the differences were most marked. Thus, broader germplasm screening for varieties with high NUE under limited N resources using pots and facilities such as greenhouses could be an easier, useful tool to select genotypes aimed to improve durum wheat crop yield and minimize environment contamination due to excessive N fertilization in field-based trials.

Durum wheat grain carbohydrates tend to be stored as mono and disaccharides (glucose, sucrose, and maltose), not as starch, when the applied N is high

Durum wheat grain non-structural carbohydrate concentrations were highly dependent on N dose and genotype. Soluble sugars (glucose, maltose, and sucrose) were more abundant in grain at high than at low N application. On the contrary, starch concentration decreased with increasing N availability. It can be concluded that, under the high N treatment, grain carbohydrates tend to be stored as mono and disaccharides (glucose, sucrose, and maltose) not as starch, suggesting a slowed down starch synthesis. Results from different crops agree with our findings. Zadeh et al. (2013) reported that starch in rice could be increased due to a moderate reduction in N. Starch in developing and mature maize kernel was negatively correlated with N availability, whereas the response of glucose and fructose in developing maize ears toward N supply was opposite to starch (Ning et al., 2018). Similarly, Galani et al. (1991) reported that N fertilizer increased soluble carbohydrates, mainly sucrose, in sweet sorghum. Matching results were also presented by Almodares et al. (2009) and Asthir et al. (2017) in sweet sorghum and wheat, respectively. Taken together, all these findings suggest that high N dose stimulates soluble sugars biosynthesis and reduces their conversion into starch. It has been reported that, N fertilization increases the triose-phosphate/phosphate translocation activity, as well as sucrose-phosphate synthase1 (*ZmSps1*), leading to more C flux to sucrose synthesis than to starch accumulation in maize leaves (Ning et al., 2018). Polysaccharides, hexoses, and disaccharides concentrations in durum wheat grain were higher in high-yielding genotypes under both N doses. It can be hypothesized a higher photosynthetic capacity of their leaves and C fixation into carbohydrates in those genotypes. Regarding the technological quality of durum wheat, there is no evidence that an increase in the content of soluble sugars affects its aptitude for making pasta. On the other hand, it could have a negative effect on how "healthy" the resulting pasta is, since increasing the soluble sugars increases the glycaemic index. It must be taken into account that pasta is considered a food with a low glycaemic index (Chiavaroli et al., 2018).

N supply stimulated the synthesis of storage proteins, gliadins, and glutenins, in durum wheat grains but only in the high-yielding genotypes

As above discussed, a higher N supply resulted in an accumulation of N in the durum wheat grain, putatively in form of grain protein. Gliadins and glutenins are storage proteins with the latter being more important than gliadins for obtaining good dough properties (Wieser and Kieffer, 2001; Zhang et al., 2007; Li et al., 2008). In line with previous reports (González-Torralba et al., 2011; Stepien and Wojtkowiak, 2013), our results showed that not only gluten concentration is increased but also its composition is impacted by N fertilization. Although the 3 fractions of gliadins increased, the highest N-mediated increase was observed in ω-gliadins. In the case of glutenin fractions, both HMW and LMW increased with a higher N dose being the increase a little bit higher for the HMW fraction. In bread wheat, this greater increase in the synthesis of ω-gliadins and HMW glutenins has been related to the proportion of amino acids that contain S (Cys and Met) in both types of proteins (Altenbach et al., 2011). An interesting output of our work is that protein accumulation during grain filling seems to have also a genetic component. Low-yielding genotypes showed the highest concentrations of gliadins and glutenins (and consequently of their fractions) under both treatments, however, high-yielding genotypes appears to be more sensitive to N supply (raising N dose does not affect gluten content in grains of low-yielding plants). Under low N dose, the accumulation of carbohydrates in high-yielding genotypes was much larger than that observed in the low-yielding ones, which can explain the low concentration of gliadins and glutenins in their grains. The larger gluten content found in high-yielding genotypes grown under high N fertilization in the current work should be related to an increased N uptake and/or N remobilization to grain in these genotypes. Also, since it is a relatively late N contribution (post-anthesis), the ability of the plant to use that N to produce more grain is limited, using it to synthesize reserve proteins. Gliadin to glutenin ratio was not affected by the N treatment when considered all genotypes as a whole (although the ratio decreased at high N in genotypes 6 and 9), which indicates that this trait is controlled genetically. These results agree with those reported by Johansson et al. (2001) who found that this ratio was only influenced by the cultivar. In the literature, there are examples of increases (Jia et al., 1996), decreases (Kharel et al., 2011) and no changes (Pechanek et al., 1997; Wieser and Seilmeier, 1998) of the gliadin to glutenin ratio in response to high N.

A high nitrogen supply increases durum wheat grain mineral concentrations

From the literature, a clear conclusion on how N supply affects macro- and micronutrients concentrations in wheat grain cannot be obtained. On one hand, there are reports showing that late N application at heading increased macro- and micronutrients (Zn, Fe, and Mg) grain concentrations (Feil and Bänziger, 1993; Ma et al., 2018). On the other hand, Dolijanovic et al. (2019) concluded that a reduced N application (60 vs. 120 kg ha^{-1}) had a positive effect on the concentration of nutritionally important minerals (Ca, Cu, Fe, K, Mg, Mn, P, and Zn). In line with this, Smith et al. (2018) reported that grain concentration of P, K, Ca, Mg, Mn, and Zn declined as crop yields increased in response to N fertilization. These latter results appear to be contradictory with the fact that, according to our results, increasing the N dose post-anthesis enhances slightly grain yield and does raise grain mineral concentrations for both high- and low-yielding genotypes. Causes for the enhanced grain mineral concentrations under higher N supply have been ascribed to a higher root growth that promotes nutrient uptake (Feil and Bänziger, 1993; Svecnjak et al., 2013). In bread wheat, grain mineral concentrations tend to decrease as yields increase, therefore, breeding for yield improvement may reduce wheat nutritional quality (Guttieri et al., 2015). When comparing among genotypes, it should be highlighted that there was a large variability with respect to grain nutrients, which indicates that grain mineral composition is in part controlled genetically. Low-yielding genotypes had the highest mineral concentrations (K, P, Mg, S, Na, Ca, Mn, Fe, Zn, and Cu) under both N treatments, suggesting a “concentration” effect. Also, the lower values of grain nutrients that had the high-yielding genotypes can be related to a “dilution” effect due to their higher yields regardless of the N applied. Finally, other reports have shown no changes with N supply in most nutrients analyzed (P, K, Mg, and Na), only Ca increased (Jaskulska et al., 2018). Our results with N fertilization from anthesis to maturity are in line with those of Zhao et al. (2009) investigating water availability at post-anthesis. These authors found that the concentration of Zn and Fe has a significant positive correlation with grain P and protein.

A high nitrogen supply increases total grain polyphenols, due to the hydrolysable fraction, but with no impact on the anti-radical activity

Few studies have analyzed the effect of N fertilization on polyphenols in grains, being their results divergent. Engert et al. (2011) and Ma et al. (2015) found that total phenolics increased in wheat grains due to N fertilization, while N availability affected negatively total phenolics in tef grains (Tietel et al., 2020) and sesame seeds (Elhanafi et al., 2019). Stumpf et al. (2019) found, on the contrary, that total phenolic concentration was not affected by N treatment in wheat grains. These differences could be explained by the different amounts of N fertilizer and/or the genotype used for the experiments. Our results agree with the two former of these studies. In response to N fertilization increase, grain polyphenols concentration and composition were significantly modified. A clear interaction in soluble and bio-accessible polyphenols was observed; i.e., decrease in soluble polyphenolsin high-yielding genotypes with no change in the low-yielding ones, and decrease in bio-accessible polyphenols in low-yielding genotypes compared to a slight increase in the high-yield ones. When analyzing the variation of polyphenol fractions in response to a rise in the N fertilization dose, hydrolysable polyphenols explain to a large extent the augmentation in grain total polyphenols. Our results on soluble polyphenols contrast with those of Stumpf et al. (2019) and Langenkämper et al. (2006), who reported that soluble phenolics were higher in grains of unfertilized wheat compared to fertilized plants. The increase in total polyphenols could be ascribed to the sufficient availability of phenylalanine under high N application. This amino acid is a key metabolite in the synthesis of phenols, together with its use in the route for protein biosynthesis (Margana, 1977). In soybean, N fertilization has been related with phyto-hormones production and phenolics as growth-promoting compounds (Taie et al., 2008). Ma et al. (2015) suggested that the antioxidant activity in wheat grain could be increased with an adequate N application. Our data indicate that fertilization had no effect on the anti-radical activity when considering all genotypes as a whole, in agreement with previous reports (Kosik et al., 2014; Stumpf et al., 2019). However, there was a genotype x treatment interaction. At low N, the low-yielding genotypes had more anti-radical activity, but, with high N, it was the other way around.

CONCLUSIONS

Nitrogen fertilization applied from anthesis to maturity had small effects on durum wheat grain yield but had a major impact on grain quality. Traits such as grain yield and TGW were used to differentiate high- and low-yielding genotypes. A higher N supply increased grain N concentration, but decreased NUE. A higher N availability during grain filling resulted in an overall enhancement in nutritional grain quality. Storage proteins, gliadin and glutenin, soluble sugars, minerals, and phenolic compounds were increased in mature grains. Results on grain yield and quality were genotype-dependent, particularly were characteristically different in high- and low-yielding genotypes. A crop like durum wheat has quality requirements that are based, in part, on a high protein content in the grain. To achieve this protein content, an extra supply of N will be needed, which will inevitably reduce the NUE, but, it cannot be considered as intrinsically negative. Therefore, to meet the future demands of global population, screening for genotypes with an adequate balance between high stable yields, and satisfactory nutritional values and NUE could be a suitable alternative.

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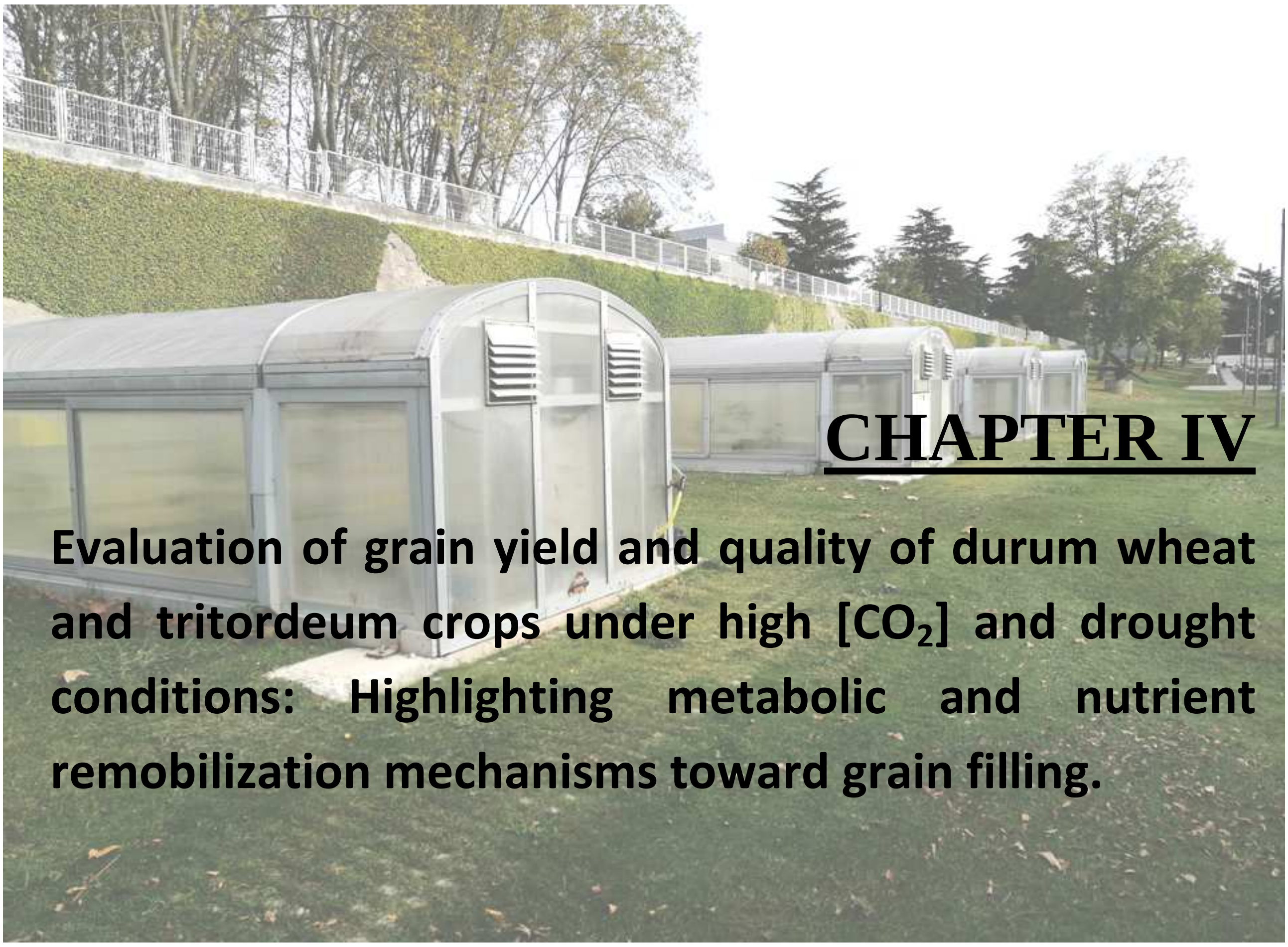
SUPPLEMENTARY MATERIAL

Table S1: Pedigree and selection history of the 20 durum wheat genotypes obtained from the International Maize and Wheat Improvement Center (CIMMYT, Mexico).

Genotypes	Pedigree
1	ALTAR 84/STINT//SILVER_45/3/GUANAY/4/GREEN_14//YAV_10/AUK/5/GUAYACAN INIA/… (CDSS11B00002S-0133Y-055M-2Y-0M)
2	BYBLOS/7/WID22256/5/ALTAR 84/STINT//SILVER_45/3/GUANAY/4/GREEN_14//YAV_10/… (CDSS12Y00252S-09Y-018M-2Y-0M)
3	BYBLOS/7/WID22256/5/ALTAR 84/STINT//SILVER_45/3/GUANAY/4/GREEN_14//YAV_10/… (CDSS12Y00252S-09Y-018M-9Y-0M)
4	CBC 509 CHILE/6/ECO/CMH76A.722//BIT/3/ALTAR 84/4/AJAIA_2/5/KJOVE_1/7/AJAIA_12/… (CDSS12Y00717T-035Y-017M-1Y-0M)
5	SILVER_14/MOEWE//BISU_1/PATKA_3/3/PORRON_4/YUAN_1/9/USDA595/3/D67.3/RABI//CRA/4/… (CDSS11B00068S-066Y-064M-10Y-0M)
6	CBC 509 CHILE/6/ECO/CMH76A.722//BIT/3/ALTAR 84/4/AJAIA_2/5/KJOVE_1/7/AJAIA_12/… (CDSS11B00319T-044Y-028M-39Y-0M)
7	ALTAR 84/STINT//SILVER_45/3/GUANAY/4/GREEN_14//YAV_10/AUK/5/SOMAT_4/INTER_8/6/… (CDSS11B00351T-072Y-051M-20Y-0M)
8	ADAMAR_15//ALBIA_1/ALTAR 84/3/SNITAN/4/SOMAT_4/INTER_8/5/SOOTY_9/RASCON_37/6/… (CDSS11B00356T-045Y-025M-41Y-0M)
9	SOOTY_9/RASCON_37//GUAYACAN INIA/11/BOOMER_33/ZAR/3/BRAK_2/AJAIA_2//… (CDSS12Y00165S-072Y-032M-23Y-0M)
10	SILVER_14/MOEWE//BISU_1/PATKA_3/3/PORRON_4/YUAN_1/9/USDA595/3/D67.3/RABI//CRA/4/… (CDSS11B00138S-078Y-055M-15Y-0M)
11	GERUFTEL-1//GUAYACAN INIA/2*SNITAN (CDSS10Y00291S-099Y-044M-5Y-2M-06Y-0B)
12	CBC 509 CHILE/SOMAT_3.1//BOOMER_18/LOTUS_4/6/SOMAT_3/PHAX_1//TILO_1/… (CDSS10Y00493T-099Y-035M-9Y-4M-06Y-0B)
13	CMH83.2578/4/D88059//WARD/YAV79/3/ACO89/5/2*SOOTY_9/RASCON_37/6/1A.1D 5+1-06/… (CDSS10B00122T-099Y-011M-4Y-0M-06Y-0B)
14	MOHAWK/6/LOTUS_5/F3LOCAL(SEL.ETHIO.135.85)/5/CHEN… (CDSS11Y00431S-099Y-032M-13Y-0M-06Y-0B)
15	MOHAWK/6/LOTUS_5/F3LOCAL(SEL.ETHIO.135.85)/5/CHEN… (CDSS11Y00438S-099Y-029M-16Y-0M-06Y-0B)
16	LABUD/NIGRIS_3//GAN/3/AJAIA_13/YAZI/10/PLATA_10/6/MQUE/4/USDA573//QFN/AA_7/3/… (CDSS07Y00042S-099Y-099M-15Y-1M-04Y-0B)
17	MÂALI/5/LOTUS_5/SORD_1/3/CANELO_8//SORA/2*PLATA_12/4/YAZI_1/AKAKI_4//SOMAT_3/3/… (CDSS06B00054S-099Y-099M-1Y-1B-04Y-0B)
18	MÂALI/6/MUSK_1//ACO89/FNFOOT_2/4/MUSK_4/3/PLATA_3//CREX/ALLA/5/OLUS*2/ILBOR//… (CDSS07Y00784D-2B-07Y-07M-7Y-4B-04Y-0B)
19	YAZI_1/AKAKI_4//SOMAT_3/3/AUK/GUIL/GREEN/5/2*NETTA_4/DUKEM_12//RASCON_19/3/SORA/… (CDSS04B00346T-0TOPY-3Y-0M-4Y-3Y-0B)
20	C F4 20 S/4/YAZI_1/AKAKI_4//SOMAT_3/3/AUK/GUIL//GREEN/5/CANELO_9.1//SHAKE_3/… (CDSS09B00035S-099Y-069M-8Y-4M-06Y-0B)



CHAPTER IV

Evaluation of grain yield and quality of durum wheat and tritordeum crops under high [CO_2] and drought conditions: Highlighting metabolic and nutrient remobilization mechanisms toward grain filling.

INTRODUCTION

Cereal grains including wheat, maize, and rice are considered primary crops as they are staple foods to most of the population across the globe. An increase by 70% to 100% in cereal food supply is required to feed the predicted world population of 9.8 billion people by 2050 (Godfray et al., 2010). Among the major cereal crops that are widely cultivated, wheat is considered as a stable diet for more than one third of the world population since it provides more calories and protein to human diet than any other cereal crop (Abd-El-Haleem et al., 2009).

According to the Intergovernmental Panel on Climate Change (IPCC, 2007), reduction in precipitation is predicted in some regions of the world (such as southern Europe) and will have effects on the soil moisture status and groundwater level (Kang et al., 2009). All these changes have a direct impact on wheat grain yield affecting worldwide food security. On the other hand, atmospheric CO_2 concentration ($[CO_2]$) has increased by more than 40% since the beginning of the industrial revolution and is expected to double by the end of this century (IPCC, 2013). Human activities release carbon dioxide to the atmosphere through fossil fuel burning (among others) which is considered as the major source of CO_2 and contributes by more than 75% to atmospheric CO_2 (Sivaramanan, 2015). It has been reported by NASA Global Climate Change (2015) that current CO_2 levels exceed 400 ppm and the rate of increase is expected to be more than 2.75 ppm per year.

The primary effect of a short-term exposure to elevated $[CO_2]$ includes an initial stimulation of photosynthesis, which may eventually contribute to a higher biomass (Stitt and Krapp, 1999; Long et al., 2006). High $[CO_2]$ also induces a stomatal closure leading to a better leaf water status. However, frequently, growth over the long-term under elevated $[CO_2]$ leads to a down-regulation of photosynthetic capacity, which has been related to a decline in Rubisco protein content and activity, together with a higher carbohydrate accumulation and a decline in N concentration and protein content in wheat (Aranjuelo et al., 2011, 2013; Vicente et al., 2015). On the other hand, it has been well documented that drought stress affect negatively yields of many species via decreasing the photosynthetic rate (Chen et al., 1993; Brevedan and Egli, 2003).

Grain filling, and therefore grain yield, is a target process conditioning final grain yield. Further, this process is mainly sustained by the assimilation and management of carbon/ nitrogen (and other compounds) (Zhou et al., 2016). Gebbing and Schnyder (1999) reported that the metabolic demand during grain filling in wheat is met by both CO_2 and nitrogen (N) assimilation in leaves (in addition to refixation of respired CO_2 by glumes) and remobilization of pre-anthesis stem reserves. Accordingly, leaves are classically considered as the main nitrogen contributor to kernels due to their large protein content. The fact that Rubisco might represent up to 50% of the total soluble protein (TSP) and 25-30% of total leaf nitrogen (Buchanan-Wollaston et al., 2003; Parry et al., 2003; Feller et al., 2008; Aranjuelo et al., 2013) implies that it can be considered as a major N storage form. During senescence, leaf proteins, mainly Rubisco, are degraded into free amino acids that temporarily are stored in the leaves at first, with asparagine, aspartate, glutamine, and glutamate considered as the major amino acid forms. Then, these amino acids are translocated to grains via phloem (Zhang et al., 2017). Similarly, according to Peeters and Van Laere (1994), aspartic acid, glutamic acid, and alanine make up about 60% of all free amino acids in naturally senescing flag leaves of wheat. Adding to hydrolysis of leaf proteins, amino acids are also synthesized from photosynthates and inorganic N (Tegeder and Rentsch, 2010), and the first nitrogenous molecule produced from the inorganic nitrogen is glutamine or glutamic acid, which is then transaminated to produce other amino acids or N-containing compounds (Pratelli and Pilot, 2014).

As commented above, grain yield and quality (referring to protein content) are mainly dependent on assimilates which are translocated from vegetative tissues before anthesis. In fact, nitrogen uptake from the soil, assimilation, and allocation from source to sink organs are affected by environmental conditions which considerably impact nitrogen use efficiency (NUE) of cereal crops. NUE is a concept that has been used to characterise plant behaviour regarding different levels of nitrogen availability (Szilvia et al., 2018) and is defined as the amount of grain produced per unit of N taken up. The determination of NUE in cereals enable the assessment of agronomic management and environmental factors related to N use as grain yield, nitrogen accumulation and nitrogen harvest index, which are the key indicators of NUE (Huggins and Pan, 2003). In fact, the increase in air temperature and $[CO_2]$ is affecting yield potentials and are predicted to have substantial influences in the future by altering timing of phenology or favoring photosynthetic processes determining N utilization efficieny (Semenov and Shewry, 2011; Asseng et al., 2019). Climate change will also impact soil water resources and nutrient availability, requiring optimized root related traits favoring high N uptake efficiceny. In this perspective, the improvement of NUE could be considered as a selection tool for genotypes better adapted to long-term climate change.

While, traditionally, the flag leaf has been assigned as the main photosynthetic organ supporting grain filling, different studies (Richards, 2000; Xiao et al., 2012; Zhou et al., 2014) have not detected any correlation between flag leaf photosynthetic rates (per unit area basis) and grain yield. Furhter, recent findings suggest that ear photosynthetic activity represents a key source of photoassimilates that sustain grain filling (Tambussi et al., 2005, 2007; Sánchez-Bragado et al., 2014; Zhou et al., 2014). The reported contributions to grain filling vary widely, with estimates ranging from about 10% to 76% of the total assimilates contributing to grain filling (Gebbing and Schnyder, 2001; Tambussi et al., 2007; Aranjuelo et al., 2011; Sanchez-Bragado et al., 2014). Actually, the relative contribution of each organ to grain filling may reflect genetic diversity and is probably strongly affected by growing conditions (Sanchez-Bragado et al., 2014). Therefore, a deeper understanding of the effect of drought stress, elevated [CO_2], and the interaction between them on the role of wheat leaves and ears during grain filling is needed.

During leaf senescence, Howarth et al. (2008) reported that amino acid biosynthesis was regulated in favour of glutamine production at the expense of a number of other amino acids, and it was the major transported form of amino acid imported during early development of wheat grain. Grain filling in cereals depends also on carbon (C) that is derived from photosynthetic assimilation and the remobilization of prestored carbohydrates from source organs. The main storage form of non-structural carbohydrates in stem is starch that is degradated to glucose and then the sucrose is resynthesized when C is remobilized to the grains (Beck and Ziegler, 1989). For wheat, grain formation is depending on leaf photoassimilates from flowering to grain maturity, which are transported to kernels mainly as sucrose (Lemoine, 2000; Lalonde et al., 2004; Reynolds et al., 2012). Sucrose is synthesized in the cytoplasm of leaves from glucose and fructose and then it is loaded into the phloem and transported to sink tissues during grain filling, where it is considered as the main source of carbon and energy.

In Mediterranean area, water deficit usually occurs at anthesis and grain-filling stage (Del pozo et al., 2016; Hochman et al., 2017) when the reproductive phase is extremely sensitive to plant water status (Saeedipour and Moradi, 2011). As a consequence, enhanced leaf senescence by accelerating loss of leaf chlorophyll and soluble proteins, and shortening in grain-filling period are promoted (Rawson et al., 1983; Wardlaw and Willenbrink, 2000; Yang et al., 2001).

The interactive effects between drought stress and increasing [CO_2] on plants metabolism and physiological mechanisms are scarce. In fact, climate change is a multifactorial stress that has varying effects on plant responses at the molecular level as well as at the developmental processes and physiological mechanism (Soares et al., 2019), thus, the understanding of how water scarcity, with emphasis to elevated [CO_2], is impacting cereal yields and grain nutritional quality together with their associated adaptative response, is of paramount importance tool to mitigate the cumulative effect of climate change as time progresses (Fanzo et al., 2018). Adding to that, it has been reported that plant responses to high [CO_2] or water deficit are influenced by the duration and level of the environmental factor, the growth stage and the genetic variability (Medina et al., 2016).

Within a current and near future context in which plants will be growing in challenging environmental conditions it is crucial to promote the identification of the more resource efficient crops. Plant genetic resources for agriculture might be one of the biological bases of world food security (Villegas et al., 2010). A decrease of the genetic diversity in major crops (i.e. wheat, maize, rice) is increasing the vulnerability of agriculture to predicted climate change scenarios (Kotschi, 2007; Poudel et al., 2020). This has urged scientists to develop more crop varieties suiting the changing climate in order to obtain sufficient yield for the increasing population with adequate grain nutritional value. Tritordeum plants have been developed as a relevant cereal with high grain nutritional value as well as its lower gluten levels (Kakabouki et al., 2020). Additionally, tritordeum marks high adaptability in the Mediterranean area (Villegas et al., 2010). The new tritordeum genotypes from the current breeding program have not been extensively tested at field level in a set of environments with different yield potentials. Tritordeum presents a large genetic pool and more than 250 primary lines exist today (Erlandsson, 2010). Growth conditions potentially impact C_3 grain yields and determine the nutritional value of the final product. Therefore, evaluating the mechanisms and the physiological processes involved in assimilates partitioning in plants under abiotic stress is mandatory for breeding programs.

Therefore, the aim of this work was to evaluate (alone and interacting) the impact of [CO_2] (ambient [CO_2] vs elevated [CO_2]) and water availability (full irrigation vs moderate drought stress) on durum wheat and tritordeum (i) grain yields/quality, together with the analyses on (ii) the contribution of flag leaves, basal leaves and ears (glumes) on grain development of durum wheat and tritordeum cultivars with different nitrogen use efficiency (NUE).

MATERIAL AND METHODS

Plant material and experimental design

The study was conducted with four high yielding durum wheat genotypes (received from the International Maize and Wheat Improvement Center (CIMMYT, Mexico) and six tritordeum lines (provided by Agrasys S.L.) (Table S1). Durum wheat and tritordeum genotypes were identified as lines with high and low nitrogen use efficiency (NUE) in a previous experiment (Ben Mariem et al., 2020b) in which 28 genotypes (Durum wheat: 20 genotypes, Tritordeum: 8 genotypes) were exposed to two nitrogen fertilization levels. According to the results obtained in that study (Table S2), durum wheat genotypes G3 and G9 had lower NUE than genotypes G6 and G18. Similarly, tritordeum genotypes G22, G24, and G25 had recorded the lowest NUE under both nitrogen treatments when compared to genotypes G21, G23, and G26. Seedlings were vernalized during two weeks in a cold room at 4°C and then transplanted to 3L pots containing a peat/perlite/vermiculite 2:2:1 (v/v/v) substrate mixture. After sowing, the plants were transferred to 4 greenhouses located at the University of Navarra (UNAV) in Pamplona (Spain) and were exposed to changing [CO_2] and irrigation conditions.

During vegetative stage, all plants were watered at pot capacity by ½ Hoagland nutrient solution. Ten days before anthesis stage, plants grown in greenhouse (3) and (4) were watered at 50% of pot capacity until plants reached maturity stage, while the other plants (grown in greenhouse (1) and (2)) were maintained at control irrigation conditions until the end of the experiment. Drought stress was applied during flowering and post-anthesis grain-filling period because the typical Mediterranean climate is characterized by low precipitation in spring, increasing the risks of occurrence of water deficit during this growth stage of durum wheat (Costa et al., 2013; Páscoa et al., 2017). Four treatments were applied and described as following:

- **WW-Amb.CO_2**: Well watered (full irrigation) plants grown at ambient [CO_2] (400 ppm) conditions.
- **WW-Elev.CO_2**: Well watered (full irrigation) plants grown at high [CO_2] (700 ppm) conditions.
- **WS-Amb.CO_2**: Water stressed (watered at 50% of pot capacity) plants grown at ambient [CO_2] (400 ppm) conditions.

- **WS-Elev.CO_2**: Water stressed (watered at 50% of pot capacity) plants grown at high [CO_2] (700 ppm) conditions.

For each treatment, 3 pots were used per genotype with 2 plants per pot and the experiment was conducted according to completely randomized design. Plants were irrigated 3 times per week, two times with½ Hoagland solution and once with water. In order to characterize post-anthesis contribution sustaining grain filling, basal leaves, flag leaves, and entire ears (glumes) without grains (the grains were removed) of durum wheat were sampled at anthesis and soft dough stages to analyze their carbohydrate concentrations, total soluble protein, amino acid concentrations, and Rubisco (large sub-unit) degradation. At maturity, durum wheat and tritordeum plants were harvested and grains were collected to determine yield and quality traits of the different genotypes.

Grain yield and thousand-grain weight

Grain yield was determined for each genotype as the weight of total grains produced per plant. Thousand-grain weight (TGW) was determined by calculating the weight of 20 grains and then converted to the weight of 1000 grains as follows:

$$\text{TGW(g)} = (\text{weight of 20 grains (g)} \times 1000)/20$$

Grain carbon and nitrogen concentrations and nitrogen use efficiency (NUE)

Approximately 1mg of milled grains were weighed and stored in tin capsules (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA) to determine C and N concentration (% of dry weight (DW)) using an elementalanalyzer (FlashEA1112, ThermoFinnigan, Waltham, MA, USA) equipped with a MAS200 Rautosampler, as it is described in Ben Mariem et al. (2020b).

Nitrogen use efficiency (NUE) of production was determined as the ratio of grain yield to the total grain N content according to Hawkesford and Riche (2020).

Grain micro- and macronutrients analyses

Grains were ground to a fine powed and 100mg (approximately) were used to determine mineral concentrations in durum wheat and tritordeum grains by inductively coupled plasma/optical emission spectrometry (ICP/OES, iCAP 6500 Duo, Thermo Fisher Scientific, Waltham, MA, USA).

Non-Structural carbohydrate concentrations

Soluble sugar concentrations (glucose, fructose, and sucrose) were determined in 25mg of milled grains and 80mg of frozen fresh matter of basal leaves, flag leaves, and glumes supernatants (previously ground in amortar with liquid nitrogen) after ethanol extraction, using an ionic chromatographer (ICS-3000, Thermo Scientific^{TM}, Waltham, MA, USA). The pellet was used to determine starch concentration. The quantification was performed using the amyloglucosidase test kit (R-Biopharm, AG; Darmstadt, Germany) and measuring the absorbance at 340nm with a spectrophotometer.

Amino acid concentrations

Amino acids were determined in 20mg of milled grains and 50mg of frozen fresh matter of basal leaves, flag leaves, and glumes (previously ground in amortar with liquid nitrogen) after derivatization with a ACCQ-Fluor™ Reagent kit (Waters, USA) based in borate buffer, acetonitrile and AQC derivatizing reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using high performance liquid chromatography (HPLC).

Total soluble protein concentration in wheat leaves and glumes

Soluble proteins were extracted from 200mg of frozen matter ground in a mortar with liquid nitrogen mixed with 50mg of polyvinylpolypyrrolidone (PVPP) and 600µL of citrate-phosphate buffer (20nM citrate – 160nM phosphate, pH= 6.8). After centrifugation (1 h, 12000g, 4°C), the supernatant was transferred to a 1.5mL microtube and completed with to 500µL with citrate-phosphate buffer. The concentration of total soluble protein was determined using Bradford method and measuring the absorbance at 595nm with a spectrophotometer.

Rubisco large subunit concentration in wheat leaves and glumes

In order to quantify Rubisco large subunit in durum wheat leaves and glumes at anthesis and soft dough stages, a method using stain free gels (Bio-Rad) was performed. This method allows the detection of proteins immediately after the end of the SDS-PAGE, and is based on a UV-induced trihalo-compound modification of tryptophan residues contained in proteins (for more details, see Kazmin et al., 2002). For this, the same protein extract described above was used. 15µg of proteins were denatured with Laemmli 2X buffer containing β-mercaptoethanol (5% (v/v)) during 5 min in boiling water. The resulting samples were loaded on SDS-PAGE (Mini-PROTEAN TGX Stain Free Gels 4-15%, Bio-Rad), and after 1 h of incubation, Rubisco large sub-unit bands were visualized and quantified using Image Lab software (Bio-Rad).

Statistical analysis

To explore the effect of the different treatments on yield and grain quality of durum wheat and tritordeum, one-way analysis of variance (ANOVA) (STATGRAPHICS, Centurion XV, Version 15.1.02, Madrid, Spain) was conducted using ‘treatments’ as factor having four values (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Least Significant Difference (LSD) was used to determine statistical differences among treatments for each genotype. Two-way analysis of variance has been also conducted to analyze the interaction between the genotypes and treatments within each species. The statistical analysis of carbon and nitrogen metabolites remobilization between anthesis and soft dough stages was focused on differences among treatments at both phenological stages. Hence, one-way ANOVA was performed considering the combination between the four treatments and the two growth stages (anthesis and soft dough) as one factor having therefore, eight values. Results were considered to be significant when $p<0.05$.

RESULTS

I. Grain yield and quality of durum wheat and tritordeum under drought and high [CO_2] conditions

1. Grain yield, thousand-grain weight (TGW), and nitrogen use efficiency (NUE)

Durum wheat grain yield results presented in Table 1 showed that well watered plants had higher yields than plants grown under water stress conditions. Under full irrigation conditions, high [CO_2] had no significant effect in wheat and tritordeum grain yield. On the other hand, the study also showed that, under drought stress, growth at 700 ppm increased grain yield by 24% and 98% (relative to drought treatment) for durum wheat and tritordeum, respectively. Grain yield means recorded under ambient [CO_2] and drought stress conditions showed that tritordeum seems to be more sensitive to water shortage than durum wheat (2.58 vs 4.40 g/plant). However, under drought stress combined with elevated [CO_2], tritordeum genotypes showed better response and reached more or less the same mean yield as wheat (5.12 vs 5.44 g/plant). While no significance was detected among durum wheat genotypes (p= 0.27), tritordeum lines revealed significant difference between cultivars (p<0.001). On the other hand, durum wheat and tritordeum did not show significant differences among treatments regarding thousand-grain weight (TGW) (Table 2) (p= 0.30 for both species), which could explain that the observed differences in grain yield between treatments might be attributed to grain number produced per plant rather than grain size. Averages of TGW of the two species indicated that durum wheat had higher grain weight when compared to tritordeum within each treatment (Table 2).

The increase of [CO_2] under well watered and drought conditions had improved nitrogen use efficiency (NUE) of durum wheat and tritordeum genotypes (Table 3). The results showed that drought stress has significantly increased NUE of durum wheat by 15% while no significant increase by 4% had been recorded for tritordeum, when compared to control. Under non-limited water conditions, similar NUE was observed for durum wheat and tritordeum. However, under drought stress, higher NUE was obtained in wheat genotypes when compared to tritordeum.

Table 1: Grain yield (g/plant) of **durum wheat** and **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6)**.** Statistical analysis was made by a one-way ANOVA. Different letters mean significant differences among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Species	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
Durum wheat	G3	5.12^{a} ±0.46	5.82^{a} ±0.34	4.74^{a} ±0.33	5.52^{a} ±0.43	0.44
	G9	5.63^{a} ±0.46	5.74^{a} ±0.38	3.69^{b} ±0.36	5.44^{a} ±0.39	**0.02**
	G6	5.38^{ab} ±0.47	6.70^{a} ±0.66	4.73^{b} ±0.52	5.13^{b} ±0.52	0.08
	G18	5.94^{ab} ±0.38	6.32^{a} ±0.46	4.45^{b} ±0.55	5.68^{ab} ±0.58	0.11
	Mean	5.52^{ab} ±0.18	6.15^{a} ±0.23	4.40^{c} ±0.25	5.44^{b} ±0.12	**<0.001**
	p-values (Genotypes)					0.27
	p-values (G x T)					0.52
Tritordeum	G21	6.01^{b} ±0.41	7.91^{a} ±0.53	3.13^{c} ±0.32	6.17^{b} ±0.52	**<0.001**
	G22	7.96^{a} ±0.35	7.66^{a} ±0.84	3.29^{c} ±0.51	5.51^{b} ±0.85	**<0.001**
	G23	7.53^{a} ±0.31	7.13^{a} ±0.40	2.07^{c} ±0.11	5.38^{b} ±0.84	**<0.001**
	G24	5.81^{ab} ±0.51	6.87^{a} ±0.43	2.63^{c} ±0.29	4.70^{b} ±0.51	**<0.001**
	G25	6.87^{a} ±0.48	6.76^{a} ±0.40	2.22^{c} ±0.23	4.79^{b} ±0.69	**<0.001**
	G26	6.08^{a} ±0.68	6.04^{a} ±0.56	2.12^{c} ±0.21	4.17^{b} ±0.50	**<0.001**
	Mean	6.71^{a} ±0.36	7.06^{a} ±0.27	2.58^{c} ±0.22	5.12^{b} ±0.29	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**0.04**

Table 2: Thousand grain weight (TGW, g) of **durum wheat** and **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6)**.** Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Species	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
Durum wheat	G3	39.59^{b} ±0.65	46.75^{a} ±2.17	40.88^{ab} ±1.98	39.00^{b} ±2.36	0.07
	G9	49.50^{a} ±0.94	47.30^{ab} ±2.58	45.14^{ab} ±0.54	43.66^{b} ±1.22	0.10
	G6	40.02^{a} ±1.87	43.88^{a} ±1.25	44.00^{a} ±0.43	43.00^{a} ±0.98	0.14
	G18	45.97^{a} ±1.40	46.10^{a} ±2.01	45.00^{a} ±2.96	48.90^{a} ±0.93	0.50
	Mean	43.77^{a} ±2.40	46.01^{a} ±0.75	43.75^{a} ±0.99	43.64^{a} ±2.03	0.30
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**0.02**
Tritordeum	G21	30.63^{ab} ±1.13	33.33^{ab} ±1.59	29.50^{b} ±1.54	34.00^{a} ±0.72	0.11
	G22	34.75^{a} ±0.76	35.33^{a} ±1.06	34.40^{a} ±0.86	34.00^{a} ±1.23	0.80
	G23	30.88^{a} ±1.42	31.83^{a} ±1.02	29.75^{a} ±1.25	28.20^{a} ±2.41	0.45
	G24	30.60^{ab} ±1.89	30.00^{ab} ±1.95	26.50^{b} ±0.50	33.06^{a} ±0.56	0.10
	G25	32.17^{ab} ±1.96	27.25^{b} ±1.76	33.50^{a} ±2.03	33.00^{a} ±1.61	0.09
	G26	31.75^{a} ±2.17	30.20^{a} ±1.59	30.67^{a} ±2.60	33.17^{a} ±0.67	0.71
	Mean	31.79^{a} ±0.65	31.33^{a} ±1.15	30.72^{a} ±1.18	32.57^{a} ±0.89	0.30
	p-values (Genotypes)					**0.002**
	p-values (G x T)					0.11

Table 3: Nitrogen use efficiency (NUE, g(grains)/g(N)) of **durum wheat** and **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6)**.** Statistical analysis was made by a one-way ANOVA. Different lettersmean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Species	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
Durum wheat	G3	26.14^{b} ±2.00	28.58^{ab} ±0.85	32.54^{a} ±0.62	29.71^{ab} ±1.52	0.11
	G9	28.14^{b} ±0.87	28.48^{b} ±0.99	29.52^{ab} ±0.79	33.24^{a} ±2.06	0.06
	G6	26.86^{b} ±2.00	32.69^{a} ±0.30	31.80^{a} ±0.86	30.19^{ab} ±0.84	**0.04**
	G18	24.71^{b} ±0.93	26.22^{b} ±1.02	28.08^{ab} ±1.39	30.84^{a} ±0.91	**0.01**
	Mean	26.46^{c} ±0.72	28.99^{b} ±1.35	30.49^{ab} ±1.03	30.99^{a} ±0.78	**<0.001**
	p-values (Genotypes)					**0.02**
	p-values (G x T)					0.12
Tritordeum	G21	26.32^{b} ±0.71	28.85^{a} ±0.18	27.50^{ab} ±0.33	27.63^{ab} ±0.75	**0.02**
	G22	26.91^{b} ±0.47	30.39^{a} ±0.64	27.53^{b} ±0.45	28.36^{b} ±0.93	**0.01**
	G23	28.09^{b} ±0.99	27.77^{b} ±0.55	28.22^{b} ±0.60	31.92^{a} ±1.28	**0.01**
	G24	22.78^{b} ±1.79	26.15^{ab} ±0.89	26.44^{ab} ±1.35	27.69^{a} ±0.56	0.08
	G25	26.68^{a} ±0.48	27.96^{a} ±0.77	26.49^{a} ±1.27	27.69^{a} ±0.90	0.61
	G26	25.01^{b} ±0.47	27.52^{a} ±0.97	25.55^{ab} ±0.97	26.53^{ab} ±0.64	0.12
	Mean	25.96^{b} ±0.75	28.11^{a} ±0.58	26.96^{b} ±0.39	28.30^{a} ±0.76	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**0.04**

2. Grain carbon concentration, nitrogen concentration, and C/N ratio

Grain carbon concentration significantly increased in wheat and tritordeum plants when high [CO_2] was applied (Table 4). Moreover, regardless of anlyzed crop, drought stress did not show a negative effect on carbon concentration. On the other hand, the results showed that, while in case of durum wheat plants grown under high [CO_2] grain nitrogen concentration had no significant effect (Table 5), in case of tritordeum plants N concentrationwas lower under 700 ppm. In case of wheat plants, the only significance was found between well-watered condition and drought stress with higher means recorded under irrigated treatment. On the other hand, grain nitrogen concentration of triordeum cultivars did not show significant difference between irrigated and drought conditions. High [CO_2] negatively affected nitrogen concentration with an average decrease by 8% and 5% under full irrigation and limited water conditions, respectively. When comparing between the two species, grain nitrogen concentration seems to be higher in tritordeum than in durum wheat genotypes.

Table 4: Grain carbon concentration (%) of **durum wheat** and **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6)**.** Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Species	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
Durum wheat	G3	41.94^{ab} ±0.18	42.38^{a} ±0.15	41.35^{b} ±0.44	42.52^{a} ±0.32	**0.04**
	G9	41.72^{c} ±0.08	42.44^{a} ±0.07	41.98^{bc} ±0.21	42.22^{ab} ±0.14	**0.01**
	G6	41.83^{b} ±0.21	42.54^{a} ±0.14	41.98^{ab} ±0.23	42.39^{a} ±0.16	**0.04**
	G18	42.18^{b} ±0.06	42.67^{a} ±0.13	42.37^{ab} ±0.12	42.73^{a} ±0.10	**0.01**
	Mean	41.92^{b} ±0.10	42.51^{a} ±0.06	41.92 ^{b}±0.21	42.46^{a} ±0.11	**<0.001**
					p-values (Genotypes)	**0.008**
					p-values (G x T)	0.32
Tritordeum	G21	42.52^{a} ±0.12	42.93^{a} ±0.08	42.59^{a} ±0.11	42.73^{a} ±0.48	0.73
	G22	42.33^{b} ±0.09	43.13^{a} ±0.09	42.53^{b} ±0.07	42.48^{b} ±0.19	**<0.001**
	G23	42.35^{b} ±0.12	43.15^{a} ±0.13	41.92^{c} ±0.06	42.46^{b} ±0.13	**<0.001**
	G24	42.19^{b} ±0.09	43.10^{a} ±0.10	42.26^{b} ±0.24	42.42^{b} ±0.12	**<0.001**
	G25	42.32^{b} ±0.10	43.00^{a} ±0.08	42.23^{b} ±0.16	42.70^{a} ±0.11	**0.002**
	G26	42.10^{c} ±0.12	42.82^{ab} ±0.17	42.50^{bc} ±0.24	43.21^{a} ±0.14	**<0.001**
	Mean	42.30^{c} ±0.06	43.02^{a} ±0.05	42.34^{c} ±0.10	42.67^{b} ±0.12	**<0.001**
					p-values (Genotypes)	0.38
					p-values (G x T)	**0.02**

Table 5: Grain nitrogen concentration (%) of **durum wheat** and **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6)**.** Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at p<0.05 as determined by LSD test. Values in bold indicate significance (p<0.05). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Species	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
Durum wheat	G3	3.46^{ab} ±0.23	3.45^{a} ±0.07	3.06^{b} ±0.04	3.30^{ab} ±0.17	0.12
	G9	3.59^{a} ±0.16	3.33^{ab} ±0.09	3.40^{ab} ±0.09	3.12^{b} ±0.18	0.14
	G6	3.80^{a} ±0.33	3.18^{b} ±0.06	3.15^{b} ±0.09	3.04^{b} ±0.08	**0.03**
	G18	3.79^{a} ±0.20	3.54^{a} ±0.12	3.56^{ab} ±0.16	3.25^{b} ±0.10	0.09
	Mean	3.66^{a} ±0.08	3.37^{ab} ±0.16	3.29^{bc} ±0.11	3.17^{c} ±0.06	**0.002**
	p-values (Genotypes)					0.053
	p-values (G x T)					0.10
Tritordeum	G21	3.80^{a} ±0.08	3.47^{c} ±0.02	3.69^{ab} ±0.06	3.57^{bc} ±0.10	**0.02**
	G22	3.72^{a} ±0.07	3.30^{b} ±0.07	3.66^{a} ±0.05	3.63^{a} ±0.13	**0.01**
	G23	3.57^{a} ±0.13	3.65^{a} ±0.07	3.55^{a} ±0.07	3.22^{b} ±0.12	**0.02**
	G24	4.46^{a} ±0.29	3.93^{ab} ±0.14	3.88^{ab} ±0.17	3.64^{b} ±0.09	0.052
	G25	3.76^{a} ±0.07	3.59^{a} ±0.09	3.82^{a} ±0.17	3.63^{a} ±0.12	0.43
	G26	4.02^{a} ±0.06	3.61^{b} ±0.13	3.94^{a} ±0.15	3.78^{ab} ±0.09	0.09
	Mean	3.89^{a} ±0.13	3.59^{b} ±0.09	3.76^{a} ±0.06	3.58^{b} ±0.08	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					0.053

As carbon concentration had increased and nitrogen had decreased under high [CO_2], grain C/N ratio showed the same tendency as carbon concentration (Table 6). Drought conditions had increased slightly but not significantly C/N ratio in grains of wheat and tritordeum. Contrarily to nitrogen concentration, durum wheat grain showed higher mean values when compared to tritordeum.

Table 6: C/N ratio in **grains of durum wheat** and **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Species	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
Durum wheat	G3	12.24^{ab} ±0.75	11.97^{b} ±0.27	13.48^{a} ±0.20	12.62^{ab} ±0.61	0.20
	G9	11.73^{b} ±0.49	12.05^{b} ±0.33	12.39^{ab} ±0.29	13.74^{a} ±0.78	0.07
	G6	11.28^{b} ±0.91	13.77^{a} ±0.13	13.34^{a} ±0.30	12.72^{a} ±0.26	**0.02**
	G18	11.30^{b} ±0.62	11.83^{ab} ±0.72	11.98^{ab} ±0.51	13.18^{a} ±0.40	0.20
	Mean	11.64^{b} ±0.23	12.40^{ab} ±0.46	12.80^{a} ±0.37	13.06^{a} ±0.26	**0.003**
	p-values (Genotypes)					0.31
	p-values (G x T)					0.14
Tritordeum	G21	11.22^{c} ±0.22	12.39^{a} ±0.08	11.55^{bc} ±0.21	12.01^{ab} ±0.26	**0.004**
	G22	11.39^{b} ±0.19	13.11^{a} ±0.27	11.64^{b} ±0.18	11.78^{b} ±0.39	**0.001**
	G23	11.89^{b} ±0.39	11.85^{b} ±0.22	11.83^{b} ±0.24	13.28^{a} ±0.54	**0.03**
	G24	9.66^{b} ±0.63	11.03^{a} ±0.38	10.93^{ab} ±0.41	11.67^{a} ±0.30	**0.03**
	G25	11.29^{a} ±0.19	12.02^{a} ±0.32	11.18^{a} ±0.50	11.82^{a} ±0.36	0.34
	G26	10.49^{c} ±0.14	11.91^{a} ±0.40	10.86^{bc} ±0.41	11.46^{ab} ±0.27	**0.03**
	Mean	10.99^{b} ±0.32	12.05^{a} ±0.28	11.33^{b} ±0.16	12.00^{a} ±0.26	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**0.04**

3. Grain non-structural carbohydrate concentrations (soluble sugars and starch)

Monosaccharide concentrations (glucose and fructose) in wheat grains were negatively affected by drought stress (Figure 1). Comparing to control, a mean decrease by 31% and 45% had been detected for glucose and fructose, respectively (Table 7). The increase of [CO_2] had also significantly decreased glucose and fructose concentrations in grains and the effect was larger under drought stress (Figure 1; Table 7). Same observations were found in tritordeum grains (Figure 2; Table 7). It should be noted that the concentration of these monosacharides was higher in tritordeum than in durum wheat grains. On the other hand, grain sucrose concentration had been significantly and positively affected by the increase of [CO_2]. Further, this effect was higher under well-watered conditions than under drought and results showed an increase (relative to control) by 17% and 6%, respectively (Table 7). Concerning tritordeum, the [CO_2] effect was higher under limited water conditions than under irrigated treatment in which 19% and 6% increases were observed, respectively (Table 7). Maltose concentration had also been significantly affected by drought (Figures 1 and 2) and results showed a mean decrease of 48% and 45% in durum wheat and tritordeum grains, respectively. The rising [CO_2] had also decreased maltose concentration and different pattern was observed between durum wheat and tritordeum. More precisely, higher decrease was recorded when high [CO_2] was applied with full irrigation than with drought stress by 71% and 55%, respectively, in durum wheat grains (Table 7). However, maltose concentration had been diminished by 43% and 66% in tritordeum grains under well-watered and limited conditions, respectively.

Grain starch concentration was not significantly affected by drought and high [CO_2] in durum wheat ($p= 0.09$) but a small decrease was found under limited water conditions (Figure 3, Table 7). In case of tritordeum plants, drought did not show significant negative effect on starch concentration in grains (Figure 3, Table 7), while under high [CO_2] starch concentration decreased significantly by 16% and 10% (relative to control) under well watered and drought conditions, respectively.

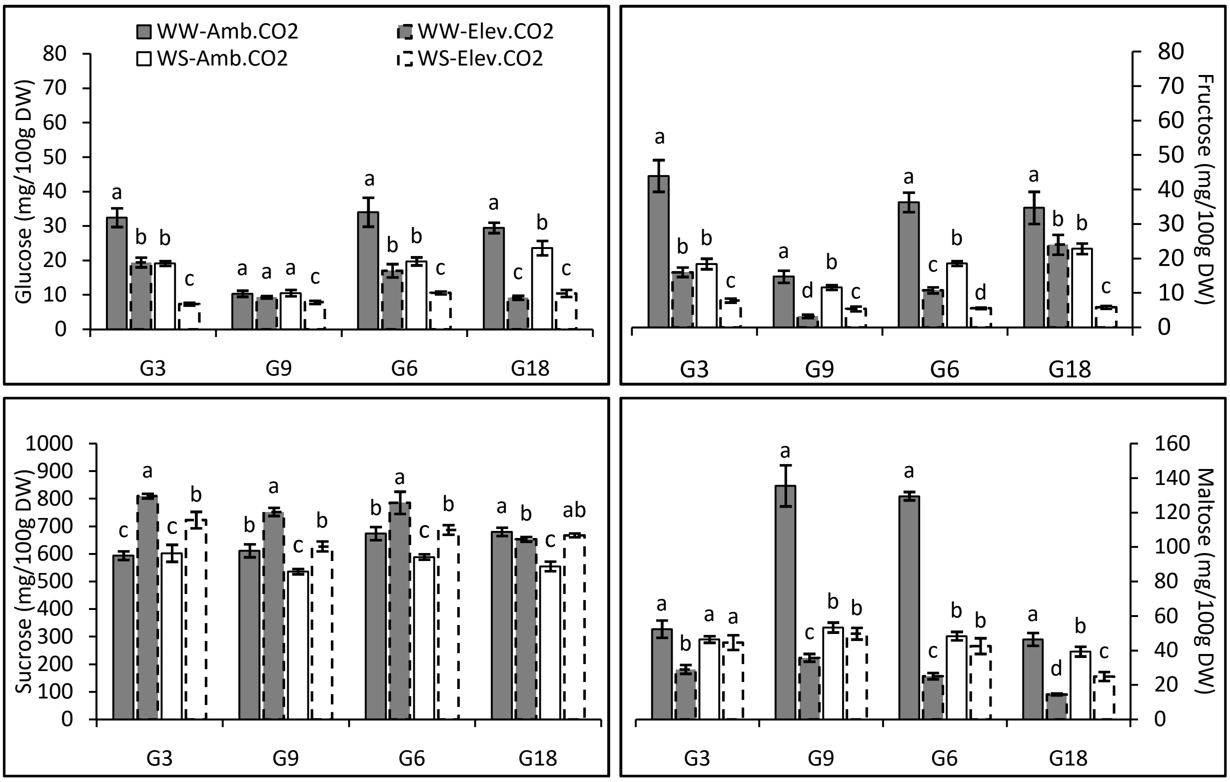


Figure 1: Grain soluble sugar concentrations of **durum wheat genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at p<0.05 as determined by LSD test.

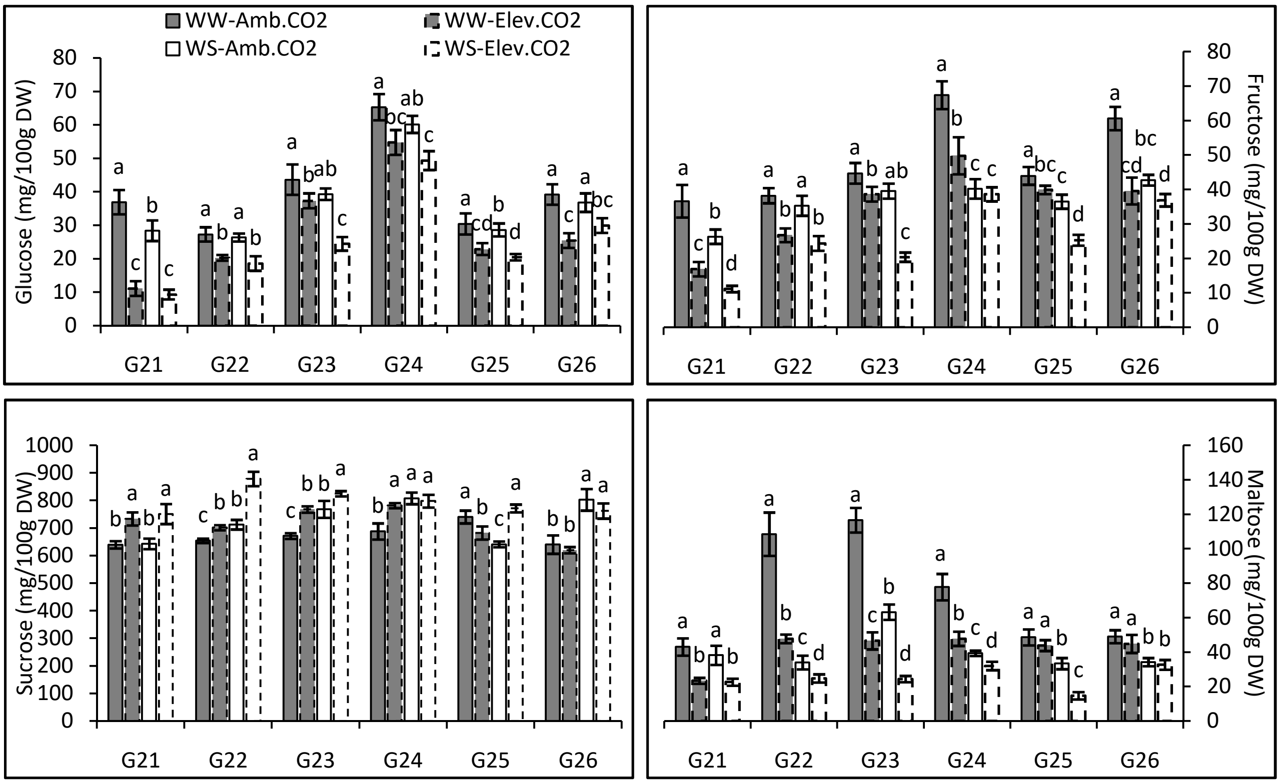


Figure 2: **Grain soluble sugar concentrations** of **tritordeum genotypes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at p<0.05 as determined by LSD test.

Table 7: Means of non-structural carbohydrate concentrations in grains of **durum wheat** and **tritordeum** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments at p<0.05 as determined by LSD test. Values in bold indicate significance (p<0.05).

Treatments	**Durum wheat**					**Tritordeum**				
	Glucose (mg/100g DW)	**Fructose (mg/100g DW)**	**Sucrose (mg/100g DW)**	**Maltose (mg/100g DW)**	**Starch (µmol/g DW)**	**Glucose (mg/100g DW)**	**Fructose (mg/100g DW)**	**Sucrose (mg/100g DW)**	**Maltose (mg/100g DW)**	**Starch (µmol/g DW)**
WW-Amb.CO_2	26.57^{a} ±5.49	32.41^{a} ±6.23	639.69^{c} ±21.87	90.93^{a} ±24.06	1851^{a}±101.60	40.45^{a} ±5.53	48.57^{a} ±5.12	672.38^{c} ±15.63	73.91^{a} ±13.24	1754^{a} ±78.35
WW-Elev.CO_2	13.74^{c} ±2.63	13.50^{c} ±4.38	750.19^{a} ±34.45	26.08^{d} ±4.44	1790^{ab} ±62.50	28.62^{c} ±6.27	35.25^{b} ±4.74	714.56^{b} ±24.42	42.35^{b} ±3.85	1471^{c} ±55.14
WS-Amb.CO_2	18.24^{b} ±2.75	17.85^{b} ±2.32	570.49^{d} ±15.28	46.85^{b} ±2.90	1713^{b} ±42.40	36.58^{b} ±5.16	36.74^{b} ±2.36	729.39^{b} ±30.90	40.37^{b} ±4.67	1722^{a} ±63.13
WS-Elev.CO_2	9.05^{d} ±0.84	6.13^{d} ±0.57	675.99^{b} ±19.89	40.42^{c} ±5.40	1799^{ab} ±71.84	25.33^{d} ±5.55	26.10^{c} ±4.22	797.80^{a} ±19.49	25.16^{c} ±2.71	1570^{b} ±38.65
p-values (Treatments)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.09	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

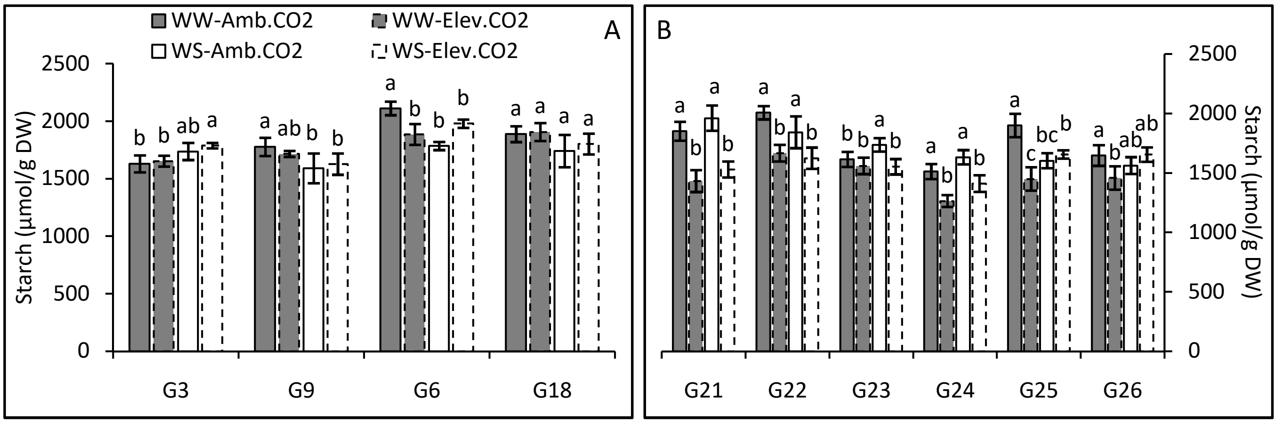


Figure 3: Grain starch concentration of **durum wheat** (A) and **tritordeum genotypes** (B) grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test.

4. Grain amino acid concentrations and composition

Drought stress and high [CO_2] significantly affected grain total amino acids of durum wheat and tritordeum (Tables 8 and 9). Under well-watered treatment, high [CO_2] decreased total amino acids concentration by 56% and 52% in wheat and tritordeum grains, respectively. However, total amino acid concentration was reduced by 46% and 13% in wheat and tritordeum grains, respectively, when [CO_2] was combined with drought. On the other hand, under ambient [CO_2], drought stress increased total amino acid concentration by 25% and 85% in durum wheat and tritordeum grains, respectively.

Glutamic acid and glutamine concentrations in wheat and tritordeum grains where higher under drought stress (Tables 8 and 9). However, the lowest concentrations of glutamic acid and glutamin were detected under high [CO_2] combined with full irrigation in durum wheat grain as well as in tritordeum. Tables 8 and 9 also showed that proline amino acid levels were lower than the ones detected for glutamic acid and glutamine. Drought stress stimulated the accumulation of proline in durum wheat and tritordeum grains. As reported for glutamic acid and glutamin, the rised [CO_2] tended to decrease proline concentration when it was applied under well-watered treatment as well as limited irrigation. As indicated previously concerning nitrogen concentration in grains, total amino acid concentration seems to be higher in tritordeum genotypes than in durum wheat.

Table 8: Grain total amino acids, glutamic acids, glutamine, and proline concentrations (nmol/g DW) of **durum wheat** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at p<0.05 as determined by LSD test. Values in bold indicate significance (p<0.05). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Amino acids	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***P-values***
Total amino acids	G3	4.98^{b} ±0.09	2.54^{c} ±0.11	5.62^{a} ±0.06	2.44^{c} ±0.08	**<0.001**
	G9	3.66^{b} ±0.15	1.46^{d} ±0.03	4.99^{a} ±0.20	2.09^{c} ±0.03	**<0.001**
	G6	4.92^{a} ±0.18	2.12^{c} ±0.10	5.23^{a} ±0.32	2.64^{b} ±0.09	**<0.001**
	G18	4.67^{b} ±0.17	1.83^{d} ±0.08	6.88^{a} ±0.46	2.71^{c} ±0.14	**<0.001**
	Mean	4.56^{b} ±0.31	1.99^{d} ±0.23	5.68^{a} ±0.42	2.47^{c} ±0.14	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**
Glutamic acid	G3	0.224^{b} ±0.006	0.084^{c} ±0.006	0.361^{a} ±0.020	0.191^{b} ±0.003	**<0.001**
	G9	0.203^{b} ±0.007	0.074^{d} ±0.004	0.328^{a} ±0.016	0.153^{c} ±0.004	**<0.001**
	G6	0.235^{b} ±0.003	0.097^{d} ±0.007	0.366^{a} ±0.019	0.205 ^{c}±0.010	**<0.001**
	G18	0.254^{b} ±0.015	0.080^{d} ±0.004	0.385^{a} ±0.021	0.172^{c} ±0.010	**<0.001**
	Mean	0.229^{b} ±0.011	0.084^{d} ±0.005	0.360^{a} ±0.012	0.180^{c} ±0.011	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					0.16
Glutamine	G3	0.200^{b} ±0.002	0.064^{d} ±0.006	0.319^{a} ±0.022	0.105^{c} ±0.002	**<0.001**
	G9	0.187^{b} ±0.008	0.050^{d} ±0.001	0.308^{a} ±0.015	0.100^{c} ±0.001	**<0.001**
	G6	0.197^{b} ±0.004	0.087^{d} ±0.007	0.312^{a} ±0.018	0.118^{c} ±0.011	**<0.001**
	G18	0.239^{b} ±0.021	0.057^{d} ±0.002	0.366^{a} ±0.002	0.127^{c} ±0.013	**<0.001**
	Mean	0.206^{b} ±0.011	0.064^{d} ±0.008	0.326^{a} ±0.013	0.112^{c} ±0.006	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					0.08

Table 8: (Continued).

Proline	G3	0.072^{a} ±0.002	0.067^{a} ±0.006	0.056^{b} ±0.002	0.031^{c} ±0.003	**<0.001**
	G9	0.033^{b} ±0.001	0.022^{b} ±0.002	0.060^{a} ±0.007	0.024^{b} ±0.001	**<0.001**
	G6	0.073^{a} ±0.006	0.045^{b} ±0.004	0.053^{b} ±0.010	0.037^{b} ±0.005	**<0.001**
	G18	0.053^{b} ±0.004	0.029^{c} ±0.003	0.095^{a} ±0.016	0.046^{b} ±0.004	**<0.001**
	Mean	0.058^{b} ±0.009	0.041^{c} ±0.010	0.066^{a} ±0.010	0.034^{c} ±0.005	**<0.001**
					p-values (Genotypes)	**<0.001**
					p-values (G x T)	**<0.001**

Table 9: Grain total amino acids, glutamic acids, glutamine, and proline concentrations (nmol/g DW) of **tritordeum** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at p<0.05 as determined by LSD test. Values in bold indicate significance (p<0.05). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Amino acids	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***P-values***
Total amino acids	G21	4.01^{b} ±0.09	1.87^{c} ±0.10	6.89^{a} ±0.50	2.33^{c} ±0.05	**<0.001**
	G22	4.59^{b} ±0.20	2.08^{c} ±0.14	10.52^{a} ±0.60	2.73^{c} ±0.12	**<0.001**
	G23	6.49^{b} ±0.45	2.58^{c} ±0.14	13.55^{a} ±1.38	3.37^{c} ±0.17	**<0.001**
	G24	7.16^{b} ±0.25	3.75^{c} ±0.13	13.99^{a} ±0.74	6.84^{b} ±0.48	**<0.001**
	G25	3.99^{c} ±0.14	2.40^{d} ±0.16	7.45^{a} ±0.45	5.60^{b} ±0.28	**<0.001**
	G26	4.28^{b} ±0.12	2.00^{c} ±0.09	3.93^{b} ±0.13	5.47^{a} ±0.22	**<0.001**
	Mean	5.09^{b} ±0.56	2.45^{d} ±0.28	9.39^{a} ±1.63	4.39^{c} ±0.75	**<0.001**
					p-values (Genotypes)	**<0.001**
					p-values (G x T)	**<0.001**

Table 9: (Continued).

Glutamic acid	G21	0.245^{b} ±0.009	0.092^{d} ±0.007	0.461^{a} ±0.045	0.153^{c} ±0.008	**<0.001**
	G22	0.259^{b} ±0.022	0.091^{c} ±0.008	0.602^{a} ±0.061	0.169^{c} ±0.005	**<0.001**
	G23	0.290^{b} ±0.020	0.114^{c} ±0.004	0.766^{a} ±0.113	0.238^{bc} ±0.006	**<0.001**
	G24	0.349^{b} ±0.021	0.160^{c} ±0.012	0.749^{a} ±0.065	0.357^{b} ±0.028	**<0.001**
	G25	0.215^{c} ±0.010	0.104^{d} ±0.007	0.499^{a} ±0.036	0.325^{b} ±0.020	**<0.001**
	G26	0.200^{b} ±0.006	0.070^{c} ±0.005	0.264^{a} ±0.013	0.282^{a} ±0.013	**<0.001**
	Mean	0.259^{b} ±0.022	0.105^{c} ±0.012	0.557^{a} ±0.078	0.254^{b} ±0.034	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**
Glutamine	G21	0.196^{b} ±0.003	0.049^{d} ±0.004	0.351^{a} ±0.031	0.101^{c} ±0.003	**<0.001**
	G22	0.171^{b} ±0.006	0.055^{d} ±0.005	0.396^{a} ±0.050	0.110^{c} ±0.007	**<0.001**
	G23	0.201^{b} ±0.009	0.068^{c} ±0.003	0.525^{a} ±0.052	0.118^{bc} ±0.006	**<0.001**
	G24	0.255^{b} ±0.015	0.110^{c} ±0.007	0.365^{a} ±0.014	0.253^{b} ±0.016	**<0.001**
	G25	0.157^{c} ±0.008	0.059^{d} ±0.003	0.373^{a} ±0.032	0.233^{b} ±0.018	**<0.001**
	G26	0.160^{b} ±0.007	0.060^{c} ±0.005	0.156^{b} ±0.014	0.224^{a} ±0.019	**<0.001**
	Mean	0.190^{b} ±0.015	0.067^{c} ±0.009	0.361^{a} ±0.048	0.173^{b} ±0.029	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**
Proline	G21	0.041^{b} ±0.003	0.051^{b} ±0.004	0.108^{a} ±0.016	0.035^{b} ±0.002	**<0.001**
	G22	0.095^{b} ±0.006	0.055^{b} ±0.005	0.261^{a} ±0.040	0.062^{b} ±0.007	**<0.001**
	G23	0.128^{b} ±0.015	0.055^{c} ±0.005	0.277^{a} ±0.034	0.061^{c} ±0.006	**<0.001**
	G24	0.141^{bc} ±0.006	0.105^{c} ±0.009	0.195^{b} ±0.042	0.285^{a} ±0.028	**<0.001**
	G25	0.049^{b} ±0.006	0.056^{b} ±0.007	0.156^{a} ±0.028	0.117^{a} ±0.008	**<0.001**
	G26	0.035^{b} ±0.002	0.052^{b} ±0.005	0.090^{a} ±0.010	0.091^{a} ±0.007	**<0.001**
	Mean	0.082^{c} ±0.019	0.062^{d} ±0.009	0.181^{a} ±0.032	0.109^{b} ±0.037	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**

5. Grain mineral composition and concentrations

Macro- and micronutrient concentrations had been evaluated under drought and high [CO_2] conditions. Phosphorus (P) and calcium (Ca) concentrations decreased significantly under drought stress in durum wheat grains, while magnesium (Mg) and potassium (K) concentrations declined but not significantly when comparing to control treatment (Table 10). On the other hand, these macro-elements had been significantly reduced in tritordeum grains and recorded an average decrease by 22%, 10%, 20% and 20% in K, P, Mg, and Ca concentrations, respectively (Table 11). The increase in [CO_2] impacted negatively the concentration of P, K, Mg, and Ca under irrigated and drought treatments for both species. The micronutrient molybdenum (Mo) concentration was increased under water-limited conditions in durum wheat and tritordeum grains. Similarly, high [CO_2] induced significantly the accumulation of this element in grains and the highest concentrations were detected under drought conditions (0.237mg/100g and 0.267mg/100g DW in durum wheat and tritordeum, respectively). Zinc (Zn) and iron (Fe) concentrations were not significantly affected by drought stress in durum wheat grains (Table 10), but were significantly increased in tritordeum grains (Table 11). The increasing [CO_2] significantly decreased Zn and Fe concentrations (with respect to control) by 25% and 14%, respectively, in durum wheat grains, and by 7% and 8% in tritordeum seeds.

Table 10: Grain mineral concentrations of **durum wheat** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Minerals	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	**p-values**
K (mg/100g)	G3	488.89^{a} ±33.10	365.65^{b} ±21.75	452.37^{a} ±18.14	448.70^{a} ±31.09	**0.02**
	G9	386.17^{a} ±18.15	417.13^{a} ±23.08	408.79^{a} ±12.13	433.75^{a} ±35.15	0.58
	G6	502.15^{a} ±30.55	423.26^{b} ±11.95	458.95^{ab} ±14.37	486.38^{ab} ±23.44	0.19
	G18	522.08^{a} ±31.09	409.42^{b} ±21.86	434.31^{b} ±36.70	356.28^{b} ±22.98	**0.005**
	Mean	474.83^{a} ±30.33	403.87^{b} ±13.05	438.61^{ab} ±11.22	431.29^{b} ±27.34	**0.003**
					p-values (Genotypes)	**0.04**
					p-values (G x T)	**0.006**

Table 10: (Continued).

P (mg/100g)	G3	482.15^{a} ±11.47	422.20^{bc} ±23.41	452.56^{ab} ±24.12	392.75^{c} ±5.91	**0.02**
	G9	400.57^{a} ±17.34	411.85^{a} ±16.48	424.22^{a} ±21.85	401.62^{a} ±18.51	0.80
	G6	472.57^{a} ±28.55	366.39^{b} ±11.28	433.17^{a} ±12.68	461.10^{a} ±12.23	**0.01**
	G18	500.73^{a} ±19.53	450.44^{ab} ±18.47	431.58^{b} ±19.23	339.32^{c} ±21.16	**0.001**
	Mean	464.01^{a}±21.94	412.72^{bc}±17.46	435.38^{b}±6.05	398.70^{c}±24.94	**<0.001**
	p-values (Genotypes)					0.15
	p-values (G x T)					**<0.001**
Mg (mg/100g)	G3	148.69^{a} ±5.11	124.24^{b} ±5.40	144.91^{a} ±5.39	113.08^{b} ±6.58	**0.002**
	G9	119.41^{a} ±3.44	119.36^{a} ±6.49	129.68^{a} ±5.72	120.42^{a} ±10.68	0.74
	G6	141.59^{a} ±11.37	101.65^{b} ±3.16	131.62^{a} ±4.35	127.67^{a} ±7.51	**0.02**
	G18	154.34^{a} ±12.84	130.18^{ab} ±4.35	131.05^{ab} ±6.08	111.09^{b} ±6.88	**0.02**
	Mean	141.01^{a}±7.66	118.86^{b}±6.15	134.31^{a}±3.56	118.06^{b}±3.78	**<0.001**
	p-values (Genotypes)					0.15
	p-values (G x T)					**0.03**
Ca (mg/100g)	G3	46.1^{a} ±2.3	30.8^{c} ±1.5	37.2^{b} ±1.8	28.8^{c} ±0.6	**<0.001**
	G9	31.1^{a} ±2.0	25.7^{b} ±1.5	30.0^{ab} ±1.8	30.1^{ab} ±1.3	0.15
	G6	30.7^{ab} ±1.2	29.1^{b}±1.3	34.2^{a} ±1.6	31.1^{ab} ±1.5	0.14
	G18	30.6^{a} ±2.1	27.1^{ab} ±1.8	24.1^{b} ±1.5	23.1^{b} ±1.3	**0.04**
	Mean	34.63^{a}±3.82	28.16^{c}±1.12	31.39^{b}±2.85	28.26^{c}±1.79	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**

Table 10: (Continued).

Mo (mg/100g DW)	G3	0.133^{c} ±0.006	0.188^{ab} ±0.006	0.162^{bc} ±0.014	0.212^{a} ±0.016	**0.001**
	G9	0.178^{b} ±0.009	0.186^{b} ±0.009	0.145^{c} ±0.007	0.218^{a} ±0.008	**<0.001**
	G6	0.191^{b} ±0.016	0.210^{b} ±0.015	0.178^{b} ±0.022	0.296^{a} ±0.017	**0.001**
	G18	0.202^{ab} ±0.026	0.151^{b} ±0.005	0.237^{a} ±0.021	0.222^{a} ± 0.013	**0.03**
	Mean	0.176^{b}±0.015	0.184^{b}±0.012	0.181^{b}±0.020	0.237^{a}±0.020	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**
Zn (mg/100g DW)	G3	3.76^{a} ±0.04	2.58^{c} ±0.13	3.04^{b} ±0.16	2.36^{c} ±0.13	**<0.001**
	G9	2.45^{a} ±0.14	2.49^{a} ±0.10	2.90^{a} ±0.24	2.59^{a} ±0.35	0.38
	G6	3.15^{a} ±0.23	1.63^{b} ±0.15	2.14^{b} ±0.19	2.21^{b} ±0.22	**<0.001**
	G18	3.38^{a} ±0.08	3.21^{b} ±0.09	2.33^{c} ±0.11	2.39^{c} ±0.12	**<0.001**
	Mean	3.18^{a} ±0.34	2.48^{bc} ±0.33	2.65^{ab} ±0.22	2.39^{c} ±0.08	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**
Fe (mg/100g DW)	G3	3.68^{ab} ±0.17	3.31^{bc}±0.18	4.00^{a}±0.09	3.05^{c}±0.21	**0.01**
	G9	3.40^{b}±0.26	2.61^{c}±0.14	4.24^{a}±0.16	3.10^{bc}±0.29	**<0.001**
	G6	3.79^{a}±0.37	2.37^{c}±0.16	3.70^{a}±0.16	3.54^{b}±0.07	**0.02**
	G18	4.23^{a}±0.35	3.57^{ab}±0.15	3.65^{ab}±0.21	3.32^{b}±0.25	**0.14**
	Mean	3.77^{a} ±0.17	2.97^{b} ±0.28	3.90^{a} ±0.14	3.25^{b} ±0.11	**<0.001**
	p-values (Genotypes)					0.17
	p-values (G x T)					**0.02**

Table 11: Grain mineral concentrations of **tritordeum** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means ±standard error (n=6). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments for each genotype at $p<0.05$ as determined by LSD test. Values in bold indicate significance ($p<0.05$). Two-factor ANOVA was made to study G x T interaction (G: Genotype, T: Treatment).

Minerals	**Genotypes**	**WW-Amb.CO_2**	**WW-Elev.CO_2**	**WS-Amb.CO_2**	**WS-Elev.CO_2**	***p-values***
K (mg/100g DW)	G21	576.55^{ab} ±35.70	600.39^{a} ±22.23	463.96^{c} ±29.69	505.61^{bc} ±35.72	**0.03**
	G22	561.58^{a} ±29.42	479.01^{ab} ±31.62	486.03^{ab} ±25.73	437.41^{b} ±27.64	**0.04**
	G23	672.15^{a} ±9.48	450.90^{b} ±5.55	477.03^{b} ±25.84	518.75^{b} ±32.40	**0.001**
	G24	676.65^{a} ±24.75	582.16^{b} ±14.31	457.45^{c} ±27.49	505.66^{c} ±25.37	**<0.001**
	G25	658.35^{a} ±31.60	564.52^{ab} ±28.20	515.67^{b} ±33.22	555.65^{b} ±29.62	**0.02**
	G26	537.93^{a} ±27.21	546.58^{a} ±33.79	437.15^{b} ±16.23	401.63^{b} ±17.91	**0.001**
	Mean	613.87^{a} ±25.30	537.26^{b} ±24.28	472.88^{c} ±10.98	487.45^{c} ±23.21	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**0.008**
P (mg/100g DW)	G21	418.01^{bc} ±12.71	497.56^{a} ±13.87	386.86^{c} ±12.75	446.41^{b} ±15.63	**0.001**
	G22	419.27^{a} ±17.24	417.35^{a} ±20.26	435.56^{a} ±15.31	396.08^{a} ±22.38	0.55
	G23	465.48^{a} ±27.15	429.41^{a} ±21.23	414.62^{ab} ±20.61	366.86^{b} ±21.45	**0.04**
	G24	482.42^{a} ±26.56	491.08^{a} ±32.16	393.81^{b} ±17.68	462.85^{ab} ±26.04	0.14
	G25	483.90^{a} ±17.02	424.02^{b} ±5.70	419.41^{b} ±16.78	485.53^{a} ±18.99	**0.01**
	G26	446.73^{ab} ±19.46	467.92^{a} ±12.47	405.38^{b} ±19.04	431.54^{ab} ±12.47	0.08
	Mean	452.64^{ab} ±12.08	454.56^{a} ±14.50	409.27^{c} ±7.26	431.55^{bc} ±17.85	**<0.001**
	p-values (Genotypes)					**0.03**
	p-values (G x T)					**0.003**

Table 11: (Continued).

Mg (mg/100g DW)	G21	150.17^{a} ±3.68	129.05^{b} ±8.57	115.42^{b} ±6.51	130.30^{b} ±4.63	**0.008**
	G22	152.74^{a} ±5.39	127.30^{bc} ±4.32	137.64^{ab} ±4.58	116.51^{c} ±6.61	**0.001**
	G23	154.27^{a} ±12.17	131.62^{ab} ±7.34	133.93^{ab} ±6.76	125.11^{b} ±5.71	0.11
	G24	162.09^{a} ±6.96	155.65^{a} ±6.39	127.96^{b} ±12.66	149.09^{ab} ±6.55	0.06
	G25	173.71^{a} ±3.87	129.84^{cd} ±0.72	124.14^{d} ±6.02	139.45^{b} ±6.59	**<0.001**
	G26	154.49^{a} ±4.79	135.33^{b} ±3.54	122.42^{c} ±5.04	129.98^{bc} ±3.11	**<0.001**
	Mean	157.91^{a} ±3.55	134.80^{b} ±4.32	126.92^{c} ±3.29	131.74^{bc} ±4.62	**<0.001**
	p-values (Genotypes)					**0.002**
	p-values (G x T)					**<0.001**
Ca (mg/100g)	G21	37.04^{a} ±1.36	35.41^{a} ±1.50	28.46^{b} ±2.37	28.77^{b} ±1.01	**0.002**
	G22	36.48^{a} ±1.44	29.63^{bc} ±1.91	34.82^{ab} ±2.16	27.01^{c} ±0.94	**0.007**
	G23	44.29^{a} ±1.57	36.57^{b} ±2.16	43.87^{a} ±2.11	36.76^{b} ±1.89	**0.03**
	G24	42.50^{a} ±2.21	35.42^{b} ±1.15	29.49^{bc} ±1.78	23.63^{c} ±0.60	**<0.001**
	G25	40.02^{a} ±0.88	31.61^{b} ±1.86	26.60^{b} ±1.14	30.34^{b} ±1.69	**<0.001**
	G26	33.93^{a} ± 2.15	31.13^{a} ±1.54	25.22^{b} ±1.39	22.40^{b} ±1.39	**<0.001**
	Mean	39.04^{a} ±1.60	33.29^{b} ±1.16	31.41^{b} ±2.83	28.15^{b} ±2.12	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**
Mo (mg/100g)	G21	0.162^{b} ±0.011	0.205^{a} ±0.010	0.146^{b} ±0.004	0.199^{a} ±0.015	**0.008**
	G22	0.196^{b} ±0.008	0.232^{ab} ±0.006	0.271^{a} ±0.018	0.243^{a} ±0.018	**0.01**
	G23	0.199^{b} ±0.012	0.237^{ab} ±0.011	0.204^{b} ±0.012	0.266^{a} ±0.013	**0.004**
	G24	0.184^{b} ±0.014	0.290^{a} ±0.011	0.219^{b} ±0.023	0.276^{a} ±0.019	**0.001**
	G25	0.197^{bc} ±0.017	0.222^{b} ±0.015	0.164^{c} ±0.015	0.271^{a} ±0.014	**0.001**
	G26	0.233^{bc} ±0.009	0.253^{b} ±0.012	0.213^{c} ±0.015	0.344^{a} ±0.012	**<0.001**
	Mean	0.195^{c} ±0.009	0.240^{b} ±0.012	0.203^{c} ±0.018	0.267^{a} ±0.019	**<0.001**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**

Table 11: (Continued).

Zn (mg/100g)	G21	2.66^{b} ±0.16	2.49^{b} ±0.06	3.20^{a} ±0.27	2.67^{b} ±0.08	**0.04**
	G22	2.43^{a} ±0.14	2.36^{a} ±0.13	2.58^{a} ±0.07	2.37^{a} ±0.15	0.54
	G23	2.46^{b} ±0.21	2.87^{b} ±0.12	3.69^{a} ±0.27	2.39^{b} ±0.09	**<0.001**
	G24	2.77^{ab} ±0.16	2.53^{b} ±0.10	3.19^{a} ±0.16	2.36^{b} ±0.25	**0.02**
	G25	3.13^{b} ±0.24	2.43^{b} ±0.14	2.82^{ab} ±0.10	2.71^{ab} ±0.12	**0.06**
	G26	3.00^{ab} ±0.24	2.49^{b} ±0.14	3.32^{a} ±0.19	2.75^{b} ±0.01	**0.02**
	Mean	2.74^{b} ±0.12	2.53^{c} ±0.07	3.13^{a} ±0.16	2.54^{c} ±0.08	**<0.001**
	p-values (Genotypes)					**0.004**
	p-values (G x T)					**<0.001**
Fe (mg/100g)	G21	3.43^{a} ±0.17	3.47^{a} ±0.12	3.29^{a} ±0.19	3.59^{a} ±0.21	0.70
	G22	4.04^{a} ±0.18	3.27^{c} ±0.17	3.80^{ab} ±0.13	3.48^{bc} ±0.10	**0.008**
	G23	3.40^{b} ±0.18	3.38^{b} ±0.15	4.47^{a} ±0.28	2.78^{c} ±0.08	**<0.001**
	G24	4.05^{a} ±0.12	3.70^{bc} ±0.10	4.21^{a} ±0.21	3.36^{c} ±0.27	**<0.001**
	G25	3.53^{a} ±0.13	3.33^{a} ±0.16	3.78^{a} ±0.12	3.56^{a} ±0.23	0.34
	G26	3.65^{ab} ±0.17	3.38^{b} ±0.11	3.94^{a} ±0.16	3.62^{ab} ±0.10	0.13
	Mean	3.68^{b} ±0.12	3.42^{c} ±0.06	3.92^{a} ±0.16	3.40^{c} ±0.13	**0.01**
	p-values (Genotypes)					**<0.001**
	p-values (G x T)					**<0.001**

II. Carbon and nitrogen remobilization of durum wheat leaves and ears during grain filling

1. Carbohydrate concentrations in wheat leaves and ears at anthesis and soft dough stages under drought and high[CO_2] conditions

- ***Basal leaf***

Glucose and fructose concentrations were significantly higher under moderate drought stress in wheat basal leaves at anthesis (Figure 4). The increase in [CO_2] applied with full irrigation decreased, but not significantly, the concentration of these monosaccharides. However, the increase of [CO_2] under drought stress stimulated significantly the accumulation of glucose and fructose in basal leaves of wheat.

On the other hand, at soft dough stage, the concentration of these soluble sugars had decreased in all treatments except under elevated [CO_2]. The results showed also that the rates of glucose and fructose reduction between anthesis and soft dough stages were similar under drought as under control treatment. Sucrose concentration was higher under limited water conditions when compared to full irrigated conditions at anthesis stage. The increase in [CO_2] seemed to have larger effect when it was applied under drought stress where sucrose concentration had increased significantly while no effect was detected under irrigated treatment. Similarly to what was observed in flag leaves, sucrose concentration had declined significantly under drought conditions at soft dough stage (Figure 4). Nevertheless, basal leaves maintained similar accumulation of sucrose at both phenologic stages under well-watered treatments. Results presented in Figure 4 also showed that starch concentration was high in basal leaves of plants full irrigated, and the growth at [CO_2] stimulated starch accumulation in basal leaves at anthesis. Contrarily to flag leaves, starch concentration increased at soft dough stage in basal leaves compared to anthesis. When comparing among treatments, the obtained results indicated that drought stress did not affect starch concentration at basal leaves when compared to the control, while high [CO_2] impacted starch concentration, which was significantly increased under both water regimes.

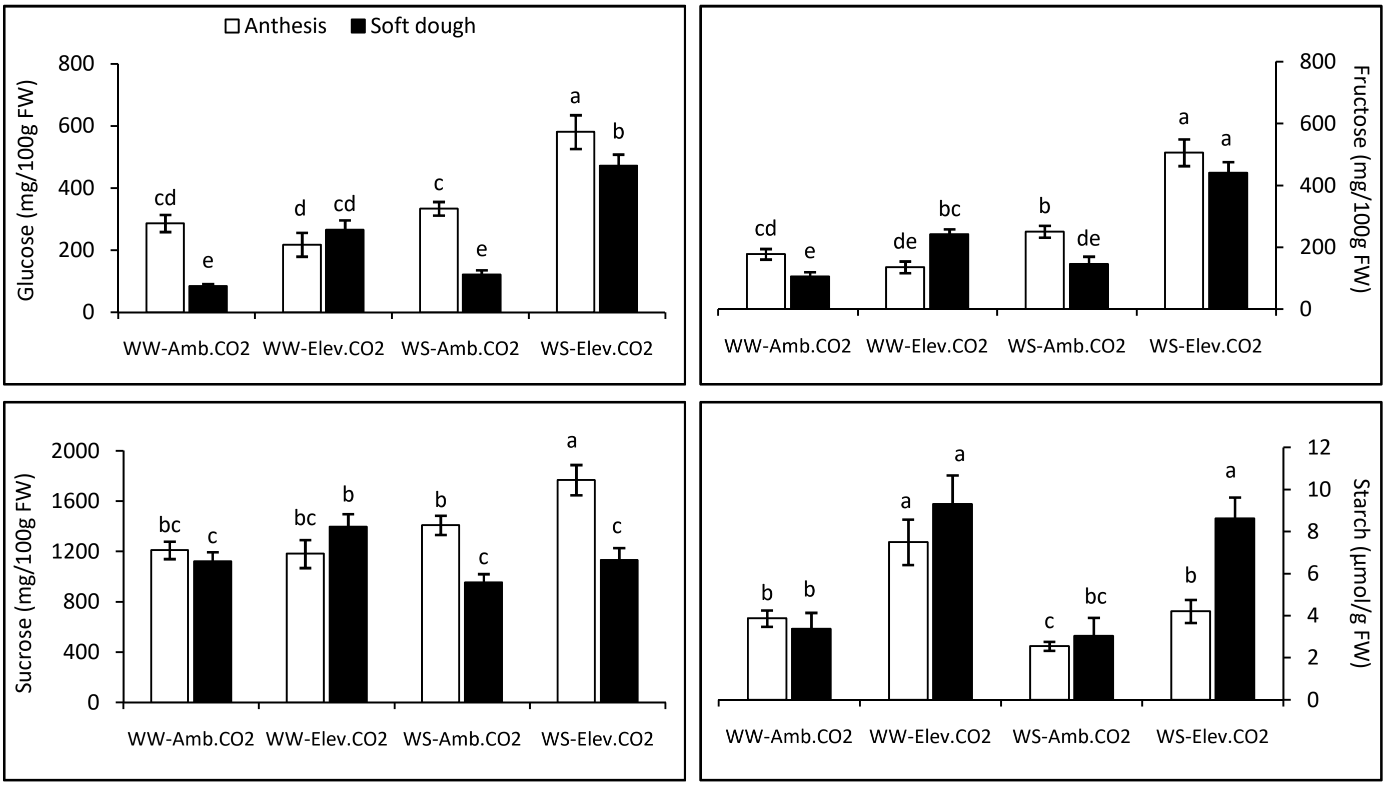


Figure 4: Non-structural carbohydrate concentrations in **durum wheat basal leaves** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and phenotypic stages at $p<0.05$ as determined by LSD test.

- ***Flag leaf***

At anthesis, in case of durum wheat flag leaves, glucose and fructose concentrations were higher under limited water conditions than under well-watered treatment (Figure 5). The increasing [CO_2] did not show significant effect on these monosaccharide concentrations in flag leaves under full irrigation conditions as well as under drought stress. At soft dough stage, glucose and fructose concentrations decreased significantly under drought stress and the highest rate of reduction was recorded under drought and ambient [CO_2]. Irrigated plants submitted to elevated [CO_2] did not show variation in glucose and fructose concentrations between anthesis and soft dough stages. Glucose concentration in flag leaf decreased significantly under full irrigation treatment, while no significant change had been detected regarding fructose concentration. Sucrose concentration in wheat flag leaf had been also increased under moderate drought stress conditions at anthesis when compared to well-watered treatment (Figure 5). However, sucrose concentration tended to decline under limited irrigation at soft dough stage while itremained stable or increased in flag leaves of irrigated plants. High [CO_2] did not affect significantly sucrose concentration when plants reached soft dough stage. Results presented in Figure 5 showed that starch concentration is very low in flag leaves when compared to soluble sugar concentration. Irrigated plants showed higher starch concentration than plants subjected to drought stress. In plants grown at high [CO_2], flag leaf starch concentration increased significantly under both irrigation treatments. At soft dough stage, high [CO_2] effect was similar to the one described at anthesis stage. However, no significant differences were found between well irrigated and drought conditions. The rates of decrease in starch concentration after anthesis (Figure 5) were higher under irrigated treatments than under drought stress (46%, 39%, 14%, and 28% under control, high [CO_2], drought stress and drought stress with high [CO_2], respectively).

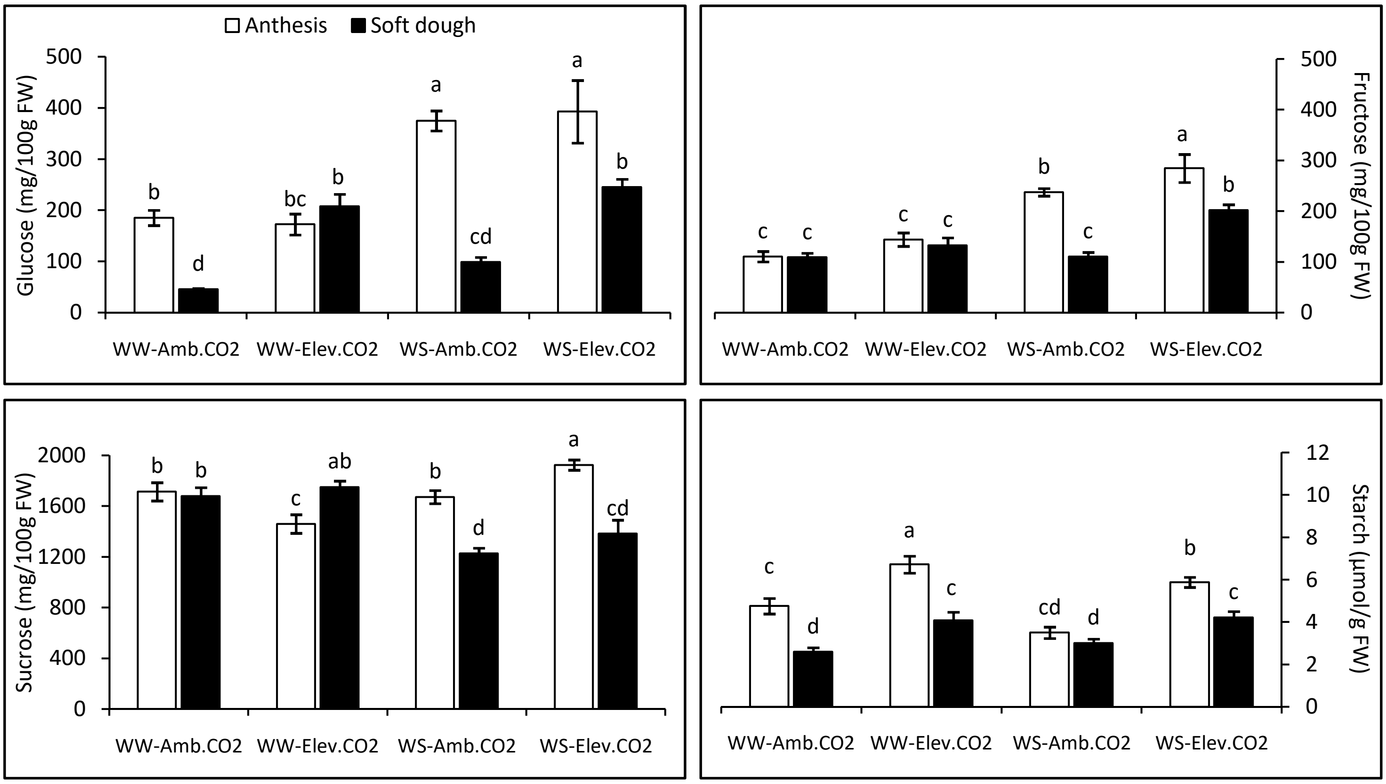


Figure 5: Non-structural carbohydrate concentrations in **durum wheat flag leaves** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and

- ***Glumes***

Similarly as flag and basal leaves, glucose, and fructose concentrations were higher under limited water conditions at anthesis (Figure 6). The increase in [CO_2] seemed to have no significant effect on the concentrations of these sugars when it was applied under both water regimes. When plants reached soft dough stage, glucose and fructose concentrations were decreased under all treatments except under high [CO_2]. It should be indicated that under drought stress conditions the rate of decrease is larger than under control for both soluble sugars. Sucrose concentration was not significantly influenced by the different treatments neither was changed between anthesis and soft dough stages (p= 0.10). Regarding starch concentration, no significant effect was detected under drought stress when comparing to control.

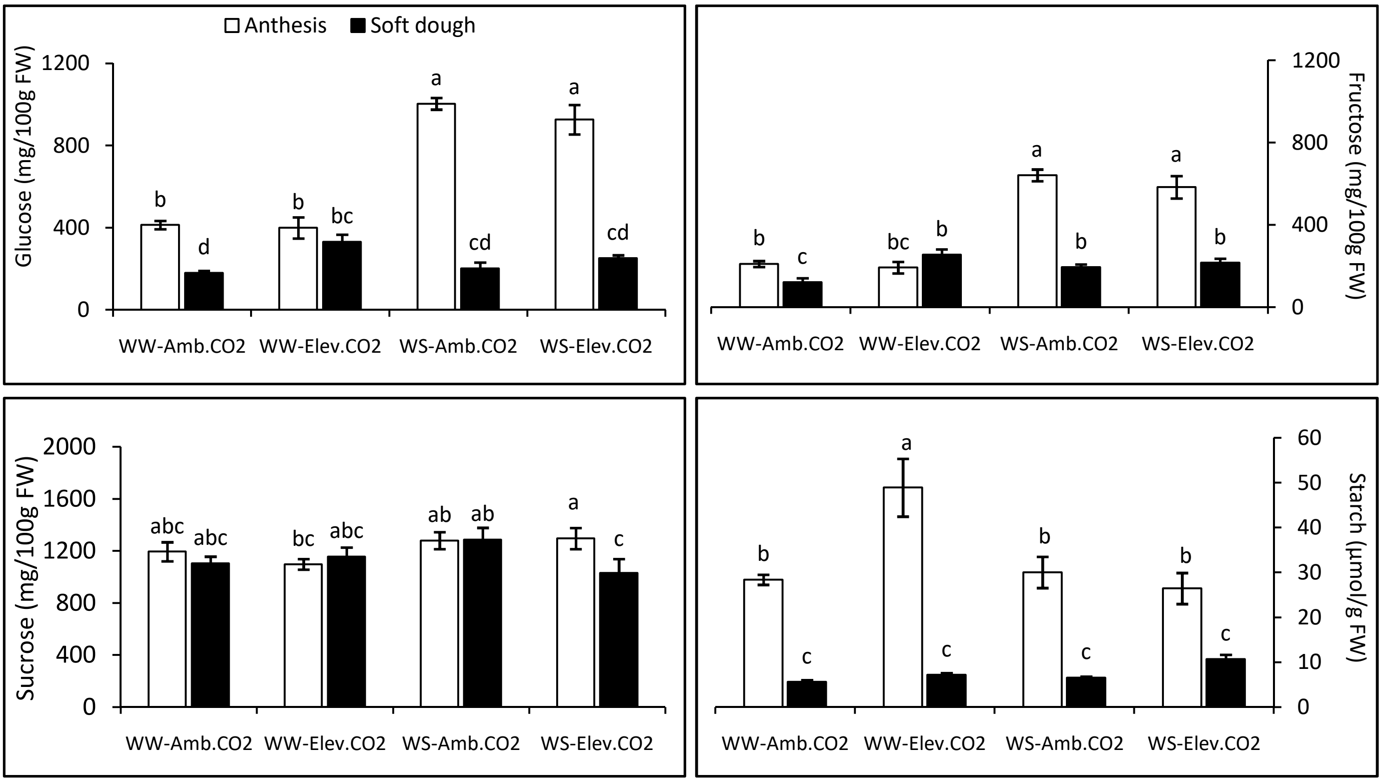


Figure 6: Non-structural carbohydrate concentrations in **durum wheat glumes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and phenotypic stages at p<0.05 as determined by LSD test.

The increase in air [CO_2] significantly increased starch concentration in glumes only under full irrigation treatment. At soft dough stage, starch concentration was significantly reduced under all treatments with no significance detected among them. It should be mentioned that the decrease in starch concentration after anthesis was higher in glumes than in flag leaves, and the reduction was largely higher under irrigated treatments than under drought stress (80%, 85%, 78%, 59% under control, high [CO_2], drought stress and drought stress with high [CO_2], respectively).

2. Nitrogenous metabolites in wheat leaves and ears at anthesis and soft dough stages under drought and high [CO_2] conditions

2.1 Total soluble protein and Rubisco large subunit concentrations

- ***Basal leaf***

The results presented in Figure 7 showed that, at anthesis, no significant effect was detected on total soluble protein concentration among the different treatments. At soft dough stage, a significant increase was detected in plants grown at high [CO_2] and 100% irrigation. However, the opposite effect was observed under drought conditions in which soluble protein concentration was reduced by 51% relative to control. Our study showed that the highest decline (76%) in total protein concentration between anthesis and soft dough stages was recorded under high [CO_2] and limited water irrigation conditions. Furthermore, Rubisco large subunit concentration in basal leaves was lower than that in flag leaves at anthesis stage (Figure 7). Drought stress also reduced (by 18%) significantly Rubisco concentration. On the other hand, under elevated [CO_2], Rubisco large subunit concentration significantly decreased by 23% and 54% (relative to control) under full irrigation and limited irrigation regime, respectively. At soft dough stage, Rubisco concentration decreased under all treatments and recorded a decrease by 59%, 50%, 62%, and 66% under control, high [CO_2], drought stress and drought stress with high [CO_2], respectively. These results indicated that the increasing [CO_2] under drought stress stimulated the degradation and remobilization of proteins from basal leaves when compared to non-stressed conditions.

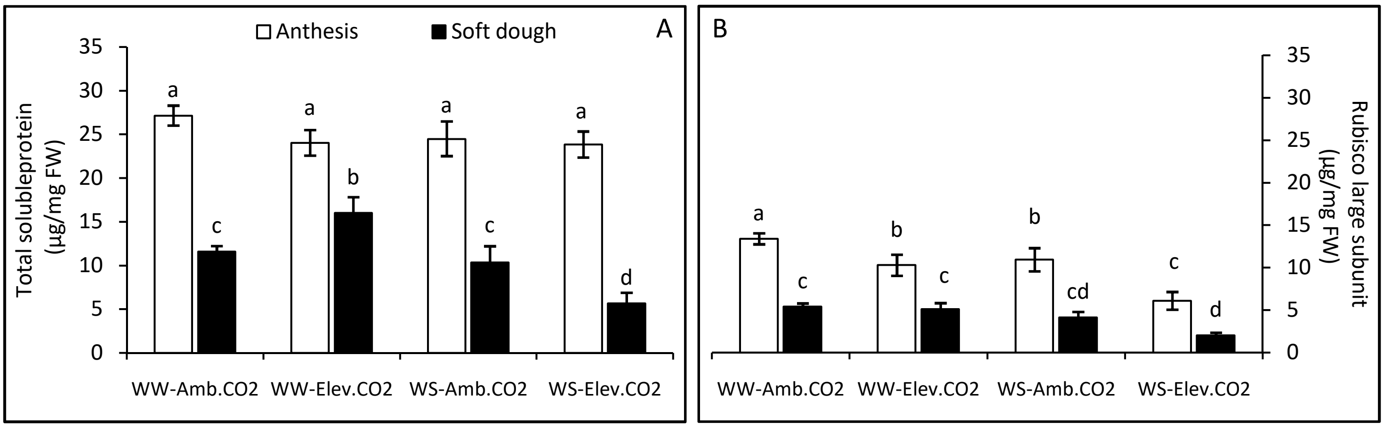


Figure 7: Total soluble protein concentration (A) and **Rubisco large subunit concentration** (B) in **durum wheat basal leaves** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and phenotypic stages at $p<0.05$ as determined by LSD test.

- ***Flag leaf***

Total soluble protein concentration in flag leaves of full-irrigated wheat plants was higher than under drought stress at anthesis and soft dough stages (Figure 8). Increasing [CO_2] did not affect soluble protein concentration under well-watered treatment. On the other hand, regardless of [CO_2], drought stress reduced total protein concentration in flag leaves. At soft dough stage, total soluble protein concentration was decreased by 66% and 78% in ambient and high [CO_2] treatments respectively. These results suggest that limited water availability induced higher protein remobilization from flag leaves than under non-stressed conditions. Rubisco large subunit determination (Figure 8) showed that drought stress induced significant reduction in Rubisco concentration in flag leaves when compared to the control ambient [CO_2]. On the other hand, raising [CO_2] did not significantly affect flag leaf Rubisco concentration under both water treatments. At soft dough stage, Rubisco concentration had been reduced significantly under all treatments and the highest rate was found under drought treatments by an average of 79% since anthesis, while 72% was recorded under control. These results suggested that drought stress might accelerate the degradation of Rubisco protein in wheat flag leaves.

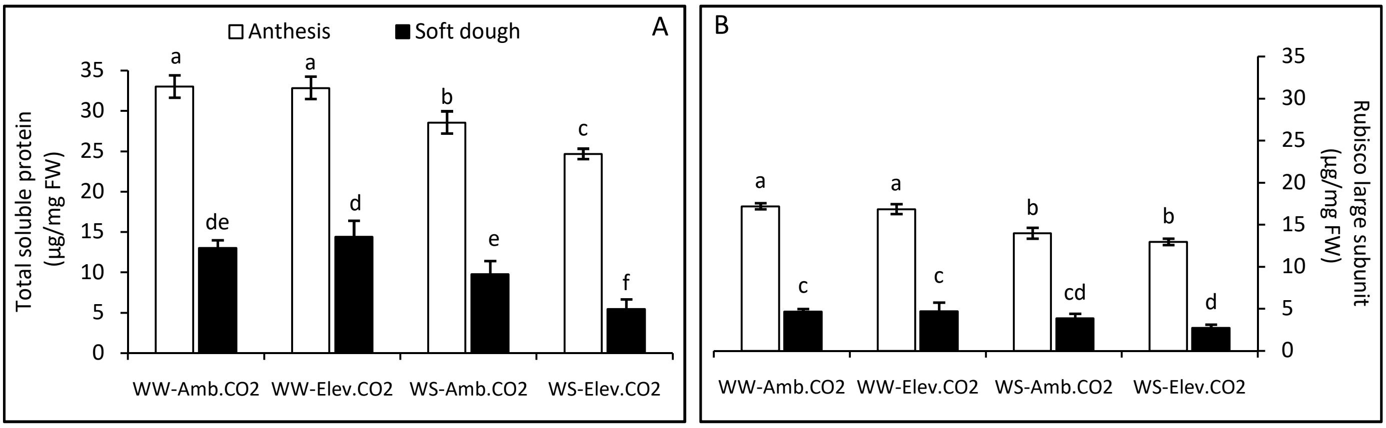


Figure 8: Total soluble protein concentration (A) and **Rubisco large subunit concentration** (B) in **durum wheat flag leaves** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and phenotypic stages at $p<0.05$ as determined by LSD test.

- ***Glumes***

Our analyses showed that total soluble protein concentration in wheat glumes was higher under moderate water deficiency than under full irrigation conditions at anthesis (Figure 9). This concentration tended to decrease significantly at soft dough stage and no significant difference was detected among treatments. Rubisco concentration was not affected by high [CO_2] or by drought stress at anthesis (Figure 9). However, when plants reached soft dough stage, Rubisco large subunit concentration in glumes had significantly been reduced and no significant difference was detected among the treatments.

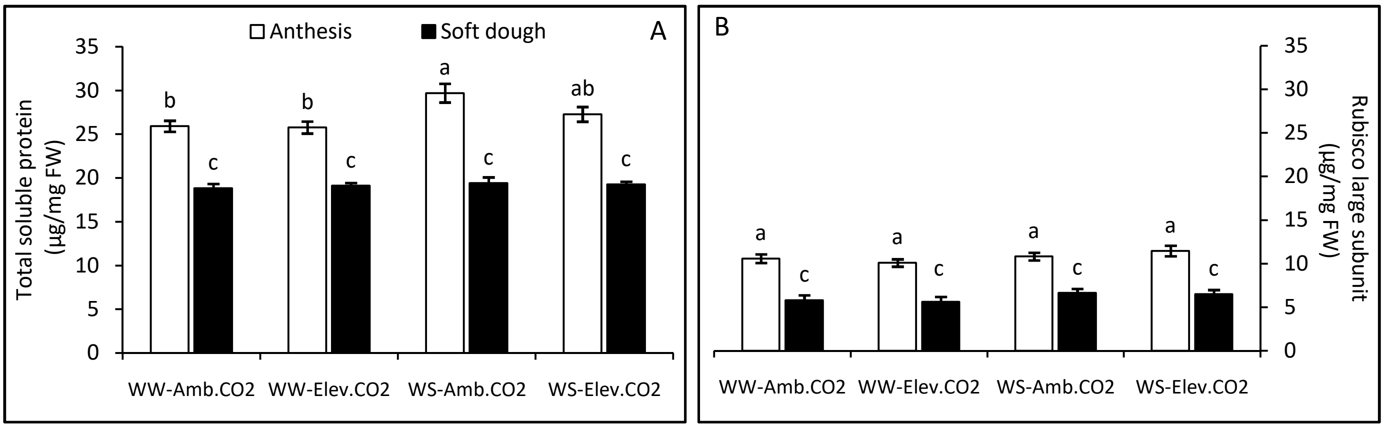


Figure 9: Total soluble protein concentration (A) and **Rubisco large subunit concentration** (B) in **durum wheat glumes** grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and phenotypic stages at $p<0.05$ as determined by LSD test.

2.2 Total amino acids concentration

- ***Basal leaf***

As reported in flag leaves, total amino acids concentration was higher under drought stress treatments, but, elevated [CO_2] negatively affected total amino acids at anthesis under both water regimes (Figure 10). At soft dough stage, basal leaves accumulated amino acids and the total concentration has significantly increased under all treatments and the largest increase was observed under drought stress with high [CO_2] (Figure 10).

- ***Flag leaf***

Total amino acids concentration in wheat flag leaves was evaluated under different conditions of CO_2 and water regimes and results are presented in figure 10. At anthesis, significant differences were observed among treatments. In general terms, compared to fully irrigated plants, drought stress significantly increased total amino acids when compared to irrigated conditions. At soft dough stage, total amino acids concentration remained stable after anthesis under all treatments except for drought stress with high [CO_2] where total amino acids concentration decreased significantly. The elevated [CO_2] showed negative effect on total amino acids concentration under both water regimes and phenological stages.

- ***Glumes***

Total amino acids concentration in wheat glumes was increased under limited water conditions at anthesis and soft dough stages (Figure 10). The increase of [CO_2] did not affect significantly total amino acids concentration under full irrigation treatment when compared to the control. However, when applied with limited water conditions, high [CO_2] increased significantly total amino acids concentration in wheat glumes at both phenological stages. It should be mentioned also that total amino acids concentration had not been significantly changed between anthesis and soft dough stages.

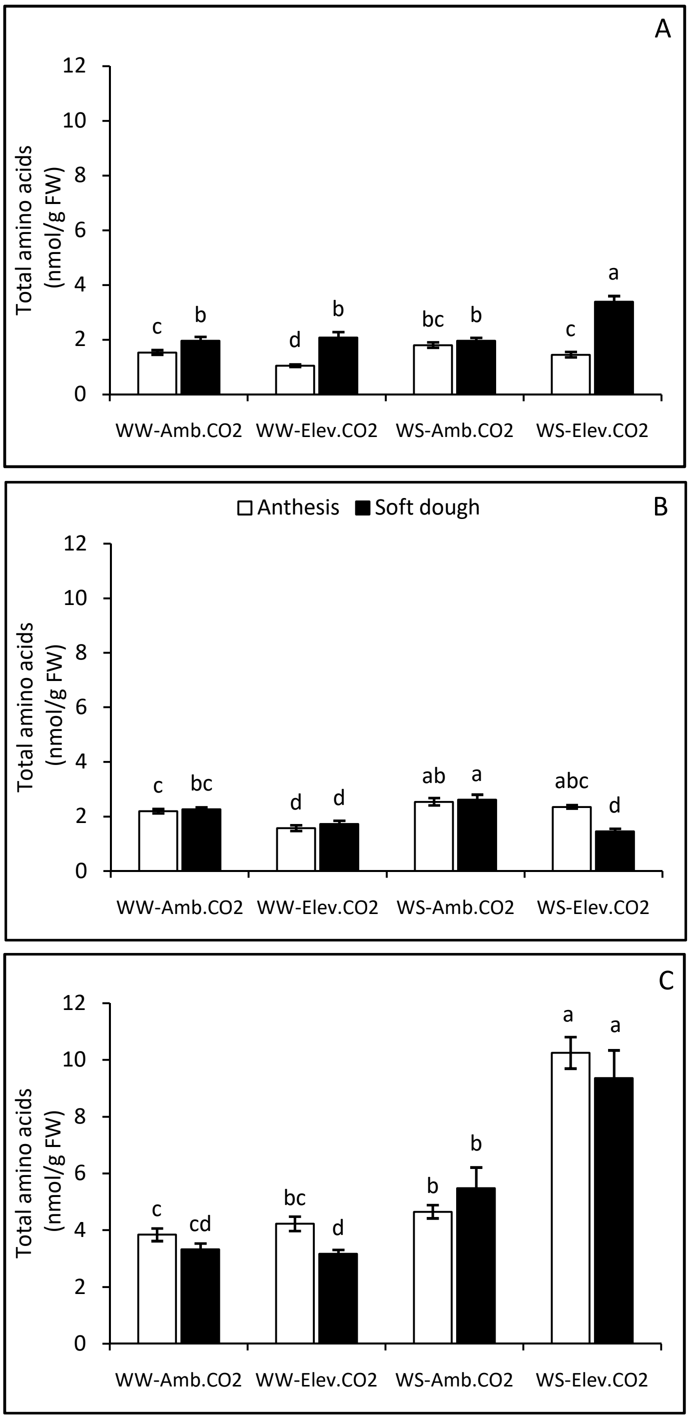


Figure 10: Total amino acids concentration in **basal leaves** (A), **flag leaves** (B), and **glumes** (C) of wheat grown under four different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12). Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and phenotypic stages at $p<0.05$ as determined by LSD test.

2.3 Amino acid profile

Amino acid determinations carried out in leaves and ears highlighted relevant alterations linked to [CO_2], water availability and phenology of durum wheat and tritordeum plants.

- ***Basal leaf***

At anthesis, the concentrations of aspartic acid, glutamic acid, serine, alanine, and proline decreased significantly in basal leaves of plants grown under higher [CO_2], while no effect was found in glycine, glutamine, and GABA (γ-aminobutyric acid) concentrations (Table 12). On the other side, drought stress showed a different pattern in basal leaf amino acids composition characterized by significant increase of glycine, GABA, and proline whereas aspartic acid and glutamic acid concentrations had been significantly reduced but at a lesser extent than that under high [CO_2]. The interaction between drought and elevated [CO_2] resulted mainly in decreasing aspartic acid, glutamic acid, and serine concentrations, and stimulating GABA accumulation in basal leaves. The other amino acids were not significantly affected comparing to the control. At soft dough stage, most of the amino acids concentrations did not significantly vary since anthesis under CO_2 enrichment treatment. Only glycine, alanine, and proline concentrations had been increased. It is worthy to mention that in general, amino acids concentrations were lower under high [CO_2] than under control as remarked at anthesis. Same trend was observed under drought stress as at anthesis, and the results showed that glutamine, alanine, GABA, and proline concentrations had been significantly increased since anthesis, while the rest of amino acids tended to decrease. The interaction between drought and high [CO_2] showed significant effect only on decreasing glutamine and increasing alanine concentrations when compared to the control. Adding to that, the comparison between anthesis and soft dough stages revealed that only glycine and alanine concentrations had been significantly increased, whereas no significant variation was found for the rest of amino acids.

- ***Flag leaf***

Among the analysed amino acids presented in Table 12, aspartic acid, glutamic acid, serine, alanine, and proline concentrations had been significantly decreased in well-watered plants under high [CO_2] at both anthesis and soft dough stages. Aside from proline, drought stress had also led to a decrease in these amino acids but at a lesser extent than high [CO_2]. However, glycine and GABA (γ-aminobutyric acid) concentrations had been increased not only by drought but also by increasing atmospheric [CO_2], with a larger increase recorded under drought stress. At anthesis, the interaction between drought and high [CO_2] caused significant increase in glycine and GABA concentrations when compared to the control. Nevertherless, the concentrations of aspartic acid, glutamic acid, serine, and alanine had decreased significantly while no significant decrease was found regarding glutamine and proline. It should be noted that, compared to control, the observed decrease in amino acids concentrations under elevated [CO_2] was largely higher than the reduction observed under combined effect with drought suggesting that the effect of increasing [CO_2] was more remarkable under well-watered treatment that under drought. At soft dough stage, the concentrations of all the indicated amino acids had been decreased under drought stress combined with high [CO_2] and showed the lowest values comparing to the other treatments. Therefore, a high remobilization of these amino acids to sink organs could be suggested.

- ***Glumes***

Oppositly to basal and flag leaves and aside from GABA, the increase in [CO_2] did not affect significantly amino acids concentrations in glumes at anthesis when compared to control, as it is presented in Table 12. At soft dough stage, the concentrations of all amino acids tended to decrease comparing to anthesis except proline that showed a significant increase. Drought did not have a significant effect on most of amino acid concentrations at anthesis when compared to the control. However, alanine concentration had been significantly decreased while GABA had been accumulated in wheat glumes. Generally, drought had enhanced the accumulation of all amino acids in glumes at soft dough stage. The combination between drought and high [CO_2] increased significantly all amino acid concentrations that reached the highest values comparing to other treatments at both phenological stages (Table 12). From anthesis to soft dough stage, significant decrease was observed in glutamic acid, serine, glycine, alanine, and proline concentrations indicating higher remobilization of these amino acids towards grains.

Table 12: Amino acid concentrations (nmol/g DW) in **wheat leaves** and **glumes** under different treatments (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2) at anthesis and soft dough stages. The values represent the grand mean of the four durum wheat genotypes (G3, G9, G6, and G18). Means ±standard error (n=12)**.** Statistical analysis was made by a one-way ANOVA. Different letters mean significant difference among treatments and stages at p<0.05 as determined by LSD test. Values in bold indicate significance (p<0.05).

Samples	**Treatments**	**Aspartic acid**	**Glutamic acid**	**Serine**	**Glycine**	**Glutamine**	**Alanine**	**GABA**	**Proline**
Basal leaf	**Anthesis**								
	WW-Amb.CO_2	0.094^{a} ±0.004	0.166^{a} ±0.009	0.079^{a} ±0.009	0.030^{d} ±0.002	0.064^{c} ±0.003	0.687^{c} ±0.051	0.067^{c} ±0.005	0.042^{c} ±0.004
	WW-Elev.CO_2	0.062^{d} ±0.002	0.084^{d} ±0.005	0.042^{d} ±0.001	0.030^{d} ±0.001	0.060^{c} ±0.003	0.392^{d} ±0.014	0.063^{c} ±0.002	0.012^{d} ±0.001
	WS-Amb.CO_2	0.079^{b} ±0.004	0.114^{bc} ±0.007	0.078^{a} ±0.007	0.050^{a} ±0.004	0.077^{bc} ±0.004	0.583^{c} ±0.045	0.157^{b} ±0.016	0.125^{a} ±0.008
	WS-Elev.CO_2	0.067^{cd} ±0.003	0.086^{d} ±0.009	0.044^{d} ±0.002	0.034^{cd} ±0.002	0.076^{bc} ±0.009	0.601^{c} ±0.057	0.111^{b} ±0.028	0.033^{c} ±0.003
	Soft dough								
	WW-Amb.CO_2	0.085^{ab} ±0.008	0.138^{b} ±0.015	0.059^{bc} ±0.004	0.039^{bcd} ±0.003	0.096^{b} ±0.008	0.998^{b} ±0.074	0.058^{c} ±0.005	0.050^{c} ±0.008
	WW-Elev.CO_2	0.055^{d} ±0.003	0.083^{d} ±0.008	0.045^{cd} ±0.004	0.048^{ab} ±0.006	0.068^{c} ±0.005	1.202^{b} ±0.174	0.045^{c} ±0.002	0.031^{c} ±0.004
	WS-Amb.CO_2	0.066^{cd} ±0.004	0.094^{cd} ±0.009	0.065^{b} ±0.002	0.043^{abc} ±0.003	0.170^{a} ±0.003	0.844^{b} ±0.059	0.186^{a} ±0.003	0.084^{b} ±0.004
	WS-Elev.CO_2	0.075^{bc} ±0.004	0.099^{cd} ±0.007	0.050^{cd} ±0.003	0.048^{ab} ±0.006	0.069^{c} ±0.017	1.844^{a} ±0.110	0.077^{bc} ±0.027	0.044^{c} ±0.013
	p-values	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table 12: (Continued).

Flag leaf	**Anthesis**								
	WW-Amb.CO$_2$	0.129^{a} ±0.006	0.206^{a} ±0.009	0.119^{a} ±0.010	0.027^{d} ±0.001	0.110^{bc} ±0.006	1.077^{a} ±0.054	0.074^{c} ±0.004	0.087^{bc} ±0.008
	WW-Elev.CO$_2$	0.079^{cd} ±0.006	0.084^{d} ±0.004	0.052^{de} ±0.004	0.033^{cd} ±0.001	0.077^{c} ±0.005	0.705^{c} ±0.060	0.181^{b} ±0.019	0.025^{e} ±0.003
	WS-Amb.CO$_2$	0.104^{b} ±0.006	0.133^{bc} ±0.010	0.097^{b} ±0.007	0.042^{b} ±0.002	0.102^{bc} ±0.008	1.040^{ab} ±0.053	0.308^{a} ±0.024	0.196^{a} ±0.024
	WS-Elev.CO$_2$	0.096^{b} ±0.005	0.123^{c} ±0.009	0.072^{c} ±0.004	0.037^{bc} ±0.001	0.101^{bc} ±0.012	0.900^{b} ±0.048	0.295^{a} ±0.023	0.060^{cd} ±0.006
	Soft dough								
	WW-Amb.CO$_2$	0.103^{b} ±0.005	0.155^{b} ±0.010	0.066^{cd} ±0.003	0.034^{c} ±0.002	0.195^{a} ±0.030	1.011^{ab} ±0.050	0.075^{c} ±0.006	0.077^{bc} ±0.010
	WW-Elev.CO$_2$	0.089^{bc} ±0.007	0.093^{d} ±0.007	0.050^{e} ±0.003	0.035^{c} ±0.002	0.138^{b} ±0.022	0.655^{c} ±0.069	0.085^{c} ±0.008	0.041^{de} ±0.006
	WS-Amb.CO$_2$	0.090^{bc} ±0.005	0.146^{b} ±0.008	0.076^{c} ±0.007	0.055^{a} ±0.005	0.210^{a} ±0.016	1.053^{ab} ±0.087	0.154^{b} ±0.013	0.093^{b} ±0.012
	WS-Elev.CO$_2$	0.068^{d} ±0.003	0.077^{d} ±0.004	0.055^{e} ±0.002	0.036^{c} ±0.002	0.080^{c} ±0.006	0.607^{c} ±0.042	0.103^{c} ±0.008	0.051^{de} ±0.005
	p-values	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table 12: (Continued).

Glumes	**Anthesis**								
	WW-Amb.CO_2	0.111^{cd} ±0.005	0.213^{bc} ±0.009	0.122^{c} ±0.009	0.061^{bc} ±0.012	0.317^{c} ±0.025	2.007^{b} ±0.255	0.059^{d} ±0.003	0.168^{d} ±0.010
	WW-Elev.CO_2	0.100^{cd} ±0.005	0.203^{bc} ±0.012	0.117^{c} ±0.010	0.066^{ab} ±0.011	0.262^{c} ±0.031	2.314^{b} ±0.251	0.094^{c} ±0.007	0.135^{d} ±0.006
	WS-Amb.CO_2	0.085^{d} ±0.004	0.140^{c} ±0.014	0.086^{c} ±0.008	0.045^{bcd} ±0.004	0.213^{c} ±0.021	1.212^{c} ±0.135	0.111^{c} ±0.005	0.169^{d} ±0.015
	WS-Elev.CO_2	0.276^{a} ±0.013	0.477^{a} ±0.057	0.323^{a} ±0.028	0.088^{a} ±0.014	0.834^{a} ±0.066	5.998^{a} ±0.613	0.718^{a} ±0.063	0.886^{a} ±0.133
	Soft dough								
	WW-Amb.CO_2	0.139^{c} ±0.013	0.134^{c} ±0.008	0.096^{c} ±0.006	0.037^{cd} ±0.003	0.344^{bc} ±0.019	0.975^{d} ±0.043	0.068^{d} ±0.004	0.404^{b} ±0.077
	WW-Elev.CO_2	0.089^{cd} ±0.006	0.051^{d} ±0.004	0.093^{c} ±0.009	0.033^{d} ±0.003	0.304^{c} ±0.050	0.774^{d} ±0.017	0.143^{c} ±0.018	0.281^{c} ±0.038
	WS-Amb.CO_2	0.223^{b} ±0.038	0.215^{bc} ±0.035	0.177^{b} ±0.025	0.050^{bcd} ±0.003	0.512^{b} ±0.031	1.680^{c} ±0.341	0.327^{b} ±0.042	0.565^{b} ±0.047
	WS-Elev.CO_2	0.258^{ab} ±0.039	0.274^{b} ±0.021	0.197^{b} ±0.014	0.056^{bcd} ±0.006	0.823^{a} ±0.091	2.802^{b} ±0.217	0.809^{a} ±0.044	0.511^{b} ±0.087
	p-values	**<0.001**	**<0.001**	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

DISCUSSION

I. Effect of CO_2 enrichment and drought stress on grain yield components of wheat and tritordeum

- **Grain yield, thousand-grain weight (TGW), and nitrogen use efficiency (NUE)**

In the present study, grain yields of wheat and tritordeum were increased under elevated air CO_2 concentration as previously reported in many studies (Wu et al., 2004; Ainsworth and Long, 2005; Högy et al., 2009a; Cai et al., 2016; Ben Mariem et al., 2020a). However, drought stress applied during flowering period had drastic negative effect on cereal grain yield. These results are in agreement with previous studies carried out under ambient [CO_2] conditions (Kiliç and Yagbasanlar, 2010; Gevrek and Atasoy, 2012; Méndez-Espinoza et al., 2019; Pour-Aboughadareh et al., 2020). The current study also showed that thousand-grain weight (TGW) of wheat and tritordeum were not affected by these two environmental factors while significant differences were associated with genotypic variability. Similar results were found by Högy et al. (2009a) in bread wheat. Additionally, Wu et al. (2004) found a slight increase in thousand-grain weight of wheat under high [CO_2] with a larger effect on the number of grains produced per plant, thus, it has been concluded that the response of wheat yield to CO_2 enrichment has been attributed to an increase in grain number. Adding to that, number of ears and above-ground biomass weight results found in this experiment (Table S3) were increased under high [CO_2], which could contribute to explain the increase of grain yield. In agreement with Bunce (2017), our study showed that both, increases in the number of seed heads per m^2 and in the number of seeds per head, could be key parameters in yield increases at elevated [CO_2] in wheat. Similarly to elevated [CO_2], the stability observed in TGW under drought stress may also explain that the observed decrease in grain yield of wheat and tritordeum might not be due to smaller grain size, but it is more related to a reduction in number of grains produced. Arguello et al. (2016) and Mathew et al. (2018) reported that thousand-kernel weight presents in general high heritability and is less affected by the water deficit. Nevertheless, it has been reported in other studies that drought stress negatively affected TGW of wheat (Gevrek and Atasoy, 2012; Pour-Aboughadareh et al. (2020). Villegas et al. (2010) claimed that wheat and tritordeum kernel weights were low under drought conditions, thus, resulting in low yield. They reported also that tritordeum kernel weight was lower than that of wheat. This finding is matching with our results. This difference is attributed to short grain-filling period due to a delay in anthesis date (Villegas et al., 2010). The authors affirmed also that, when drought occurred at the first part of the cycle, grain yield was mostly explained by the number of grains produced per spike rather than kernel weight. Similar results were found by Senapati et al. (2018), who reported that in wheat, the number of fertile grains setting was reduced by drought stress during reproductive development.

The interaction between high [CO_2] and water scarcity improved the negative effect caused by drought stress (as a single limiting factor) on grain yield. It seems that CO_2 enrichment could alleviate the negative effect of water deficit on plants. It has been reported by Wu et al. (2004) and Medina et al. (2016) that the interactive effects of increasing [CO_2] and drought stress depended on the genotype and the severity of water stress. Nitrogen use efficiency (NUE) has been influenced by changing [CO_2] concentration and water availability conditions. CO_2 enrichment and moderate drought stress have increased NUE and similar results were found in Li et al. (2003) and Dier et al. (2019) in bread wheat grown under high [CO_2]. However, it has been reported by Hoseinlou et al. (2013) and Szilvia et al. (2018) that NUE was decreased under severe drought stress in spring barley and wheat, respectively. This may indicate that NUE is dependent on the intensity of water stress. Nitrogen use efficiency is also determined genetically (Hawkesford and Riche, 2020). Actually, NUE seems to be stable between wheat and tritrodeum under irrigated treatments, but when drought stress was applied, wheat increased the efficiency in using nitrogen, resulting in higher grain yield under limited water environments. Nitrogen use efficiency of wheat was more affected by drought stress, whereas tritordeum showed more sensitivity to increasing [CO_2]. Adding to that, the ranking of genotypes with high and low NUE under different nitrogen fertilization levels had been changed in the current study. In fact, and similarly to genotype G6, durum wheat genotypes G3 and G9 showed higher efficieny in nitrogen use under high [CO_2] and drought conditions than genotype G18 (selected as genotype with high NUE from a previous experiment). Concerning tritordeum, low NUE genotypes G24 and G25 exhibited also in this experiment low NUE under high [CO_2] and drought comparing to other genotypes. However, genotype G22 showed high NUE similar to G21 and G23 that maintained suitable efficiency in nitrogen use under such conditions.

II. Effect of CO_2 enrichment and drought stress on grain quality of wheat and tritordeum

➢ Nitrogen metabolism

- **Sink organs: Grains**

In both species, the increase in atmospheric [CO_2] tended to reduce N concentration in mature grains, but, it should be mentioned that significant effect was only observed in tritordeum, although other studies reported significant effect in wheat (Fangmeir et al., 1999; Wu et al., 2004; Verillo et al., 2017; Soba et al., 2019). Several hypotheses have been suggested to explain this reduction: (i) a dilution effect due to an excess accumulation of carbon (mainly as carbohydrates) leading to reduced grain N concentration; (ii) a decrease in N-uptake rate; (iii) inhibition of N assimilation under elevated [CO_2] (Taub and Wang, 2008; Bloom et al., 2010). Drought has also shown similar effect on grain N concentration, as CO_2 enrichment, in wheat genotypes while insignificant decrease was observed in tritordeum lines. This could be explained by a dilution effect due to high wheat grain yield than tritordeum under drought stress. The interaction between water deficit and high [CO_2] did not improve nitrogen concentration in wheat and tritordeum grains. On the other hand, total free amino acids concentration in grains was strongly increased by drought stress and reduced by increasing [CO_2]. This is in agreement with many previous studies in wheat (Högy et al., 2009b; Soba et al., 2019; Tcherkez et al., 2020). Besides quantity, grain amino acids composition is affected by environmental factors. As gluten proteins are rich in glutamine and proline (Högy et al., 2009b), their concentrations were significantly decreased under high [CO_2]. Glutamic acid (an important amino acid involved in the biosynthesis of proteins) showed also the same tendency. This could explain the decrease in gluten concentration in grains due to increasing [CO_2] reported in many previous studies. Other studies reported a decrease in specific amino acids such as glutamine, lysine, arginine, and proline (DaMatta et al., 2010) or a general decrease in both essential and non-essential amino acids (Högy et al., 2010). Similarly, our study revealed a decrease in glutamic acid, glutamine, and proline concentrations in durum wheat and tritordeum grains under high [CO_2].

- **Source organs: Leaves and glumes**

The variations of several amino acids highlight a relationship among N translocation compounds-N storage and [CO_2] (Arp et al., 1998). It has been reported that, during grain filling, the assimilates are provided by photosynthesis in the leaves (Evans et al., 1975) and spikes (Tambussi et al., 2007; Maydup et al., 2010), and by the redistribution of reserves stored in vegetative tissues during the pre- and/or post-anthesis periods, which are translocated to the growing grains (Schnyder, 1993; Zhang et al., 2010). In wheat, Gaju et al. (2014) reported that leaf and stem are the most important nitrogen sources of N remobilization, contributing about 75% of the N originating from N remobilization. Depending on environmental conditions, 40% to 90% of the grain nitrogen originates from N remobilization and thus 10% to 60% from post-anthesis N uptake (Kong et al., 2016). The mechanism by which elevated [CO_2] decreases grain nitrogen concentration is still elusive, but results obtained regarding total protein and Rubisco degradation in wheat leaves and ears could explain in some part, the remobilization processes under drought stress and high [CO_2] between anthesis and soft dough toward grain filling. It is widely known that after heading, the ear is responsible for depositing nitrogen, whereas the other parts serve to feed this organ (Lopes et al., 2006). In agreement with this, it is notable according to our results that, at soft dough stage, total soluble protein was accumulated in ear and its concentration exceeded that in leaves. But, it should be mentioned that no effect of drought or increasing [CO_2] was detected on total protein accumulation in ears. However, the contribution to grain filling of flag leaf, which is Rubisco-derived N, was high under drought stress treatment and higher when drought was applied with elevated [CO_2]. It is obvious from the results that CO_2 enrichment did not increase Rubisco concentration in leaves as it is reported by Aranjuelo et al. (2015), who claimed that proteomics conducted on leaves sampled during anthesis in wheat cultivated under high [CO_2] showed that Rubisco content did not increase. Basal leaf contributed also to N remobilization but at lesser extent that flag leaf. Bahrani and Joo (2010) stated that flag leaf plays an important role as a source of nitrogen that is later remobilized to the grain. Therefore, we can suppose that the increase in Rubisco-derived N remobilization in water deficit conditions between anthesis and soft dough stages indicates early leaf senescence and faster assimilates remobilization to grain filling, leading to shortening of grain-filling period (Wardlaw and Willenbrink, 2000; Yang et al., 2002, Saeedipour and Moradi, 2011).

Adding to that, the reallocation of leaf N to grain filling is boosted under water deficit combined with high [CO_2] conditions. However, irrigated plants may have longer grain-filling period thus, the remobilization of nitrogen could be derived from Rubisco degradation and post anthesis N uptake. Consequently, grain nitrogen accumulation is higher under irrigated treatments. On the other hand, it seems that drought stress stimulates the accumulation of nitrogen as amino acids in wheat and tritordeum grains. Along with the lowered protein and Rubisco concentrations during grain filling in wheat leaves under water deficit, concentration of total amino acids has changed. Actually, as a response to drought stress, soluble proteins of flag leaves (and basal leaves) tend to decline during grain filling and water stress accelerates the decline (Saeedipour and Moradi, 2011) causing premature senescence of flag leaf (Rawson et al., 1983). As a consequence, total amino acids concentration increased as a result of protein degradation (Huang and Jander, 2017) to ensure osmotic adjustment and maintain cell turgor (Turner, 2018). Similar results were reported by Gámez et al. (2019) in leaves of quinoa plants grown under drought stress conditions. The accumulation of amino acids in leaves has been reported in different plant species under water deficit (Obata and Fernie, 2012; Hill et al., 2013). Among the amino acids that have been increased under limited water conditions, glycine, glutamine, alanine, GABA, and proline were accumulated in flag leaves and basal leaves. The increase of [CO_2] has also affected amino acid concentrations and composition in leaves and ears. At anthesis, total amino acids concentration decreased in leaves (both flag leaf and basal leaf) and remained constant in ears. The decrease in total amino acids concetration in flag leaves is in agreement with earlier reports in durum wheat (Vicente et al., 2015; Gámez et al., 2020). Due to the degradation of proteins in ear and the high Rubisco-N derived remobilization from leaves, total amino acids concentration in ears largely increased under drought, and more accumulation occurred when high [CO_2] was applied.

- **Carbon metabolism**
  - **Sink organs: Grains**

Our study showed that, in general terms, oppositely to nitrogen, growth under elevated [CO_2] increased carbon concentration in wheat and tritordeum grains regardless of water conditions. However, drought stress under ambient [CO_2] did not affect carbon concentration in grains in comparison to control. Absence of significant differences on starch content, pointed to the fact that the enhancement observed in grain carbon concentration of plants grown at elevated [CO_2] is related to an increase in non-structural carbohydrates such as sucrose. Similar results were reported by Högy et al. (2009a). Therefore, it could be concluded that the increase of carbon concentration in grains is mainly explained by the increase of sucrose rather than starch. Under drought stress conditions, sucrose concentration has decreased significantly in wheat and tritordeum grains, with a larger decrease observed in wheat. Nevertheless, starch concentration was not largely affected by limited irrigation, which could indicate that the decline observed in hexose concentrations (glucose and fructose) were used mostly for starch synthesis. When [CO_2] was applied under water deficit, higher decrease in glucose and fructose concentrations was found in favour of sucrose and starch synthesis. As a result of increasing grain carbon concentration and decreasing nitrogen concentration, C/N ratio has raised in high [CO_2] conditions. Similar results were reported by Verrillo et al. (2017), Soba et al. (2019) and Ben Mariem et al. (2020a) in wheat mature grains.

- **Source organs: Leaves and glumes**

During grain filling in C_3 cereals, flag leaf and ear are believed to play major roles as sources of assimilates, sustaining grain development (Sanchez-Bragado et al., 2014). Our study showed that, hexose concentrations (glucose and fructose) were increased at anthesis due to drought stress at flag leaves, basal leaves and ears. Wall et al. (2006) reported that the accumulation of total non-structural carbohydrates is positively correlated with stomatal aperture in wheat, presumably because osmoregulation that enables wheat leaves to tolerate drought. However, starch concentration in leaves was lower under limited water availability than under well-watered treatment. Similarly, Villadsen et al. (2005) and Damour et al. (2008) found that leaf starch content decreased in response to abiotic stress in barley and lychee tree. In fact, starch is the major carbohydrate storage in plants, and under challenging environmental conditions, as water deficit, plants generally remobilize starch to provide energy and carbon when photosynthesis could be potentially limited (Thalmann and Santelia, 2017). The activation of starch degradation under stress is a common plant response in many species and does contribute to sugar accumulation. The released sugars (with other derive metabolites) support plant growth under stress and function as osmoprotectants to mitigate the negative effect of the stress (Krasensky and Jonak, 2012).

In overall terms, aside from starch, drought stress stimulated the remobilization of glucose, fructose, and sucrose from flag leaves and basal leaves between anthesis and soft dough. Saeedipour and Moradi (2011) affirmed that water deficit can enhance carbon remobilization reserves from vegetative tissues during grain filling. Glucose and fructose concentrations had been also decreased under water deficit conditions indicating an initiation of hexoses remobilization to grains. Actually, the photosynthates of grains are derived from the photosynthetic product before anthesis (Jia et al., 2015) and under drought stress conditions, flag leaves undergo accelerated senescence, which might be due to the enhancement in photosynthate transport to grains (Saeidi et al., 2012). Adding to that, starch showed also significant decrease at soft dough stage in wheat ears, and it should be indicated that glucose and fructose remobilization from ears was largely higher than that of leaves. This could indicate that ears contribution to grain filling was greater in comparison with that of leaves. In fact, Aranjuelo et al. (2011) reported that in durum wheat, only a small amount of the soluble sugars coming from the carbon fixed by the leaf arrived at the ear, and the rest was stored as structural carbon compounds and starch, and then respired. They assumed that the carbon synthesized in the ear was used for grain filling. Adding to that, Evans et al. (1975) claimed that the ear may have a photosynthetic contribution during grain filling and Araus et al. (1993) found that ear photosynthetic products exhibit and obvious increase in the contribution rate to grain under water deficit. Existing studies found that non leaf organs are resistant to water deficit, and present important sources of photosynthetic carbon assimilation under drought condition during grain filling (Sánchez-Díaz et al., 2002; Kong et al.,2010). Under non-limiting water conditions, the remobilization of hexoses in wheat leaves and ears was lower in comparison to that under drought stress between anthesis and soft dough stages. Sucrose did not show a variation between the two stages and starch remobilization was highly increased in flag leaves and ears, with higher contribution observed in ears. Under irrigated treatment, Sanchez-Bragado et al. (2014) found that the contribution of the ear was greater in comparison of that of the shoot and the contribution of flag leaf was minor, with the additional advantage that the structures of the ear are physically closer than the flag leaf to the growing kernels (Evans et al., 1975). Thus, we can conclude that under well-watered conditions the main source of carbon remobilization is starch and the main organ contributing to grain filling is ear.

However, drought stress accelerates carbon remobilization in basal leaf, flag leaf, and ear, inducing higher reallocation of hexoses and sucrose from leaves and stimulates starch degradation in ears. In general, the increasing [CO_2] decreased the remobilization rate of hexoses under water deficit conditions in leaves. Starch degradation in ears was also depressed, but, an opposite effect was observed under well-watered condition. The increase of non-structural carbohydrates under high [CO_2] and full irrigation could indicate that there is photosynthetic assimilation and photosynthate accumulation in leaves and ears, which could be remobilized after soft dough stage.

- **Additional minerals in grains**

Together with C/N compounds, other minerals such as Ca, Fe, K, Mg, and P are very relevant elements from a nutritional perspective. Within this context, our study showed that durum wheat and tritordeum grains of plants grown under elevated [CO_2] were impoverished in K, P, Mg, Ca, Zn, and Fe content. These results are in agreement with many studies on wheat (Fangmeier et al., 1996; Wu et al., 2004; Loladze, 2014; Ben Mariem et al., 2020a). However, Verillo et al. (2017) reported that grain mineral content in winter wheat was not affected by increasing [CO_2] except for a decline in Zn concentration. In a FACE experiment, Högy et al. (2009a) did not find significant effect in grain micro- and macronutrients in wheat, whereas the authors found significant effect on grain minerals in another FACE experiment (Högy et al., 2009b). Högy et al. (2009a) also found significant increases in K and Pb, but Zn and Cd concentrations had largely decreased. These findings suggest both positive and negative implications for the nutritional value of wheat grain under CO_2 enrichment. Tcherkez et al. (2020) found significant reduction only in Mg concentration in wheat grains. According to our results, the negative effect of high [CO_2] seems to be equal under both water conditions. The negative effect of CO_2 enrichment on grain mineral composition could be due to, both, increased carbohydrate accumulation and reduced mass flow (Wu et al., 2004). Since most of the minerals in wheat grains originate from the redistribution from vegetative pools during grain filling, CO_2 enrichment may cause serious alterations in the concentrations of macro- and micro-elements (Hogy et al., 2009a). Our results showed also an increase in molybdenum (Mo) concentration under elevated [CO_2] in wheat and tritordeum grains. Similar result was found by Högy et al. (2009b). Molybdenum was also significantly increased under drought stress. Mo has also been reported to increase in soybean seeds in response to water defitcit (Samarah et al., 2004). Molybdenum is an essential trace element in plants that plays an important role in abiotic stress tolerance in plants (Wu et al., 2014). Wu et al. (2014) studied the effect of Mo application on wheat under drought stress and found an enhancement in relative water content and water use efficiency. Also, osmotic adjustment products such as proline, soluble protein, and soluble sugars were improved by Mo application. The results indicate that the increase of Mo in wheat plants might improve the drought tolerance by enhancing water utilization capability and the ability of osmotic adjustment. Adding to that, Mg, Zn, and Fe were not significantly affected by limited water conditions with a slight increase observed in Fe concentration in wheat grains. On the other side, a negative trend was observed for K, P, and Ca in both species. However, Chang-Xing et al. (2009) found that water deficit benefits mineral accumulation (P, K, Ca, Mg, and Zn) in wheat grain at post-anthesis stage. Significant increase in Zn and Fe concentrations were observed in tritordeum grains. Consequently, in view of these results, the different responses of cereals to environmental conditions might be genotypic dependent, and the continuing increase of atmospheric [CO_2] will have negative implications on nutritional quality of cereals.

CONCLUSIONS

The current study confirmed the fact that [CO_2] and water stress have a great impact on grain yield, quality traits and plant physiological performance of durum wheat and tritordeum plants. Growth under elevated [CO_2] had a positive effect on grain yields of wheat and tritordeum. Such increase was reflected in the enhancement of ground biomass and number of spikes per plant. On the other hand, we also observed the interaction of [CO_2] and water availability. Growth at elevated [CO_2] probed to reduce the deleterious effects of drought on grain yield. Regarding grain nutritional traits, the most striking effect of elevated [CO_2] was a lower N concentration and higher C/N ratio in grains at maturity. It seems that air CO_2 enrichment has larger effect on carbon and nitrogen metabolism in water deficit condition. Under such environments, wheat leaves senescence is accelerated and plant uses, both, proteolysis strategy (Increasing total amino acids concentration) and soluble sugar accumulation, mainly in the form of hexoses. The relative contribution of each organ to grain filling is strongly affected by growing conditions. As a response to water limited availability, early flag leaf senescence occurred and ensured higher reallocation of N derived from Rubisco degradation to the grain in comparison of that of basal leaves and ears. However, wheat ears showed to have a major role in starch degradation and hexoses remobilization during grain filling. Also, water deficit increases the concentration of total free amino acids in leaves but it showed changes in amino acids composition; it decreases the levels of some amino acids (aspartic acid, glutamic acid, and serine), maintains others (glutamine and alanine) and induces the increase of some specific ones (GABA, proline, and glycine). Regarding grain quality, drought stress induces amino acids accumulation and minerals in mature grains of wheat and tritordeum, but the increase in [CO_2] promotes the accumulation of carbon mostly as sucrose and starch, rather than N. On the other hand, tritordeum showed suitable response to CO_2 enrichment under water deficit conditions by ensuring same grain yield as durum wheat with higher N and amino acid concentrations. It showed also higher concentration in grain minerals (K, P, Mg, Zn, and Fe), thus, this species presents a high potential to have a balance between maintaining stable grain yield and grain quality under future climate scenarios characterized by water scarcity and elevated [CO_2].

Overall, the current study elucidated the behaviour of cereal crops grown under changing climate conditions focusing on post-anthesis nutrient remobilization process that occurs in different source organs and its consequent effects on grain compostion and final yield. These informations could be useful for breeders to target the traits that allow the selection of cultivars more adapted to upcoming climate changes.

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SUPPLEMENTARY MATERIAL

Table S1: Pedigree and selection history of **durum wheat (G3, G9, G6, and G18)** and **tritordeum (G21, G22, G23, G24, G25, and G26)** genotypes.

Species	Genotypes	Pedigree
Durum wheat	G3	BYBLOS/7/WID22256/5/ALTAR 84/STINT//SILVER_45/3/GUANAY/4/GREEN_14//YAV_10/… (CDSS12Y00252S-09Y-018M-9Y-0M)
	G9	SOOTY_9/RASCON_37//GUAYACAN INIA/11/BOOMER_33/ZAR/3/BRAK_2/AJAIA_2//… (CDSS12Y00165S-072Y-032M-23Y-0M)
	G6	CBC 509 CHILE/6/ECO/CMH76A.722//BIT/3/ALTAR 84/4/AJAIA_2/5/KJOVE_1/7/AJAIA_12/… (CDSS11B00319T-044Y-028M-39Y-0M)
	G18	MÂALI/6/MUSK_1//ACO89/FNFOOT_2/4/MUSK_4/3/PLATA_3//CREX/ALLA/5/OLUS*2/ILBOR//… (CDSS07Y00784D-2B-07Y-07M-7Y-4B-04Y-0B)
Tritordeum	G21	HT-621
	G22	HT-435
	G23	HT-15-54-32
	G24	HTC-29-10
	G25	BULEL-TRIT
	G26	HT-444-18

Table S2: Data of **nitrogen use efficiency (NUE)** of **durum wheat (G3, G9, G6, and G18)** and **tritordeum (G21, G22, G23, G24, G25, and G26) genotypes** fertilized by two nitrogen levels (High and Low). Means are followed by standard error (n=8). Values in bold indicate high NUE.

Species	**Genotypes**	**NUE (g(grain)/g(N))**	
		High N	Low N
Durum wheat	G3	31.77 ±1.20	39.19 ±1.84
	G9	29.52 ±0.87	36.78 ±1.44
	G6	**34.03 ±0.45**	**47.98 ±1.27**
	G18	**35.70 ±0.92**	**45.55 ±1.56**
Tritordeum	**G21**	**28.53±0.74**	**38.07±0.50**
	G22	27.62 ±0.71	38.96 ±1.29
	G23	**30.27±1.37**	**39.47±1.51**
	G24	27.70 ±1.45	36.83 ±1.43
	G25	28.07 ±0.60	33.85 ±1.26
	G26	**30.16 ±0.31**	**42.06 ±0.57**

Table S3: Data of **above-ground biomass (without ears)** and **ears number averages** of **durum wheat (G3, G9, G6, and G18)** and **tritordeum (G21, G22, G23, G24, G25, and G26) genotypes** grown under different treatmeants (WW-Amb.CO_2, WW-Elev.CO_2, WS-Amb.CO_2, and WS-Elev.CO_2). Means (n=6).

Genotypes	**Above-ground biomass (g)**				**Number of ears**			
	WW-Amb.CO_2	WW-Elev.CO_2	WS-Amb.CO_2	WS-Elev.CO_2	WW-Amb.CO_2	WW-Elev.CO_2	WS-Amb.CO_2	WS-Elev.CO_2
G3	8.95	14.12	8.73	10.94	7	8	5	8
G9	10.74	13.31	8.17	12.66	6	8	5	6
G6	9.66	13.48	8.38	10.90	6	9	5	6
G18	14.94	15.92	10.53	15.85	8	11	6	7
G21	19.96	25.30	12.28	15.70	17	20	11	12
G22	21.86	26.62	14.06	22.60	15	16	12	14
G23	18.92	27.28	9.50	18.04	13	20	12	14
G24	20.07	29.25	12.57	18.67	15	16	10	12
G25	20.33	21.84	12.04	21.22	16	16	10	13
G26	18.75	22.16	11.83	18.67	16	14	8	11

GENERAL DISCUSSION

Climate change effect on crops yield is a major point of concern to fulfil the increasing demand of a growing worldwide population. In fact, the recent global climate models predict a decrease in precipitation and an increase in temperature and CO_2 concentration ([CO_2]) during the following decades. Although durum wheat species had been described to better adapt to water stress and elevated temperature conditions than bread wheat, predicted water shortage may lead to stagnating yields. However, within a climate change context, it should be also observed that the increase in atmospheric [CO_2] has been reported to have positive effect on photosynthetic rates and, consequently in plant grain yield. Furthermore, many reports indicate that it is not possible to extrapolate plant responses to different environmental factors starting from the response deriving from a single stress (Rizhsky et al., 2004; Mittler, 2006). In this regard, in order to summarize the current knowledge of [CO_2], temperature and water availability effects on cereal crops development, a meta-analyses study was carried out in **chapter (I)**. Obtained results remarked that the individual effect of increasing [CO_2] seems to be positive on cereal grain yields associated to an increase in grain number produced per plant more than increasing the weight of the grain. However, drought and heat stresses showed negative effects on grain yield with larger effect observed under low water availability attributed mainly to a drastic decrease in grain number produced by the plant. Under field conditions, plants are subjected to a combination of many environmental factors that has a stronger detrimental effect on plant growth and productivity compared to each single stress. In fact, the obtained results showed that drought and heat stresses had adverse effects on yield components, confirming that the positive effect of high [CO_2] on grain yield will be attenuated under projected warming climate marked by water scarcity. This meta-analyses study contributed to design the working programme of the current PhD project in which the impact of environmental factors and crop management on durum wheat and tritordeum grain yields and quality traits were studied.

Crop yield. Being aware about the relevance of working in natural growth conditions, the **chapter (II)** was designed in order to analyse grain samples stored in different herbarium samples collected since 1850. The assessment of wheat yield evolution since 1850 of the broadbalk experiment in Rothamsted (UK) described in **chapter (II)**, showed a real trend of grain yield and thousand-grain weight (TGW) under field conditions without any climate simulation during 166 years. The increase in wheat yield coincided with the increase of [CO_2] and temperature, and also the introduction of new high yielding cultivars simultaneously with the increase in nitrogen fertilizers application. All these factors together, led to the improvement in wheat grain production. It should be noted that the increase in temperature to certain threshold could be beneficial to plant growth and grain set up. In fact, mean temperature values recorded in Rothamsted since 1878 are below 12°C, which indicates that wheat crop cultivated in this region is not really subjected to elevated temperatures that could affect negatively wheat grain yield. Therefore, grain yield trait is controlled by the interaction of many factors such as the location, climate conditions, nitrogen management, and also the genotype. The observed increase in grain yield despite the decrease in thousand-grain weight is supporting the previous conclusion made in regard with the increase in grain number produced rather than an increase in grain weight. Similarly to what it was observed in **chapter (II)**, the analyses of [CO_2] and drought stress on grain yield (carried out in durum wheat and tritordeum) conducted in **chapter (IV)** showed that grain yield of both species was lower than that recorded under high [CO_2], with no significant effect on TGW. It is evident that drought occurring at anthesis and during grain filling is limiting grain production and can mitigate the positive effect of the increasing [CO_2]. Regarding the impact of crop management, **chapter (III)** confirmed that, wheat yield components depend also on nitrogen fertilization amount and timing. Similarly as high [CO_2], nitrogen supply at anthesis increased durum wheat yield principally by raising number of grains produced by ear since TGW did not increase significantly. Plant response to increasing nitrogen was also controlled genotypically and differences in final yields were detected among genotypes with higher and lower nitrogen use efficiency (NUE). Late nitrogen supply also impacted positively grain quality at maturity characterized by high nitrogen, increased gluten fractions (gialdins and glutenins), high mineral concentrations, and high soluble sugar concentrations, whereas starch concentration was low.

Grain quality is a trait that depends on the end use of the grain. Generally, durum wheat is preferably used for pasta making that requires appropriate protein and gluten characteristics in grains (Sissons, 2008; Sayaslan et al., 2012). In this line, it has been reported by De Cillis (1942) that pasta produced by using vitreous grains, which possess high protein content, has better cooking quality than pasta obtained from starchy grains with low protein content. Results in **chapter (III)** showed that total polyphenols concentration has been improved by nitrogen supply, but no significant effect was found regarding anti-radical activity. Furthermore, our study also remarked that, regardless of nitrogen supply, high-yielding genotypes had lower nitrogen, gluten fractions, and mineral concentrations in grains than low-yielding cultivars probably explained by dilution of nitrogen and higher soluble sugars (glucose, maltose, and sucrose) accumulation. Thus, breeding only for increasing wheat grain yield while paying less attention to grain quality will have serious impacts on pasta dough properties. When screening the genotypes regarding to their response to nitrogen supply, we can see that genotype (G3) has an efficient use of nitrogen showing a balance between an acceptable yield and suitable grain quality under low nitrogen fertilization. Changes in grain quality have also been detected in durum wheat and tritordeum grown under future climate change scenario. Contradictory effects on carbohydrates accumulation, nitrogen and total protein concentrations, and mineral composition of grains were found when increasing [CO_2], drought stress and high temperature were applied separately (**Chapter I**). Actually, grain starch concentration tends to increase under elevated CO_2 concentration while total protein and gluten concentrations are reduced, similarly to micro and macro-nutrients. However, opposite effects on these traits were found under heat and drought stresses. As described in **chapters (II)** and **(IV)**, the overall effect of changing climate on grain quality is defined by an increase in carbohydrate concentrations (mainly as sucrose and starch) against a loss in total protein, amino acids, and mineral concentrations. It seems that increasing atmospheric [CO_2] is beneficial to improve cereal grain yields but, it is not positive for grain quality, putting additional pressure on global wheat production. These changes are determined by nutrient assimilation and remobilization from source organs to grains. The reallocation of leaves and ears nutrients between anthesis and soft dough stages in durum wheat is described in **chapter (IV)**. As a response to elevated [CO_2] and drought stress conditions, durum wheat plant tended to promote the accumulation of hexoses and sucrose in leaves and ears, over total protein and Rubisco. Besides, senescence process was accelerated increasing thereby, Rubisco degradation and accumulation of amino acids, together with soluble sugars (mainly hexoses). This mechanism is involved in osmoregulation that allows the plant to tolerate drought stress and is more accentuated under elevated [CO_2]. Basal leaf contributed also to protein degradation but at lesser extent that flag leaf.

In fact, the concentration of total amino acids accumulated in leaves and ears was maintained equally between the two stages. Meanwhile, the hexoses accumulated at pre-anthesis phase and derived from starch degradation were highly translocated from source organs to grains at post-anthesis. Therefore, under high [CO_2] and low water availability conditions, the plant tends to accelerate the remobilization of non-structural carbohydrates towards grain filling over amino acids. These mechanisms involved in the response of durum wheat to such environmental conditions could explain in some part the increase of carbon concentration, C/N ratio, sucrose, and starch in grains. Consequently, the accelerated senescence leaded to shortening of grain-filling duration, thus, affecting final yield as well as grain composition at maturity of both species under such conditions.

In **summary**, the current PhD project highlighted the fact that predicted climatic conditions will have clear impact on plant production and nutritional quality. In this regard, it is necessary, among other things, to choose plant varieties that are better adapted to the climatic conditions foreseen. For this, the development of new varieties in new growth strategies would be an interesting opportunity. Within this context, it is crucial to characterize how current and near future growth conditions might affect crop yield and quality traits. Such information might be of great value for determining crop breeding strategies taking into account this future (near and far) climate scenario. It is also mandatory to search for resilient genotypes capable of producing under these conditions and maintaining an adequate nutritional value.

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CONCLUSIONS

1. Increasing atmospheric CO_2 concentration as a single factor promotes durum wheat and tritordeum grain production through increasing number of grains per plant rather than rising thousand-grain weight. Nevertheless, the positive effect of this climatic parameter on grain yield is attenuated by other stress factors related to climate change such as extreme temperatures and low water availability.

2. Grain quality of major cereal crops, in particular wheat, is altered under changing climate, characterized by an increase in non-structural carbohydrates (mainly starch) explained by enhanced photosynthetic rates due to the increase in atmospheric CO_2 concentration, while total protein and mineral nutrient concentrations were reduced. However, opposite trend was observed under drought and heat stresses that induced an increase in total protein concentration over soluble sugar concentrations in cereal grains.

3. Shortening in grain-filling phase, due to an accelerated senescence mechanism under current and projected environmental conditions, causes fast protein degradation (mainly Rubisco) in leaves, which lead to increase total amino acids concentration. An increase in the accumulation of hexoses (glucose and fructose) in leaves occurs also under drought stress and elevated [CO_2] conditions, as a response of the durum wheat plant to ensure osmoregulation mechanism against such conditions.

4. Post-anthesis carbohydrates reallocation towards grain filling is ensured by leaves and ears, with higher contribution of the latter than the former. Comparing to amino acids, the plant tends to largely decrease soluble sugar concentrations between anthesis and soft dough stages, suggesting that the remobilization of soluble sugars from source organs to grains is probably taking place earlier and over Rubisco derived N translocation.

5. Tritordeum species showed suitable response to CO_2 enrichment under water deficit conditions by conferring same grain yield as durum wheat with higher nitrogen and amino acid concentrations and better grain mineral composition. Therefore, tritordeum has a high potential to be cultivated under the Mediterranean climate, characterized by low precipitations during grain filling and increasing [CO_2] in the atmosphere.

6. Nitrogen fertilization during post-anthesis improves durum wheat yield and quality under non-limited water availability, but induces a decrease in nitrogen use efficiency. Adding to that, the response to nitrogen supply depends on the genotype (among other factors). High yielding genotypes use nitrogen to increase mostly grain production and starch accumulation, while low yielding cultivars have better grain quality with higher protein (gluten) concentration, total polyphenols, and mineral concentration. Thus, to increase nitrogen use efficiency, an adequate nitrogen management at anthesis is necessary with screening genotypes allowing suitable response to low nitrogen supply, in terms of grain yield and quality.

7. The study of plant response to environmental factors that interfere during its growth cycle, such as nitrogen fertilization management and climatic parameters, could elucidate the molecular and physiological mechanisms conditioning plant performance under specific conditions, and this might be an efficient tool for breeding programs to develop new strategies to identify genotypes with enhancing stress tolerance and optimized yield and quality parameters.

APPENDIX

LIST OF ABBREVIATIONS

$[CO_2]$	Atmospheric CO_2 concentration
DPPH	2,2-diphenyl-1-picrylhydrazyl
FACE	Free-Air Carbon dioxide Enrichement
GABA	γ-aminobutyric acid
HMW	High molecular weight
HYG	High-yielding genotypes
LMW	Low molecular weight
LYG	Low-yielding genotypes
NUE	Nitrogen use efficiency
OTC	Open Top Chamber
TGW	Thousand-grain weight
TKW	Thousand-kernel weight
TNC	Total non-structural carbohydrates
WUE	Water use efficiency
$\Delta^{13}C$	Carbon isotope discrimination

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