Novel phenotyping and monitoring approaches to assess cereal performance under abiotic stress conditions

Nuevos enfoques de fenotipeado y monitoreo de cultivos para evaluar el rendimiento de cereales cultivados en diferentes condiciones de estrés abiótico

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Doctorando	Directores de la Tesis
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To the soul of my grandmother Aisha Abdelkader

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In the name of God the most compassionate and merciful

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Chapter 1

Introduction

1. Importance of cereals in Mediterranean agriculture

Cereals are the basis of the normal diet in most Mediterranean countries and it is estimated that they account for 35-50% of the regional populations' dietary energy consumption (Hervieu et al., 2006; Abis, 2012). Cereal consumption differs from one Mediterranean country to another due to consumer choice or the standard of living. For example in countries where incomes are low, direct human consumption of cereals is about 250 kg per person per year because cereals represent the least expensive source of calories in the diet. In countries where incomes are higher, cereals are normally replaced with meat, fruit and vegetables, which are more expensive, and thus the demand for cereals falls to 120 - 150 kg per person per year (Hervieu et al., 2006; Abis, 2012). The cereals that are the most consumed in the Mediterranean basin are durum and bread wheat. Other grains such as rice, rye, millet, barley, oats and maize, are more regional and smaller in magnitude than the first two (SLOWMED, 2015).

Durum wheat (*Triticum turgidum* L.) is a monocotyledonous plant of the Gramineae family, belonging to the genus *Triticum*. Durum in Latin means "hard", since this species has the hardest kernels of all wheats. Its high protein and gluten content, as well as its strength, make durum wheat good for manufacturing a diverse range of food products. The most common products made from durum wheat are pasta, couscous and burghul. Durum is the only tetraploid species of wheat that is widely cultivated today. At the global level, wheat production in 2013 was 715.9 million tonnes and bread wheat (*Triticum aestivum* L.) made up more than 94% of the total production, while durum wheat was placed second comprising less than 6% of global wheat production, with a production of about 37.7 million tonnes (Table 1) (FAOSTAT, 2013; AETC, 2014).

Maize (*Zea mays* L.) is also a monocotyledonous plant of the Gramineae family, belonging to the genus *Zea*. Although maize originated in Mesoamerica, it is grown in a wide range of agro-ecological environments throughout the world; from the sea level to highland temperate environments at more than 3000 m (Buckler et al., 2009).

It is a major cereal crop for both livestock feed and human nutrition (http://www.iita.org/maize), and also for bioethanol and biofuel production (Dunwell, 2000; Mackay, 2009). The world production in 2013 was around 1018 million tonnes with the top five producers being United States, China, Brazil, Argentina and Ukraine

(Table 2). Spain is listed as the 25th nation in terms of the quantity produced (4.93 million tonnes, FAOSTAT, 2013).

Table 1. Production of durum wheat in the world in millions of tonnes.

	2009	2010	2011	2012	2013
EU	8.7	9.1	8.2	7.9	7.9
Canada	5.4	3.0	4.2	4.6	6.5
U.S.	3.0	2.9	1.4	2.2	1.7
Turkey	3.1	2.9	3.0	3.0	3.0
Algeria	2.9	2.2	2.5	3.0	2.5
Morocco	1.9	1.6	1.7	1.0	1.9
World Total	40.9	34.9	36.7	35.2	37.7

*Source: (AETC, 2014).

Table 2. Production of Maize in the world in millions of tonnes.

	2009	2010	2011	2012	2013
U.S.	332.6	316.2	313.9	273.8	353.7
China	163.9	177.4	192.8	205.6	217.7
Brazil	50.7	55.4	55.6	71.1	80.5
EU	60.0	59.2	70.3	59.4	65.1
Argentina	13.1	22.7	23.8	21.2	32.1
Ukraine	10.5	11.9	22.8	20.9	30.9
World Total	820.2	851.3	887.9	872.8	1017.7

*Source: (FAOSTAT, 2015).

2. Growing conditions in the Mediterranean basin

Mediterranean regions are characterized by long, hot, dry summers and short, mild, wet winters, combined with high solar radiation especially in summer, high concentrations of rainfall in winter, and high variability of precipitation from year to year (Castri, 1981; Pereira and Chaves, 1993; Loss and Siddique, 1994). The areas of high rainfall (more than 450 mm annually) are devoted to rainfed cultivation of bread wheat and the areas of medium rainfall (250 to 450 mm) to growing durum wheat, while barley is cultivated in the driest areas (less than 250 mm) (Anderson and Impiglia, 2002). For small grain cereals such as wheat and barley, irrigation to supplement rainfall could be provided during the critical crop stress period (during grain filling) (Rodrigues et al., 2003), although crop production can still be achieved under rainfall alone (Oweis et al., 2000). This not the case for summer crops such as maize under Mediterranean regions which require high supplemental irrigation of 500-600 mm (Aguilar et al., 2007) and crop production cannot be achieved when relying only on rainfall (Rodrigues et al., 2003).

Nitrogen (N) is considered one of the most important factors determining crop production (Parry et al., 2005). Despite the fact that N constitutes only 2 to 4% of plant dry matter it is the most important plant macronutrient (Mengel and Kirkby, 2001), as N constitutes part of essential plant cell compounds from proteins to chlorophyll and genes (Srivastava and Singh, 1999).

N fertilization is one of the most commonly implemented management practices in grain production worldwide and its consumption has increased in the last 30 years by about 150% for many grain crops (Frink et al., 1999). Overall, N deficiency can decrease the carbohydrate source size by decreasing leaf growth, leaf area and duration, the photosynthetic rate per leaf area, and the source sink capacity by decreasing the size of the vegetative storage organs (Engels and Marschner, 1995), thus affecting crop yields negatively.

Under Mediterranean conditions, water shortage is known to be the most limiting factor for cereals production. As a consequence relatively little attention has been given to N fertilization in the past (Vlek et al., 1981). Thus, for some Mediterranean areas poor or inadequate nitrogen supply has been as much responsible for cereal yield losses as water availability (Passioura, 2002; Ali et al., 2006; Carvalho, 2009).

3. Constraints to cereal production in the Mediterranean basin

a. Population growth and the land and crops available for food production

Recent trends in population growth suggest that global food production is unlikely to satisfy future population needs unless the rates of crop improvement are accelerated or radical changes in the patterns of human food consumption occur (Reynolds et al. 2011). Globally, more than 2 billion people are food insecure, being either undernourished or malnourished.

Only three crops, wheat, maize, and rice, provide about 50% of the calories and 42% of the protein for human consumption in less developed countries (Braun et al., 2010). Based on projections, by 2025 the global population will increase by more than 1.5 billion and by 2050 the population will reach around 9 billion (Lutz et al., 2001; Hamilton, 2009; Godfray et al., 2010), of which approximately 4.0 billion will be living in water-scarce or water stressed countries (Engelman, 2009).

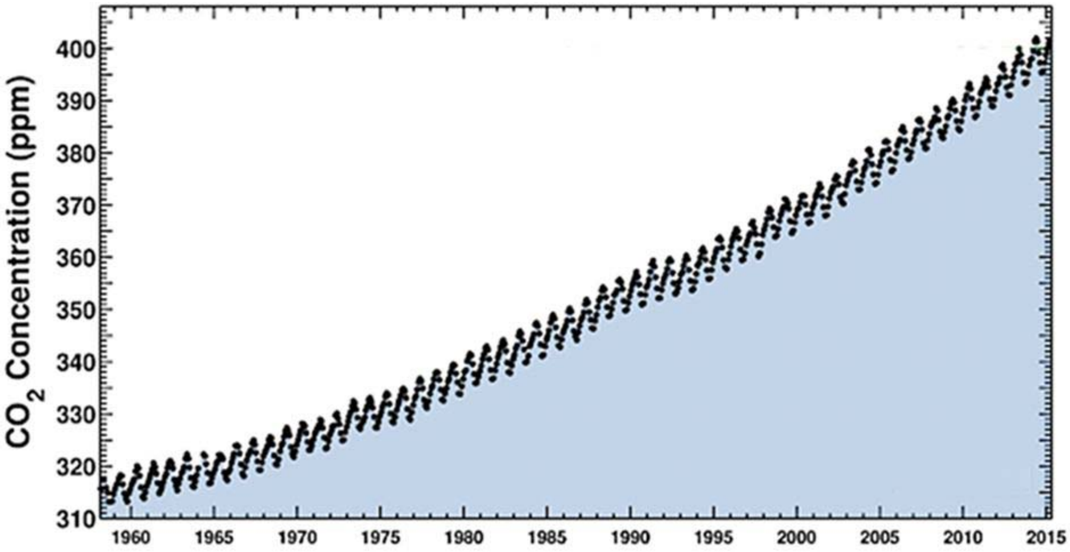
The Mediterranean region is one of the main critical areas of environmental unsustainability that has experienced negative interactions with intense human activity and agricultural exploitation (Prosperi et al., 2014; Salvati, 2014). A large part of the population in the Mediterranean region can also be considered food insecure, where out of about 500 million people at least 215 million are "qualitative and quantitative food insecure" (Prosperi et al., 2014).

Increasing the production of crops will be necessary to satisfy growing population demands and further increases in crop production will come from improvements in yield potential, as the amount of cropping land will not be increased (Albajes et al., 2013; Fischer et al., 2014). The available cropping land per capita is half of what it was in the 1960s (Engelman, 2009) and with the current rate of population growth more land will have to be allocated to build infrastructure and accommodate urbanization, as well as satisfying the increasing demands for land for bio-energy production and biodiversity protection (Campbell et al., 2008; Gahukar, 2009).

b. Global climate change

Over the past 250 years, since the beginning of the industrial revolution, the amount of carbon dioxide has increased markedly in the atmosphere, primarily because of emissions of CO_2 from the burning of fossil fuels. The CO_2 concentration in the atmosphere is increasing by about 2 ppm each year (Figure 1). Recently (in April 2015), its concentration was about 402.59 ppm (NOAA, 2015). Because CO_2 causes a greenhouse effect by absorbing infrared bands of solar radiation, the foreseen increase in atmospheric CO_2 concentrations will cause the earth to retain more heat, subsequently increasing the surface temperature of the earth. Also, the constant release of nitrous oxide (a greenhouse gas) into the atmosphere as a result of denitrification of nitrate fertilizers may play a role (Carvalho, 2009).

Figure 1. Monthly mean atmospheric carbon dioxide at Mauna Loa Observatory, Hawaii*.



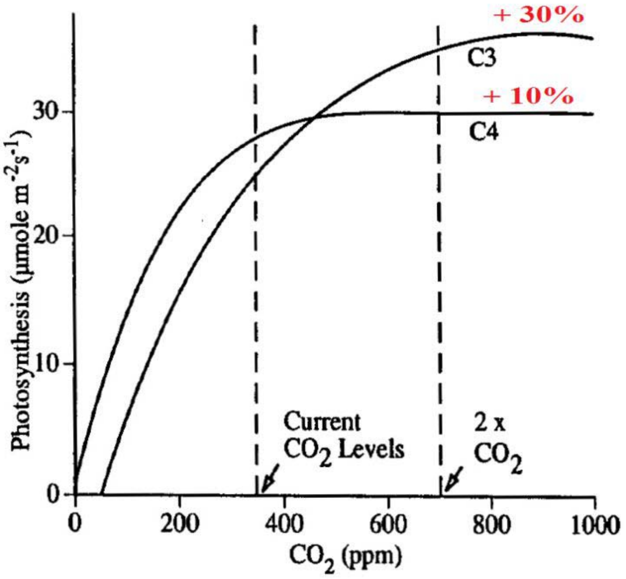
*Source: Adapted from NOAA (2015)

This increase in temperature is expected to be 0.4-0.6 °C per decade throughout the 21st Century (Centritto, 2003). For the Mediterranean regions, the prospects for the future are possibly worse than the worldwide average, with a global warming forecast of 1°C by 2025, implying less harsh winters and hotter summers. A faster increase in temperature will increase the frequency of dry summers around the Mediterranean. In the South of France and Central Spain, dry summers are expected to start three weeks earlier and end two weeks earlier, which would mean a shift of the dry season towards spring (Isendahl and Schmidt, 2006). Also, the increase in earth temperature is enough to change rainfall patterns and crop productive capabilities in many regions of the world (William, 1979). Water scarcity is an alarming problem in the Mediterranean basin due to the growth of population and the consequent increasing need of water for agriculture and other human activities. A remarkable decrease in global production occurred in 2005 due to a severe drought that was the worst since 1947 for large parts of of the Mediterranean basin. In Spain this season was the driest season recorded by the National Meteorological Institute since 1947; only 459 mm of precipitation fell compared to the historical average of 669 mm. In Southern Europe, most of the Mediterranean basin has already experienced a rainfall reduction of up to 20% in some areas during the last century, leading to increased aridity in the Mediterranean. All over Italy, a substantial decrease in the number of wet days of about 12% over the period 1880-2002 has been reported (Isendahl and Schmidt, 2006). The scarcity and irregular distribution of precipitation together, with an increased evapotranspiration (total amount of soil water losses associated with both soil evaporation and plant transpiration) is going to be a naturally recurring feature of the global climate. This would adversely affect crops and water availability, and would influence the patterns of future agricultural production. Providing constancy in evapotranspirative demand, crop evapotranspiration is positively and linearly related to grain yield (GY) in cereals; therefore water stress decreases yield (Rodrigues et al., 2003).

The period of low rainfall in the Mediterranean regions is concomitant with an increase in temperature. The time and severity of the terminal drought stress varies according to the last spring rains, temperatures, soil type, and crop growth (Loss and Siddique, 1994). During spring, rainfall will be less frequent and the increased temperatures will increase evapotranspiration, so the differences in vapour pressure deficit (VPD) between air and the wheat canopy surface will increase; as a consequence terminal drought stress may be accelerated.

Increased air CO_2 concentrations could have a positive impact on crop productivity (Seguin, 2010). The results of established photosynthesis curves of CO_2-air enrichment experiments have shown (Figure 2) an increase of about 10-20% with 550 ppm for C_3 temperate species such as wheat, rice and soybean, while it seems to be limited to 0-10% for C_4 tropical species such as maize and sorghum (Wolfe and Erickson, 1993; Easterling et al., 2007; Seguin, 2010).

Figure 2. Typical increases in photosynthesis with increases in carbon dioxide concentration



*Source: Adapted from Wolfe and Erickson (1993)

Whereas increasing CO_2 concentrations will influence plant physiology, through their effect on photosynthesis, transpiration and respiration (Centritto, 2003), the change in air temperature and plant water status will also affect these processes. Knowledge about the balance of these processes under CO_2 and temperature changes with plant water status is still being developed (Centritto, 2003).

c. *Excessive use of N fertilizer*

Overall, the potential environmental impacts of excessive use of N fertilizers are: 1) increased global warming problems due to the use of fossil fuels and emission of greenhouse gases (CO_2 and NO_x) during fertilizer production and the increase in nitrogenous gases (NO_x) released from denitrification of applied fertilizer by soil bacteria (Khalil et al., 2002; Carvalho, 2009); 2) air pollution due to ammonia (NH_3) release into the atmosphere (Renard et al., 2004); and 3) eutrophication and pollution of drinking water due to high N mobility in the soil by leaching (Glass, 2003; Cameron et al., 2013)

d. Policy impacts

The increase in cereal production in the last 50 years in the Mediterranean basin has been particularly evident in irrigated lands, by the use of improved crops varieties and other favourable agronomic practices that maximize production (e.g. the use of chemical fertilizers) (Araus, 2004). Further yield expansion in irrigated lands is not feasible due to sustainability problems, such as silting of reservoirs and canals, low levels of underground water and salinity in irrigated soils (IRRI, 1996). However, in many Mediterranean countries water resources policies have been poorly structured and organized and have negatively impacted the status of these water resources. Prices for water in Mediterranean countries are generally low compared to the European average, especially those for agricultural use, and they neither recover the cost of providing users with water nor do they stimulate water savings. Other factors to be considered are the lack of compliance with water related legislation as well as a lack of control by the responsible river basin authorities. Also, agricultural subsidies that support production and/or the development of irrigation systems, regardless of water availability (Isendahl and Schmidt, 2006) increase the problem.

e. Water use

Increasing water scarcity is threatening the economic development and the stability of many parts of the Mediterranean basin. Water consumption in the Mediterranean basin is high, especially in the hot and dry regions, where less water is available. Agriculture is the predominant user of the available freshwater resources, accounting for 75% of total water consumption in the Southern and Eastern part of the basin. As water resources shrink the competition from other sectors (e.g. industry, tertiary activities such as tourism, etc.) grows (Araus, 2004, 2013; Isendahl and Schmidt, 2006).

f. Management practices

Under rainfed conditions, soil water can be lost from the soil surface through runoff and deep infiltration through the soil. Under irrigated conditions, more than 50% of irrigation water is lost from its source to the crop roots because of inappropriate land management practices that fail to capture a large amount of the rainfall and retain it in the plant rooting zone or excessive use of irrigation water (Isendahl and Schmidt, 2006). Such losses not only lead to water waste but also to potential hazards of soil salinity and water pollution resulting from the transport of nitrate, phosphate, sediments and agro-chemicals to streams, lakes and rivers (IAEA, 2010).

4. Increasing cereal production in the Mediterranean region

Cereal production in the Mediterranean basin faces two main challenges. The first is to increase productivity, either through crop breeding of new highly productive cultivars or by implementing new agronomical practices that increase the resilience of agriculture to abiotic stresses (mainly drought and N deficiency) rather than through the expansion of arable land, which is not feasible anymore (Slafer et al., 1996; Tsialtas and Tokatlidis, 2008; Hamilton, 2009). However, this goal needs more understanding of the physiological and genetic responses of plants under water stress. The second challenge is to prevent the deterioration of water resources through contamination with soil runoff, nutrients and agrochemicals, and the subsequent related problems (silting of canals, salinity in irrigated soils, etc.).

5. How plant physiology could help plant agronomy and breeding

Agriculture defines the success of a plant as productivity per unit of land area. Environmental stresses such as drought, high temperatures and inadequate fertility impede the growth and development of both wheat and maize, impacting transpiration and nutrient uptake (Dwyer, et al. 1992; Crafts-Brandner et al., 2002; Araus et al., 2013; Araus and Carins, 2014) and thus there are decreases in the plant's ability to achieve maximum yield (Boyer, 1982). Water deficit, which is frequently accompanied by low nitrogen (N) availability, is the main constraint limiting cereal productivity in the Mediterranean regions (Loss and Siddique, 1994; Oweis et al., 2000; Araus et al., 2002; Passioura, 2002; Cabrera-Bosquet et al., 2009a). Crop management and breeding may improve the performance of cereals under such stress conditions. However, the lack of efficient tools to monitor the performance of agronomical practices or to undertake appropriate phenotyping in breeding programs limits the efficiency of both avenues.

Breeding efforts during the twentieth century for increasing crop yields in drought prone environments of the Mediterranean have depended on the selection of yield, *per se* (Jackson et al., 1996). Yield as a trait is characterized by low heritability and a high genotype x environment interaction (Jackson et al., 1996). Thus, progress in cereal breeding is too slow in arid and semiarid Mediterranean environments due to significant genotype x environment interactions, which is caused (at least in part) by annual fluctuations in water availability (Rebetzke et al., 2002). Developing cultivars that have an advantage under low water availability and N deficiency conditions is a major challenge for cereal improvement programs. Tolerance to abiotic stresses is in general a very complex character, due to the interactions between stress factors and various molecular, biochemical and physiological mechanism affecting plant growth and development. Plant physiologists have suggested that the identification and selection of physiological and/or morphological traits (Table 3), based on a good understanding of the crop under the stress at the physiological and molecular biology levels, is an effective approach to breeding for higher yield, and could be a valuable strategy for use in combination with normal methods of plant breeding and crop management practices (Araus, 1996; El-Jaafari, 2000; Razmjoo et al., 2008; Elazab et al., 2015).

Table 3. Selected physiological and/or morphological traits of practical use in plant agronomy and breeding under abiotic stresses*.

Physiological and/or morphological traits	Related to yield
Flowering/maturity date	Phenology
Ear fertility Metabolic profiles (e.g. carbohydrate levels, growth regulators) Stem Reserves.	Harvest index
Spectroradiometric techniques Spectral imaging Digital imaging	Plant growth and senescence
Canopy temperature (e.g. thermometers, thermal imaging) Stable isotopes (e.g. ^{13}C, ^{18}O)	Water status

*Source: Adapted from Araus et al. (2008).

^{13}C and ^{18}O refer to the stable isotopes of carbon and oxygen, respectively.

According to different authors (Araus et al., 2002, 2008; Lafitte, et al., 2003; Royo et al., 2005) for a secondary trait to be used in a breeding program it has to achieve most of the following criteria: 1) It must be genetically correlated with GY under the stress in the target environment; 2) It should be a highly heritable trait that is not affected very much by environment. If the heritability of yield is reduced by stress, the heritability of the secondary trait should not be reduced (or at least be less reduced) by stress than GY; 3) There must be genetic variation among lines for the trait; 4) The trait should not be associated with poor yields in the unstressed environment. However, many secondary traits may penalize yield in unstressed environments because these traits reflect a tolerance instead of avoidance to a given stress, which makes them useful traits only if selected for extremely stressed environments; 5) It must be easily and rapidly screened, and less costly than estimating GY under stress; and 6) It must have the ability to be assessed on individual plants or in small plots. Except for criteria 2 and 3 these traits may also be relevant in crop management practices.

Many secondary traits have been successful in monitoring plant status and breeding programs under abiotic stresses. However, the use of secondary traits in plant monitoring and breeding for adaptation under abiotic stresses is often limited by: (1) the lack of proper phenotyping traits that could address the stress (Araus and Cairns, 2014); (2) the fact that the relationship of the trait with aerial biomass and GY (the usual target in crop breeding) frequently changes according to the type and level of stress (Voltas et al. 1999; Araus et al. 2003*a*); and (3) phenotyping traits usually address each stress individually, while for example drought and low N deficiency often occur together (Araus et al., 2013).

Lafitte et al (2003) suggested that selection may be for an index consisting of GY plus secondary traits, and the expected progress from selection using the secondary trait and yield together must be greater than the progress made by selecting GY alone. Besides the choice of the most appropriate trait (or combination of traits) for predicting GY performance under different water regimes, it is also crucial to determine the key time(s) for their evaluation (Elazab et al., 2015). Measuring these traits at more or less frequent intervals during the crop cycle, together with measurements of the harvestable components would be unfeasible (or at best impractical) (Araus and Cairns, 2014). Moreover, the relative importance of each trait in explaining plant performance and the appropriate time to measure them may change depending on the severity, time and duration of the stress and the phenological stage when it occurs. Beyond that, a mechanistic explanation of how the different physiological traits can predict GY performance is not always clear, but the existence of a statistical relationship between the trait(s) measured and the GY is used as proof of concept for the adequacy of the trait (Elazab et al., 2015).

6. Monitoring and phenotyping techniques used in the current work

a. Stable isotopes

The use of stable isotope techniques in plant ecological research has grown steadily during the past three decades, as the analysis of stable isotopes in plant materials could provide physiological or environmental insights over the lifespan of the plant part sampled. Moreover, the rapid throughputs in automated analytical systems together with the ease of sample preparation have played a major role in the widespread use of stable isotopes in plant ecophysiological studies (Griffiths, 1996; Dawson et al., 2002; Peuke et al., 2006).

In C_3 plants the carbon isotope composition ($\delta^{13}C$) measured in plant tissues is considered one of the most promising secondary traits in wheat (and other C_3 cereals) when breeding for drought resilience (Rebetzke et al., 2002; Condon et al., 2004; Richards et al., 2011). The $\delta^{13}C$ has been reported to negatively correlate with C_i/C_a (the ratio of leaf intercellular to ambient CO_2 concentration) and positively correlated with A/E (the ratio of net assimilation to water evaporated from the transpiring organs). Therefore, the $\delta^{13}C$ is positively related to *WUE*, which is considered as the biomass produced per unit of water transpired (Farquhar and Richards, 1984; Monneveux et al*.*, 2006; Elazab et al*.,* 2012). Correlations between $\delta^{13}C$ and GY and/or aerial biomass (AB) may be either negative or positive according to the plant tissue sampled and environmental conditions tested (Condon and Richards, 1992; Voltas et al., 1999; Araus et al., 2003*b*). High $\delta^{13}C$ has been used as a selection trait for high *WUE* in commercial wheat breeding for the summer dominant rainfall growing conditions of Australia, where crop yield relies on the water accumulated before planting (Rebetzke et al., 2002; Condon et al., 2004). However, the $\delta^{13}C$ in some cases may reflect the effective water used (*EWU*) by the crop (Blum, 2009), and thus the relationship between $\delta^{13}C$ and either AB or GY may be negative (Araus et al., 1998, 2003*a*, 2013; Merah et al., 2001; Monneveux et al., 2006), meaning that genotypes that are able to maintain a higher

EWU (even if at the expense of a lower *WUE*) are the most productive (Blum, 2009; Araus et al., 2013).

In the case of a C_4 plant like maize, variations in the $\delta^{13}C$ in response to water conditions are small in comparison to C_3 plants like wheat (Henderson et al., 1999), but they are still adequate for use in maize as an indicator of water conditions during growth (Cabrera-Bosquet et al., 2009*b*; Araus et al., 2010).

The oxygen isotope composition ($\delta^{18}O$) of plant tissues is known to reflect the evaporative conditions throughout the crop cycle (Barbour et al., 2000) and thus it has been proposed as a proxy method for measuring transpiration as well as an indicator of genotypic differences in stomatal conductance (g_s) (Barbour et al., 2000; Ferrio et al., 2007; Cabrera-Bosquet et al., 2011; Elazab et al., 2012). Leaf $\delta^{18}O$ has been negatively correlated with the transpiration rate (T) (Barbour et al., 2000; Barbour and Farquhar, 2000; Ferrio et al., 2007; Cabrera-Bosquet et al., 2009*a*) and under well-watered conditions in wheat the $\delta^{18}O$ of flag leaves correlates negatively with GY and g_s (Barbour et al., 2000). Cabrera-Bosquet et al. (2009*b*) found negative correlations in maize between the $\delta^{18}O$ of grains and the GY under well-watered and moderate water stress conditions, whereas under severe water stressed conditions the correlations were positive. However, the correlation of $\delta^{18}O$ analysed in mature grains of wheat with GY is frequently weak or absent (Ferrio et al., 2007; Araus et al., 2013).

b. *Root traits*

Plant roots are the key organs in the plant responsible for the absorption of water and nutrients (Shen et al., 2013). Root growth is known to vary according to soil texture and structure, together with related traits such as the availability of water (Asseng et al., 1998; Elazab et al., 2012) and N (Comfort et al., 1988; Carvalho, 2009; Shen et al., 2013; Wang et al., 2014).

Concerning root traits, their response to drought stress is still a challenging subject for research. The laborious work required for the study of the root system has prevented the adoption of root characteristics as routine phenotyping traits for crop breeding. Therefore, it is necessary to develop precise enough but easy-to-handle techniques for estimating root traits associated with water use and crop yield.

The capacity of roots to extract water and nutrients from a given volume of soil is dependent on root architectural traits such as the length and weight distribution of roots with depth (Tinker and Nye, 2000; King et al., 2003; Carvalho et al., 2014). Thus, root weight density (RWD) and root length density (RLD) are frequently used in root studies (Zhang et al., 2009; Ayad et al., 2010; Elazab et al., 2012; Carvalho et al., 2014; Wang et al., 2014) to describe the root weight and root length, respectively, within a soil volume. Currently, RLD is the most commonly used trait to describe root distribution (Ayad et al., 2010; Zaman-Allah et al. 2011; Elazab et al., 2012; Carvalho et al., 2014) and it is an important parameter to model water and nutrient uptake (Barraclough, 1989; King et al., 2003; Vadez et al, 2008; Ayad et al., 2010; Carvalho et al., 2014). Overall, the results of wheat and barley field experiments indicate that a RLD of 1 cm cm^{-3} is a critical value for extraction of 90% of the available soil water (the amount of water in the soil between field capacity and the permanent wilting point), whereas RLD values above 1 cm cm^{-3} are excessive and inter-root competition occurs (Barraclough, 1989; King et al., 2003; Carvalho et al*.,* 2014).

The specific root length (SRL) is considered one of the most important and commonly measured morphological traits. SRL is the ratio of root length to root biomass (Eissenstat, 1991, 1992; Cornelissen et al., 2003). According to Ryser (1998) and Grossman and Rice (2012) a high SRL facilitates nutrient uptake in low-nutrient environments and makes plants more competitive for soil nutrient uptake (Bonifas and Lindquist, 2009). However, roots with high SRL may have limited transport, storage and support capacity and a shorter lifespan than low-SRL roots (MaCully and Canny, 1988; Fitter, 1996; Cornelissen et al., 2003). The value of SRL in breeding programs is still difficult to assess (Løes and Gahoonia, 2004) because unlike other root traits (i.e. total root biomass and length, RLD and RWD) the previous studies on genetic variation in cereal species under different resource availabilities (water and nutrients) for the SRL are contradictory and often based on few lines or varieties (Ryser, 1998; Manske and Vlek, 2002; Løes and Gahoonia, 2004; Carvalho, 2009; Elazab et al., 2012).

c. *The use of proximal (remote) sensing techniques.*

The assessment of AB is important for monitoring crop growth because it could reflect the effect of stresses on crop growth and senescence (Araus et al., 2008; Royo and Villegas, 2011). Thus, a number of studies have revealed that spectral reflectance techniques have the potential to provide precise, non-destructive and instantaneous quantitative estimates of AB (Raun et al., 2001; Aparicio et al., 2004). The Normalized Difference Vegetation Index (NDVI), which uses the Near-Infrared (NIR) and the visible (VIS) wavelengths in its formulation, is among the most usual of spectral reflectance indices, and it is related to the photosynthetically active AB (Ferrio et al., 2005). The NDVI has been used as an indicator of AB and GY in durum wheat (Aparicio et al., 2000, 2002, 2004; Casadesús et al., 2007) and bread wheat (Gutiérrez-Rodríguez et al., 2004; Lobos et al., 2014). However, the NDVI values saturate at high green biomass densities (i.e. a high leaf area index), which means that this technique may have a low precision at key moments of the crop cycle (e.g. anthesis), especially at high plant densities under favourable agronomic conditions. Also, its accuracy decreases by the presence of reproductive organs (Aparicio et al., 2002, 2004) and the change in the canopy structure at certain stages of the plant cycle (Gitleson et al., 2002).

In recent years the use of digital Red-Green-Blue (RGB) images has been proposed as an alternative to develop vegetation indices that may replace spectroradiometrical based NDVI (Sakamoto et al., 2012; Hunt et al., 2013; Casadesús and Villegas, 2014). The price, size, and the easy use of conventional digital cameras make them viable alternatives to assess AB and GY in cereals (Mullan and Reynolds, 2010; Casadesús and Villegas, 2014). A number of studies have used digital RGB imaging to measure different colour parameters such as: greenness; intensity of green, red and blue; and derived normalized indices from the green, red and blue bands (Gitelson et al., 2002; Casadesús et al., 2007; Mullan and Reynolds, 2010; Kipp et al., 2013). Such information has allowed estimation of a wide range of crop traits in durum and bread wheat such as early vigour, leaf area index, leaf senescence, AB and GY (Gitelson et al., 2002; Mullan and Reynolds, 2010; Casadesús and Villegas, 2014). Moreover, a large number of digital RGB images (i.e. photos) can be obtained with a minimum effort (Casadesús and Villegas, 2014) and processed with suitable (either open-access or commercial photo editing) software to extract parameters related to green AB (Casadesús et al., 2007).

Objectives

This thesis seeks to explore different methodological alternatives to monitor and evaluate the grain yield, biomass production, and the photosynthetic and water status of main crops (wheat and maize).

This thesis consists of six chapters. The first chapter introduces the main production constraints in the Mediterranean regions and briefly reviews the novel physiological approaches addressed in the thesis that are potentially useful in yield prediction, breeding and agronomical management of the main cereal crops (wheat and maize) under the production constraints of the Mediterranean environment (i.e. drought, heat and nitrogen deficiency). The second and third chapters include two different lysimeter experiments carried out to investigate the role of root architecture on the crop performance of durum wheat under different combinations of water and nitrogen conditions. In that sense the relationships of root traits with different indirect evaluation approaches (stable isotopes, gas exchange, plant growth) of the root performance were investigated together with genetic variability. The fourth chapter, also in durum wheat, aims to develop a mechanistic model to visualize in a comprehensive manner the best combination of key traits to use for phenotyping under field conditions. Moreover, the fourth chapter together with the fifth chapter (investigating maize) aim to compare different proximal (remote) sensing approaches to evaluate grain yield and the biomass as affected by drought and heat. An emphasis placed on highlighting the use of vegetation indices derived from conventional digital images as an affordable low-cost evaluation technique. The final ‘sixth’ chapter is a global discussion and conclusion of the entire thesis.

The specific objectives of the thesis were:

<u>For chapters 2 and 3:</u>

1. Comparing the effect of water regime and genotype (Chapter 2) variability and the effect of water regime, nitrogen regime and genotype variability (Chapter 3) on:
    a. The aerial biomass and root growth and architecture.

    b. Different instantaneous and time-integrated gas exchange traits.

    c. The composition of different stable isotopes informing on the photosynthetic and transpirative conditions ($\delta^{13}C$ and $\delta\,^{18}O$).

2. Evaluating $\delta^{13}C$ and $\delta\,^{18}O$ as indicators of plant water status and growth.
3. Evaluating the performance of different root distribution and morphological traits as indicators of plant water status and growth.
4. The relationships between the signature of different stable isotopes and the root traits and how these relationships could serve in indirect screening for the best root traits under the studied growth conditions.

For chapter 4:

5. Investigating in field-grown durum wheat the effect of water regime and genotype on:
    a. The aerial biomass, grain yield and agronomical components.
    b. The $\delta^{13}C$ and $\delta\,^{18}O$ of leaves and mature grains.
6. Evaluating $\delta^{13}C$ and $\delta\,^{18}O$ as indicators of plant water status and growth.
7. Comparing the potential use of two different proximal sensing techniques: spectral reflectance (using portable spectroradiometer) and low-cost digital RGB imaging to determine grain yield performance.
8. Dissecting what combination of phenotypic traits can predict grain yield performance through the different agronomical yield components.

For chapter 5:

9. Comparing the performance of two different remote sensing techniques: the multispectral band imaging and the low-cost digital imaging in detecting the effects of heat stress under field conditions on final maize aerial biomass and grain yield.

Informe de los Directores de Tesis sobre el factor de impacto de los artículos publicados y participación del doctorando

El Dr. José Luis Araus Ortega y la Dra. María Dolores Serret Molins como Directores de la Tesis que lleva por título ***"Novel phenotyping and monitoring approaches to assess cereal performance under abiotic stress conditions"*** que ha desarrollado el Doctorando Abdelhalim KHALED ELAZAB,

Informan sobre el índice de impacto y la participación del Doctorando en cado uno de los artículos incluidos en la memoria de esta Tesis Doctoral.

Capítulo 2. Artículo: **"Root traits and δ^{13}C and δ^{18}O of durum wheat under different water regimes"** publicado en la revista *Functional Plant Biology* con un índice de impacto en 2012 de 2.471, correspondiente al segundo cuartil dentro de "Plant Sciences" (aunque en la actualidad se encuentra en el primer cuartil). En este estudio se cultivaron en lisímetros cuatro líneas de trigo duro recombinantes, caracterizadas por su buena adaptación agronómica en condiciones Mediterráneas, tanto en condiciones de riego (WW, por sus siglas en inglés) como de estrés hídrico (WS, por sus siglas en inglés), hasta el estadio de mitad del llenado del grano. Se midió el intercambio de gases en la hoja bandera justo antes de la cosecha. Posteriormente, se evaluaron la materia seca aérea (DM aérea, por sus siglas en inglés), la densidad de peso de la raíz (RWD, por sus siglas en inglés), la densidad de longitud de la raíz (RLD, por sus siglas en inglés) y la longitud especifica de la raíz (SRL, por sus siglas en inglés). Además se analizaron el δ^{13}C y el δ^{18}O de las raíces, de la hoja bandera y de la espiga y se establecieron las relaciones entre los parámetros estructurales de raíz y las firmas isotópicas de carbono y oxígeno. Este artículo representa una aportación novedosa y relevante en el campo del fenotipeado de cultivos para condiciones de estrés hídrico, al identificar los caracteres radiculares asociados a la firma isotópica de la planta.

Capítulo 3. Artículo: **"Interactive effect of water and nitrogen regimes on plant growth, root structure and water status of old and modern durum wheat genotypes"** enviado a la revista *Journal of Integrative Plant Biology* con un índice de impacto en 2015 de 3.448, que le sitúa dentro del primer cuartil dentro de "Plant Science". En este trabajo se cultivaron ecotipos y variedades antiguas junto con genotipos modernos de trigo duro. El cultivo se realizó en condiciones controladas pero cercanas a las condiciones de crecimiento de la planta en el campo, consistentes en lisímetros de 150 cm de alto, bajo dos regímenes de agua y nitrógeno contrastantes, bajo cubierta (pero por todo lo demás expuesto a las condiciones climáticas naturales). Se estudió el efecto de tales condiciones de crecimiento sobre la biomasa aérea (AB, por sus siglas en inglés), el crecimiento, la distribución y la morfología de las raíces, tasas de fotosíntesis y transpiración y la eficiencia del uso del agua. Estos parámetros se han relacionado con las firmas isotópicas de carbono y oxígeno. La novedad de este estudio radica en que se trata de un estudio de raíces en condiciones cercanas a las de campo, empleando un abanico de genotipos de trigo duro contrastados y estudiando las interacciones entre régimen hídrico y de abonado nitrogenado.

Capítulo 4. Artículo: **"The combined use of vegetation indices and stable isotopes to predict durum wheat grain yield under contrasting water conditions"** publicado en la revista *Agricultural Water Management* con un índice de impacto en 2015 de 2.333, perteneciente al primer cuartil dentro del área de "Agriculture". En este estudio se cultivaron cinco genotipos modernos de trigo duro en régimen de secano (RF, por sus siglas en inglés) y de riego suplementario (SI, por sus siglas en inglés) en el campo. Se midieron dos categorías de caracteres fisiológicos; (1) índices de vegetación: Índice Normalizado de Diferencia de Vegetación (NDVI, por sus siglas en inglés) y el Índice Normalizado de Diferencia de color Rojo-Verde (NGRDI, por sus siglas en inglés); y (2) las composiciones de isótopos estables de carbono y de oxígeno ($\delta^{13}C$ y $\delta^{18}O$) en diferentes órganos de la planta. La novedad de este estudio es doble. Por una parte a partir de un "análisis de sendero" (path-análisis en inglés) desarrolla un modelo conceptual que ayuda a explicar cómo los diferentes criterios de fenotipeado explican el rendimiento. Este acercamiento conceptual puede ser útil a la hora de definir qué conjunto de criterios de selección son los más adecuados bajo diferentes condiciones hídricas de cultivo. El segundo aspecto novedoso del artículo es de índole metodológico, ya que prueba que un índice como el NGRDI, formulado a partir de imágenes convencionales rojo/verde/azul (RGB, como siglas en inglés) se comporta mejor que el NDVI, evaluado mediante espectroradiometría, como índice de vegetación.

Capítulo 5. Artículo: **“Detecting terminal heat stress effects on maize biomass and grain yield by remote sensing techniques”** enviado a la revista *European Journal of Agronomy* con un índice de impacto en 2015 de 2.918 y situado en el primer cuartil dentro del área de “Agriculture”. Este estudio se realizó en condiciones de campo durante dos temporadas consecutivas de cultivo. Se aplicaron dos tratamientos térmicos antes y después de la floración (pre y post-floración, respectivamente), además del tratamiento control a temperatura ambiente. Las mediciones de teledetección se tomaron con una cámara espectral multibanda para medir el NDVI y una cámara RGB convencional para medir el NGRDI. El objetivo de este estudio era comparar la utilidad de dos técnicas diferentes de teledetección remota sobre el efecto del estrés térmico en condiciones de campo en plantas de maíz sobre la biomasa aérea y el rendimiento. El estudio demuestra que el índice NGRDI funciona mejor que el NDVI prediciendo el efecto del estrés por calor tanto en el rendimiento como en la biomasa. Estos resultados son relevantes en el sentido que demuestra que índices de teledetección de bajo coste, derivados de imágenes RGB, funcionan mejor que los formulados a partir de imágenes multiespectrales.

Cabe destacar que el Ingro. Abdelhalim KHALED ELAZAB, desde su llegada a Barcelona se ha integrado perfectamente en nuestro equipo. Ha colaborado de forma autónoma en la realización de su tesis doctoral, participando en el diseño de los mismos, toma de datos, análisis químicos, tratamientos estadísticos, elaboración de tablas y figuras, discusión de los resultados y redacción de las publicaciones. El doctorando ha demostrado una gran capacidad de trabajo, ya sea en el invernadero (en lisímetros) como en el campo o realizando análisis de laboratorio. Fruto de estos años de trabajo ha alcanzado un gran conocimiento sobre el tema, además de haber demostrado una gran iniciativa y perseverancia.

Y para que así conste a los efectos oportunos,

Dr. José Luis Araus Ortega y Dra. María Dolores Serret Molins

Chapter 2

Root traits and δ^{13}C and δ^{18}O of durum wheat under different water regimes

Elazab A, Molero G, Serret MD, Araus JL (2012). *Functional Plant Biology* **39**, 379-393.

Durum wheat genotypes were grown in lysimeters in the Experimental Field Facilities of the Faculty of Biology, University of Barcelona. 2010/2011 cycle.

Resumen

En este capítulo se estudiaron el crecimiento de las plantas de trigo duro, las características de las raíces y la composición isotópica de carbono ($\delta^{13}C$) y oxígeno ($\delta^{18}O$). Los lisímetros se ubicaron en los invernaderos de los Campos Experimentales de la Universidad de Barcelona y en ellos se cultivaron cuatro líneas endogámicas recombinantes (seleccionadas por su buena adaptación agronómica) en dos condiciones de riego: (WW, por sus siglas en inglés) y estrés hídrico (WS) hasta el estadio de medio llenado del grano. Se midieron los parámetros de intercambio de gases en la hoja bandera justo antes de la cosecha. Posteriormente, se evaluaron la materia seca aérea (DM aérea), la densidad del aparato radicular en términos de peso (RWD) y de longitud (RLD) y la longitud específica de la raíz (SRL, todos ellos por sus siglas en inglés), y se analizaron la composición isotópica de carbono y oxígeno ($\delta^{13}C$ y el $\delta^{18}O$) de las raíces, la hoja bandera y la espiga. Bajo condiciones de estrés hídrico se redujo la conductancia estomática, la transpiración acumulada de la planta y la DM aérea, mientras que se incrementó la $\delta^{13}C$ y la $\delta^{18}O$. Se encontraron diferencias debidas al genotipo en los parámetros de intercambio de gases, morfología de raíces y firmas isotópicas. La DM aérea se correlacionó positivamente con la RLD, con independencia del régimen hídrico, mientras que se correlacionó negativamente con el $\delta^{13}C$ y el $\delta^{18}O$, pero únicamente en condiciones de WW. Por otra parte, la RWD y la RLD se correlacionaron negativamente tanto con el $\delta^{13}C$ como con el $\delta^{18}O$ en condiciones de WW, pero no se observó un patrón claro en condiciones de WS. Nuestro estudio apoya el uso del $\delta^{13}C$ y el $\delta^{18}O$ como criterios indirectos para la selección de características de las raíces asociadas a una mayor captación de recursos y crecimiento en ausencia de estrés hídrico.

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Root traits and $\delta^{13}C$ and $\delta^{18}O$ of durum wheat under different water regimes

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Abstract. Plant growth, root characteristics and the stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) composition were studied in durum wheat. Four recombinant inbred lines with good agronomic adaptation were grown under well watered (WW) and water stress (WS) conditions until mid-grain filling in lysimeters. Gas exchange was measured in the flag leaf just before harvest and then the aerial dry matter (Aerial DM), root weight density (RWD) and root length density (RLD) and the specific root length (SRL) were evaluated and the $\delta^{13}C$ and $\delta^{18}O$ of the roots, the flag leaf blade and the spike were analysed. Water stress decreased stomatal conductance, plant accumulated transpiration and Aerial DM, whereas $\delta^{13}C$ and $\delta^{18}O$ increased. Genotypic differences were found for all gas-exchange and root traits and isotope signatures. Aerial DM was positively correlated with RLD, regardless of the water regime, whereas it was negatively correlated with $\delta^{13}C$ and $\delta^{18}O$, but only under WW conditions. Moreover, RWD and RLD were negatively related to both $\delta^{13}C$ and $\delta^{18}O$ under the WW regime, but no clear pattern existed under WS. Our study supports the use of $\delta^{13}C$ and $\delta^{18}O$ as proxies for selecting root traits associated with higher growth in the absence of water stress.

Additional keywords: leaf gas exchange, lysimeter, *Triticum turgidum* ssp. *durum*, root traits, $\delta^{13}C$, $\delta^{18}O$.

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Introduction

Durum wheat is the most cultivated herbaceous crop in the south and east Mediterranean basin (FAO 2008), where a lack of water is the main factor limiting its productivity. This problem will increase even more in the future in view of the gloomy predictions for the climate (of lower precipitation and higher temperatures), together with social changes (e.g. population growth and more demand from industry and tertiary activities such as tourism) that will lead to more and more competition for already scarce water resources (Araus 2004). Although developing durum wheat cultivars with improved adaptation to drought may be a sound avenue to mitigate lack of water, breeding for drought adaptation is a challenging exercise, since yield potential and adaptation to abiotic stress are very complex traits, integrating mechanisms affecting plant growth and development at different levels of organisation (molecular, biochemical, physiological and ecological) (Araus *et al*. 2002; Lafitte *et al*. 2003). Identification of physiological and morphological traits (secondary traits), based on a good understanding of the mechanisms determining crop yield potential and drought adaptation, may be an effective approach to breeding for higher yield in a wide range of environments and could be a valuable strategy for use either in conventional or molecular breeding (Araus *et al*. 2002, 2008; Razmjoo *et al*. 2008). However, the lack of physiological understanding of the traits conferring adaptation to drought and determining yield potential, as well as the proper phenotypic tools, are perceived as the main factors limiting current breeding programs (Lafitte *et al*. 2003; Slafer and Araus 2007; Araus *et al*. 2008; Richards *et al*. 2010).

One of the few examples of successful integration of a physiological tool in a breeding program deals with the use of carbon isotope composition ($\delta^{13}C$; frequently expressed as discrimination, $\Delta^{13}C$) to produce commercial bread wheat cultivars better adapted to the Mediterranean conditions of Australia (Condon *et al*. 2002; Rebetzke *et al*. 2006). Thus, $\delta^{13}C$ of dry matter is a powerful tool that provides a time-integrated record of the ratio of intercellular to ambient CO_2 (C_i/C_a) and thus of the water use efficiency (WUE) either assessed instantaneously, as the ratio of net CO_2 assimilation to transpiration, or agronomically, as dry matter accumulated to total water transpired (Farquhar and Richards 1984; Farquhar *et al*. 1989; Ehdaie *et al*. 1991; Monneveux *et al*. 2005; Slafer and Araus 2007). According to different authors (Condon and Richards 1992; Cregg and Zhang 2000; Rebetzke *et al*. 2006) the use of $\delta^{13}C$ has many advantages, including a high heritability, substantial genetic variation and a small genotype × environment interaction.

The stable oxygen isotope composition ($\delta^{18}O$) of plant tissues integrates the evaporative conditions throughout the crop cycle (Barbour *et al.* 2000). Therefore it has been proposed as a proxy method for measuring plant transpiration (Cernusak *et al.* 2007, 2009*b*; Sheshshayee *et al.* 2005; Cabrera-Bosquet *et al.* 2009*b*) as well as for reflecting genotypic differences in stomatal conductance (g_s) in wheat (Barbour *et al.* 2000; Ferrio *et al.* 2007). When analysed in dry matter, δ^{18}O might provide a time-integrated assessment of g_s and transpiration, therefore, avoiding the intrinsic limitation of instantaneous measurements of these gas-exchange traits, which exhibit daily patterns and may change dramatically in few days as water stress progresses. Moreover, the combined measurements of δ^{13}C together with δ^{18}O of the same plant tissues may help to elucidate whether variations in δ^{13}C are driven either by changes in photosynthetic capacity (A) or due to g_s, since the δ^{18}O signature of the leaf tissue reflects the transpiration rate and it is not expected to reflect changes in A (Barbour and Farquhar 2000; Farquhar *et al.* 2007; Tcherkez and Farquhar 2008). However, compared with δ^{13}C, studies dealing with the use of δ^{18}O to evaluate genotypic performance of wheat under different water regimes are scarce (Cabrera-Bosquet *et al.* 2009*a*) and the results have not always been encouraging (Ferrio *et al.* 2007). The pioneering study on the use of δ^{18}O on wheat was performed under well irrigated conditions, which have implications for potential yield rather than stress adaptation (Barbour *et al.* 2000). More recently, Cabrera-Bosquet *et al.* (2009*b*) reported in maize a negative relationship between δ^{18}O and grain yield across genotypes under fully watered and moderate water stress conditions, whereas under severe water stress conditions the relationship between δ^{18}O and grain yield tended to be positive.

Plant roots have an important role in growth and development of plants as they contribute to water and nutrient uptake, thereby sustaining photosynthetic and transpiratory gas exchange and plant growth. Despite the huge number of studies conducted on the aboveground biomass and its response to water deficit, the number of studies on roots is comparatively small. The considerable labour associated with measuring root traits and the scarcity of reliable and efficient methods are bottlenecks for breeding (Passioura 1983; Robinson 2004; Gregory 2006; Gregory *et al.* 2009; Zhang *et al.* 2009; Richards *et al.* 2010). This not only limits the routine evaluation of root traits in high throughput phenotyping under field conditions, but even prevents clear formulation of the ideotypes that are the most suited for the given environmental conditions, or a wider adoption of the use of indirect (or secondary) traits for crop phenotyping such as the stable isotopes. Further, it may be considered that roots remain as the hidden part of the plant despite the fact they play a basic role in plant water relations.

Root weight density (RWD) and root length density (RLD) and specific root length (SRL) are frequently used in root studies (Ostonen *et al.* 2007; Zhang *et al.* 2009; Song *et al.* 2010). RWD and RLD describe the amount of root weight and root length, respectively, within the soil and are particularly useful traits for describing the spatial distribution of the roots system in the soil profile. RLD currently is the most used trait in describing root distribution (Carvalho 2009; Zaman-Allah *et al.* 2011) and it is an important parameter to model water and nutrient uptake (Barraclough 1989; King *et al.* 2003; Carvalho 2009). Thus, Barraclough (1989) found in field experiments with bread wheat that a RLD below 1 cm cm^{-3} clearly decreased water uptake by the plant. According to Passioura (1983) and Gregory *et al.* (1992) a higher RLD is associated with higher water absorption from the soil and consequently improved performance of cereal crops.

SRL characterises the pattern of biomass allocation into root length (Ryser 2006). It gives an indication of root thickness, with thinner roots having a higher SRL (Eissenstat 1992). However, the results reported in the literature concerning the effect of low soil resource availability (water and nutrients) on SLR are contradictory (Ryser 1998). In a soil column experiment, Baburai Nagesh (2006) observed an increase of SRL in winter wheat under drought conditions. In the same sense, Song *et al.* (2010) in a pot experiment with spring wheat found a gradient increase in SRL from well watered to severe water stress. However, these findings opposed those by Kage *et al.* (2004) in cauliflower, which showed that SRL decreased under drought stress conditions leading to a higher dry matter deposition in the fine root fraction.

An indirect screening of large numbers of genotypes for deeper roots that increase water supply to the growing grains may be feasible by selecting lines for specific shoot traits after flowering (Richards *et al.* 2010). For example, selection can be made for genotypes that maintain green leaf area, have a high g_s, low canopy temperature or low δ^{13}C. These traits are expected to be good indicators of which genotypes have deep roots in moist soil (Olivares-Villegas *et al.* 2007; Christopher *et al.* 2008; Lopes and Reynolds 2010). However, studies that relate genotypic variability in δ^{13}C and root characteristics are scarce (White *et al.* 1990; Monti *et al.* 2006; Lopes and Reynolds 2010) and, to the best of our knowledge, nothing has been published concerning δ^{18}O and root traits despite the fact that both δ^{13}C and δ^{18}O in dry matter have been proposed as proxies for long-term WUE and transpiration respectively. Even for studies relating δ^{13}C and root traits, the results are contradictory. Thus, White *et al.* (1990) found in common bean that under rainfed conditions, genotypes with higher δ^{13}C exhibited lower RLD, but under well watered conditions the relationship was absent. Monti *et al.* (2006) reported in sugar beet a negative relationship between δ^{13}C of different plant parts and total root dry matter per plant when all the treatments were combined together; with δ^{13}C of roots being better related than δ^{13}C of leaves. These authors conclude that root δ^{13}C was a reliable indicator of root dry weight. However they only studied a single cultivar under different water regimes. In a recent study with field wheat, Lopes and Reynolds (2010) did not find that root traits (root DM at depth in their case) were related to Δ^{13}C, despite the fact that they found that canopy temperature at grain filling was negatively correlated with root DM at depth and positively correlated with grain δ^{13}C and that this isotope composition was negatively correlated with grain yield.

The aim of the present study was to assess the effect of water regime and genotype variability on root morphological and distributional traits, stable carbon and oxygen isotopes and plant growth and to look for the potential relationships existing among these traits within each water regime. A proper understanding on the relationships between root traits and stable isotopes may help the use of δ^{13}C and δ^{18}O in a more effective manner. Measurements were performed during middle grain filling since root vigour that results in greater water capture around flowering and grain filling has the potential to significantly increase grain yield (Passioura 2006). Following Richards *et al*. (2010), evaluation must be done in a managed environment where there is little late season rainfall and lines are grown with a receding soil moisture supply. The rationale behind this is that the ability to extract water from the soil under increasing soil water deficit is a major attribute of drought adaptation (Araus *et al*. 2008; Blum 2009; Richards *et al*. 2010).

Materials and methods

Plant materials and growth conditions

Four durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.) genotypes were used in this experiment. Two genotypes (RIL2108 and RIL2510) belong to a population of 112 recombinant inbred lines (RILs) derived by single seed descent from the cross ICD-MN91–0012 between the drought resistant land race, Jennah Khetifa and the commercial variety Cham 1. This cross was made in 1991 at the Tel Hadya research station (Aleppo province, Syria) by the CIMMYT/ICARDA durum breeding program for Mediterranean dryland (Nachit *et al*. 2001). The other two genotypes (KS194 and KS230) are recombinant lines from a population of 249 RILs produced by Società Produttori Sementi (Bologna, Italy) from the elite durum cvv. Svevo (S) and Kofa (K) (Maccaferri *et al*. 2008). Both Svevo and Kofa are well adapted to the Mediterranean climate. References of RILs are found in the European INCO-MED Tritimed project (http://www.rothamsted.bbsrc.ac.uk/cpi/tritimed/indexcontent1.html, accessed May 2011). These RILs were selected based in their good performance under Mediterranean conditions.

The experiment was carried out in the Experimental Field facilities of the Faculty of Biology, University of Barcelona, Spain, from the end of October 2009 to the end of March 2010. Plants were grown until mid-grain filling in lysimeters, each one consisting of a PVC tube of 0.16 m in diameter and 1 m long. Inside each PVC tube a polyethylene bag of 0.115 m in diameter and 0.85 m long was filled with coarse A13 perlite-sand aggregates for the lower 0.45 m and with fine B10 perlite-sand aggregates for the upper 0.40 m (A13 and B10 are different commercial sizes of perlite-sand aggregates; EUROPERL, Passau, Germany). The bottom end of the polyethylene bags was cut and supported by a plastic mesh to enhance water drainage and keeping perlite from being lost. Within each lysimeter, four seeds of a given genotype were sown to achieve a plant density of 385 plants m^{-2}, within the range of a real field plant density. Control lysimeters without plants were used to calculate the evaporation from the substrate surface. The experiment was arranged as a factorial randomised complete block design in four replicates with a total number of 40 lysimeters ((four genotypes + one control lysimeter without plants) × two water regimes × four replicates). Plants were placed under a rain shelter to control water regimes properly: the rest of the environmental variables were not controlled. Therefore, the plants were exposed to temperature conditions following the seasonal pattern (Table 1).

Table 1. Monthly averaged maximum (T_{max}) and minimum (T_{min}) air temperature (°C) and RH during the growing period

Month	T_{max} (°C)	T_{min} (°C)	RH (%)
October	22.9	14.7	68.5
November	19.0	10.0	66.5
December	13.9	6.1	68.7
January	11.7	4.9	71.3
February	13.1	4.9	66.0
March	14.4	6.5	68.6
Average	13.3	5.6	68.7

Irrigation was performed with half strength Hoagland's nutrient solution (Hoagland and Arnon 1938). The day before sowing the lysimeters were over saturated with half strength Hoagland's nutrient solution and then they were left for one day to drain the excess water. Container water capacity was calculated as the difference between the weights of the lysimeter with wet substrate (after drainage of excess water) and with dry substrate. For the well watered regime (WW), the lysimeters were maintained above 80% container capacity until harvest by adding the nutrient solution every ~4 days. For the water stress regime (WS), irrigation was stopped 37 days after sowing (corresponding with the beginning of tillering; the main stem + one or two tillers appeared) until substrate reached 30% of the lysimeter total water capacity (~130 days after sowing) and then lysimeters were maintained at this water content during the next 2 weeks, after which the plants were harvested. The idea underlying such a slow decrease in water content (Fig. 1) was to simulate the field conditions in a winter planting under Mediterranean conditions and avoiding the pattern typical of pot plants, where severe water stress develops in a few days. Plants were harvested four weeks after anthesis.

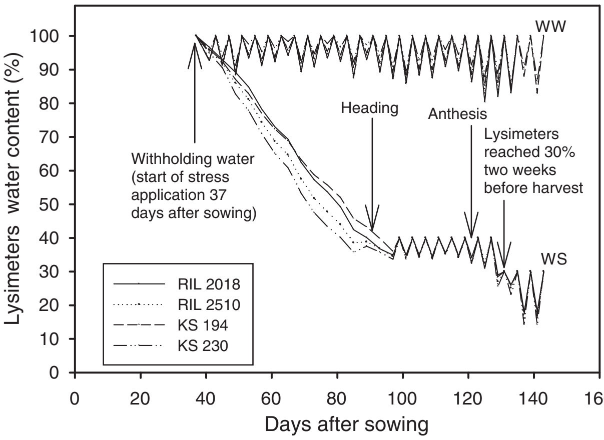

Fig. 1. Water content (%) in the lysimeters from the beginning of stress application until the end of the experiment. Plant phenology and the imposition of water stress are indicated within the figure. WW, well watered; WS, water stressed lysimeters.

Leaf gas-exchange measurements

Leaf gas-exchange measurements were conducted 3 weeks after anthesis (1 week before harvest). Measurements were made with an open IRGA Li-Cor 6400 system (Li-Cor Inc., Lincoln, NE, USA), between 10.00 and14.00 hours (solar time), under light saturated conditions (1500 μmol m^{-2} s^{-1}), 25°C, CO_2 concentration of 400 μmol mol^{-1} and a RH between 60–70%. The gas-exchange parameters measured were net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (g_s) and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a). Further the instantaneous water use efficiency ($WUE_{Instant}$) was calculated as the ratio A_{sat}/E.

Plant growth and root traits

Days to heading were measured as the days from sowing until the emergence of about half of the spike was apparent in at least 50% of the plants inside each lysimeter. Plant height, determined as the distance from substrate surface to the top end of the spikes and the number of tillers per plant, was determined 1 day before harvest. At harvest, flag leaf blades of all plants were cut and scanned (Canon PIXMA/MP140 scanner, Fukushima, Japan), images obtained at 300 dpi and the area calculated using commercial software (Digimizer 3.7, 2009, MedCalc Software, Mariakerke, Belgium). Afterwards the rest of the aboveground plant biomass was harvested and divided into three components: spikes, rest of the leaf blades and culms. Then they were dried at 60°C for 48 h and weighed. Total aerial dry matter (Aerial DM) was calculated as the total sum of spike dry matter (Spike DM), culm dry matter (Culm DM) and leaf dry matter (Leaf DM) including the flag leaf blades.

The substrate column (the polyethylene bag filled with perlite) was cut horizontally into three equal sections (of ~0.26 m each, as perlite compacted after irrigation): the upper section (0–0.26 m); the mid section (0.26–0.52 m); and the bottom section (0.52–0.78 m). Roots were cleaned of substrate with tap water, further rinsed with distilled water and then placed inside a paper envelope and dried at 60°C for 48 h. Root dry matter (Root DM) was measured for each of the three sections separately. Total root dry matter (TRoot DM) was calculated as the sum of Root DM of the three sections. Further, the ratio of Aerial DM to TRoot DM (Aerial DM/TRoot DM) was calculated.

Root weight density at the three different soil sections (RWD_{sec}, g m^{-3}) was calculated following Ma *et al.* (2000):

$$RWD_{sec} = \frac{\text{Root } DM_{sec}}{\Pi{*}PBR^2{*}PBL}, \tag{1}$$

where Root DM_s is the root dry matter in the soil section (g); PBR is the polyethylene bag radius (0.0575 m); and PBL is the polyethylene bag length of the section (0.26 m long).

The average (i.e. for the entire lysimeter) root weight density (RWDa) was calculated from the values of each of the three sections as follows:

$$RWDa = RWD_{sec1} \times \frac{\text{Root } DM_{sec1}}{\text{TRoot DM}} + RWD_{sec2} \times \frac{\text{Root } DM_{sec2}}{\text{TRoot DM}} + RWD_{sec3} \times \frac{\text{Root } DM_{sec3}}{\text{TRoot DM}}. \tag{2}$$

Specific root length at each of the three soil sections (SRL_{sec}, m g^{-1}) was determined in fresh roots as follows: 10 root segments (each one 0.20–0.25 m long) were sampled and their total lengths were measured with a ruler. Then the root segments were dried at 60°C for 48 h and weighed. Specific root length of each soil section was calculated following Głąb (2007) and Carvalho (2009):

$$SRL_{sec} = \frac{\text{TRoot } L_{seg}}{\text{TRoot } DM_{seg}}, \tag{3}$$

where TRoot L_{seg} is the total length of root segments (m); and TRoot DM_{seg} is the total dry matter of the root segments (g).

Root length of each soil section (Root L_{sec}, m) was estimated according to the following equation:

$$\text{Root } L_{sec} = SRL_{seg} \times \text{Root } DM_{sec}. \tag{4}$$

The total root length (TRoot L) was calculated as the sum of the root lengths of the three sections.

Average specific root length (SRLa) was calculated as follows:

$$SRLa = SRL_{sec1} \times \frac{\text{Root } L_{sec1}}{\text{TRoot L}} + SRL_{sec2} \times \frac{\text{Root } L_{sec2}}{\text{TRoot L}} + SRL_{sec3} \times \frac{\text{Root } L_{sec3}}{\text{TRoot L}}. \tag{5}$$

Root length density in each soil section (RLD_{sec}, m m^{-3}) was calculated following Głąb (2007) and Carvalho (2009):

$$RLD_{sec} = \frac{\text{Root } L_{sec}}{\Pi \times PBR^2 \times PBL}. \tag{6}$$

The average root length density (RLDa) was calculated as:

$$RLDa = RLD_{sec1} \times \frac{\text{Root } L_{sec1}}{\text{TRoot L}} + RLD_{sec2} \times \frac{\text{Root } L_{sec2}}{\text{TRoot L}} + RLD_{sec3} \times \frac{\text{Root } L_{sec3}}{\text{TRoot L}}. \tag{7}$$

Relative water content, cumulative transpiration and WUE

Relative water content (RWC) was measured in the same flag leaf blades scanned at harvest. For each blade, the central portion was cut and its fresh weight (FW) measured; then it was placed for 24 h at 4°C in a test tube containing distilled water and covered with aluminium foil. Its turgid weight (TW) was measured and then it was placed in a paper envelope and dried at 60°C for 48 h and its dry weight (DW) was measured. The RWC was then calculated according to Schonfeld *et al.* (1988):

$$RWC(\%) = [(FW - DW)/(TW - DW)] \times 100. \tag{8}$$

The amount of water evapotranspired in each lysimeter (W_{et}) was calculated during the growth cycle by weighing the lysimeters just prior watering. Simultaneously, direct evaporation from the soil surface (W_{ev}) was calculated by weighing the control lysimeters without plants. Then the cumulative transpiration (T_{cum}) per single plant between consecutive waterings was calculated as (W_{et} – W_{ev}) divided by the number of plants in the lysimeter. Further, the time-integrated water use efficiency of the plant was calculated as the ratio of Aerial DM to the total T_{cum} during crop growth ($\mathrm{WUE}_{\mathrm{Aerial\ DM}}$). In addition, the plant cumulative transpiration per unit of Root DM was calculated as the ratio of T_{cum} to TRoot DM (T_{cum}/TRoot DM).

N concentration and stable isotope analysis

Dry mater of spikes, flag leaf blades and roots (only from the lower substrate section) were finely milled and samples of ~1 mg were weighed into tin capsules for $^{13}C/^{12}C$ ratio (R) analysis and other samples of ~1 mg were weighed into silver capsules for $^{18}O/^{16}O$ ratio analysis.

The total N concentration and the stable carbon isotope analyses were conducted at the Scientific Facilities of the University of Barcelona. Analyses were performed using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Schwerte, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan), operating in continuous flow mode. The $^{13}C/^{12}C$ ratios (R) of plant material were expressed in δ notation (Coplen 2008), as carbon isotope composition ($\delta^{13}C$):

$$\delta^{13}\mathrm{C}(‰) = \left[\left(\frac{\mathrm{R}_{\mathrm{sample}}}{\mathrm{R}_{\mathrm{standard}}}\right) - 1\right] \times 1000, \qquad (9)$$

where sample refers to plant material and standard to Pee Dee Belemnite (PDB) calcium carbonate. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 3, polyethylene foil; IAEA CH6, sucrose; USGS 40, L-glutamic acid) were used with an analytical precision of 0.1‰.

The analysis of the ^{18}O stable isotope was conducted at Iso-Analytical Limited Laboratory, (Crewe, Cheshire, UK). The $^{18}O/^{16}O$ ratios of the different plant parts were determined by an isotope ratio mass spectrometer (IRMS, Europa Scientific Geo 20–20) with a triple Faraday cup collector array to monitor the masses 28, 29 and 30. Results were also expressed in δ notation as oxygen isotope composition ($\delta^{18}O$) values:

$$\delta^{18}\mathrm{O}(‰) = \left[\left(\frac{\mathrm{R}_{\mathrm{sample}}}{\mathrm{R}_{\mathrm{standard}}}\right) - 1\right] \times 1000, \qquad (10)$$

using secondary standards of known $^{18}O/^{16}O$ ratios (IAEA-CH-6, sucrose; IAEA-C-3, cellulose; IAEA-601, benzoic acid) calibrated against Vienna Standard Mean Oceanic Water (VSMOW) with an analytical precision of 0.2‰.

Statistical analyses

The hypothesis of zero difference between means was tested with analysis of variance (ANOVA) performed using the general linear model (GLM) procedure to calculate the effects of water regime, genotype and water regime by genotype interactions on the measured and calculated parameters. Mean separation of genotypes for the different parameters was performed by a Tukey-b multiple comparison test ($P < 0.05$). Relationships between parameters were analysed within each water regime (WS, WW) alone and both combined using Pearson linear correlation.

Data were analysed using the SPSS 16 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using SigmaPlot 11.0 for Windows (Sysat Software Inc., Point Richmond, CA, USA).

Results

The effect of water regime and genotype on growth parameters

Significant genotypic differences were found for all the growth parameters depicted in Table 2 and Table S1 (available as Supplementary Material to this paper). Water regime also had a significant effect for all studied traits except for plant height and TRoot DM. WS significantly decreased the Aerial DM and its different components, particularly Leaf DM, which decreased by ~55% compared with WW plants. Nitrogen concentration was also negatively affected by water regime. The genotype by water regime interaction was significant for Culm DM, plant height, tillers plant^{-1} and flag leaf area. In general, genotypic differences were more evident under WS than WW conditions, with genotype KS194 showing the lowest values for most of the studied traits except for tillers plant^{-1}.

The effect of water regime and genotype on photosynthetic and transpiratory gas exchange, RWC and WUE

Significant genotypic differences existed for all the traits included in Table 3, except C_i/C_a and RWC. Water regime significantly affected all studied traits except RWC. Thus, WS significantly decreased g_s, E and T_{cum}, by around 50% compared with WW plants, whereas A decreased ~30% and $\mathrm{WUE}_{\mathrm{Instant}}$ and $\mathrm{WUE}_{\mathrm{AerialDM}}$ increased. The genotype by water regime interaction was significant for all studied traits except for RWC, T_{cum} and $\mathrm{WUE}_{\mathrm{Instant}}$.

Effect of water regime and genotype on carbon and oxygen stable isotope composition

Significant genotypic differences existed for $\delta^{13}C$ and $\delta^{18}O$ in the different studied plant organs (Table 4). Water regime significantly affected $\delta^{13}C$ and $\delta^{18}O$ in all the studied plant organs except for roots $\delta^{18}O$. Compared with WW plants, WS increased (in absolute values) the $\delta^{13}C$ of the spikes, flag leaf and roots by 1.72, 1.15 and 0.35‰, respectively, and the $\delta^{18}O$ of the same organs by 0.64, 1.46 and 0.22‰ respectively. In addition, significant differences emerged between the different studied plant organs for both $\delta^{13}C$ and $\delta^{18}O$ under each water regime. Thus, $\delta^{13}C$ of all genotypes in both water regimes followed a similar pattern, where spikes showed the highest value followed by roots and flag leaves respectively. Concerning $\delta^{18}O$ and except for KS230 where the flag leaf was the most enriched in $\delta^{18}O$, the other three genotypes showed a similar gradient of enrichment in $\delta^{18}O$ from roots to spikes regardless the water regime. The genotype by treatment interaction was significant for all studied plant organs except for roots $\delta^{13}C$.

The effect of water regime and genotype on root traits

Highly significant genotypic differences existed for all studied root traits in all substrate sections (Table 5) as well as for the averaged (i.e. entire lysimeter) values (see Fig. S1, available as Supplementary Material to this paper). Water regime also caused highly significant differences for all studied root traits, except for RWDa and the RWD for the upper section (RWD1). Generally, under both water regimes in all soil sections,

Table 2. Effect of water regime and genotype on different studied growth parameters of individual plants

For each genotype and treatment data shown are the means of the four replications. Means followed by different letters were significantly different ($P < 0.05$) by Tukey's b test. WW, well watered plants; WS, water stressed plants; G, genotype; T, treatment; G × T, genotype by treatment interaction. The associated sum of squares type III and probabilities (ns, not significant; *, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.001$) are shown

T	G	Aerial DM (g plant^{-1})	TRoot DM (g plant^{-1})	Aerial DM/TRoot DM	Plant height (cm)	Tillers plant^{-1} (No. plant^{-1})	Days to heading (Days)	Flag leaf area (cm^2)
WW	KS194	3.58a	0.31a	11.39ab	58.44b	3.44a	88.25a	30.01a
	KS230	3.89a	0.32a	12.39b	54.08a	3.13a	93.75b	33.24a
	RIL2108	4.53a	0.48b	9.66a	68.96c	3.00a	89.25a	45.33b
	RIL2510	4.50a	0.49b	9.13a	66.81c	3.06a	95.75b	45.61b
	Mean	4.13	0.40	10.64	62.07	3.16	91.75	38.55
WS	KS194	1.80a	0.32a	5.69a	59.55a	1.63b	87.50a	23.55a
	KS230	2.41b	0.42ab	5.81a	58.63a	2.50c	90.75b	22.18a
	RIL2108	2.44b	0.44ab	5.67a	65.93b	1.00a	89.00ab	28.05a
	RIL2510	2.59b	0.55b	4.71a	61.33a	1.25ab	94.50c	25.08a
	Mean	2.31	0.43	5.47	61.36	1.59	90.43	24.71
ANOVA	G	3.66**	0.20***	22.19**	595.41***	3.23*	254.59***	626.22***
	T	26.42***	0.01ns	214.11***	4.07ns	19.53***	13.78*	1531.00***
	G × T	0.41ns	0.02ns	8.41ns	118.35***	2.39*	8.59ns	237.41**

Table 3. Effect of water regime and genotype on the light saturated net CO_2 assimilation rate (A_{sat}); stomatal conductance (g_s); transpiration rate (E); the ratio of intercellular to ambient CO_2 concentration (C_i/C_a); the flag leaf relative water content (RWC); plant cumulative transpiration ($T_{cum,}$); instantaneous water use efficiency ($WUE_{Instant}$) and plant time-integrated water use efficiency calculated as Aerial DM/T_{cum} ($WUE_{Aerial\ DM}$)

For each genotype and treatment data shown are the mean of the four replications. Means followed by different letters were significantly different ($P < 0.05$) by Tukey's b test. WW, well watered plants; WS, water stressed plants; G, genotype; T, treatment; G × T, genotype by treatment interaction. The associated sum of squares type III and probabilities (ns, not significant; *, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.001$) are shown

T	G	A_{sat} (μmol CO_2 m^{-2} s^{-1})	g_s (mmol CO_2 m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})	C_i/C_a	RWC (%)	T_{cum} (dm^3)	$WUE_{Instant}$ (μmol CO_2 mmol H_2O^{-1})	$WUE_{Aerial\ DM}$ (g dm^{-3})
WW	KS194	19.39b	236.7b	3.87c	0.64b	86.4a	1.63a	5.02a	2.20a
	KS230	13.60a	135.4a	2.84a	0.56a	86.2a	1.75a	4.79a	2.22a
	RIL2108	23.65c	266.3b	3.64bc	0.60ab	86.4a	1.62a	6.51b	2.78c
	RIL2510	24.55c	278.8b	3.53b	0.60ab	87.8a	1.78a	6.95b	2.54b
	Mean	20.30	229.3	3.47	0.60	86.7	1.70	5.82	2.43
WS	KS194	14.46ab	110.3ab	1.84a	0.43a	87.5a	0.75a	8.10b	2.39a
	KS230	11.30a	94.2a	1.81a	0.49a	87.8a	0.98b	6.24a	2.48a
	RIL2108	15.07b	118.1ab	1.66a	0.44a	85.4a	0.71a	9.09b	3.46b
	RIL2510	17.10b	149.2b	1.77a	0.44a	87.5a	0.78a	9.77b	3.33b
	Mean	14.48	117.9	1.77	0.45	87.0	0.81	8.30	2.91
ANOVA	G	322.36***	43466***	1.15**	0.00ns	12.8ns	0.19*	39.20***	4.15***
	T	270.61***	99233***	23.16***	0.18***	1.0ns	6.34***	49.33***	1.83***
	G × T	46.82**	13663**	1.26**	0.02**	8.6ns	0.05ns	3.09ns	0.54***

RIL2510 tended to show the highest values and KS194 the lowest values for almost all studied roots traits. Compared with the WW regime, WS clearly decreased the SRL and RLD of the three soil sections but tended to increase the RWD of the upper and middle soil sections and to decrease that of the lower section. The genotype × treatment interaction was significant for all studied root traits in different soil sections, except for the root weight density of the middle (RWD2) and lower sections (RWD3) and the specific root length in the upper section (SRL1).

Under the WS regime, the RWD and RLD of the four genotypes decreased from the upper to the lower soil section (Table 5). Under the WW regime, RLD decreased from the upper to the middle section and then remained quite similar in the lower section except in genotype KS230, which has the lowest RLD in the middle section, whereas the upper and bottom sections are quite similar. RWD under WW conditions was the highest in the upper soil section followed by the bottom and middle sections, respectively, for all genotypes except for KS194, which showed a gradient decrease in RWD from the upper to the bottom soil section. Regardless of the water regime, SRL values increased from the upper to the middle section and then remained fairly unchanged.

Relationships of gas-exchange traits with aerial biomass and stable isotopes

T_{cum} was positively correlated with Aerial DM ($r^2 = 0.44$, $P < 0.01$ and $r^2 = 0.85$, $P < 0.0001$ for WW plants and both

Table 4. Effect of water regime and genotype on the stable carbon isotope composition (δ^{13}C) and oxygen isotope composition (δ^{18}O) of roots, the flag leaf and the spike

Data shown are means of the four replications of each genotype in each treatment. Means followed by different letters were significantly different ($P < 0.05$) by Tukey's b test. WW, well watered plants; WS, water stressed plants; G, genotype; T, treatment; G × T, genotype by treatment interaction. The associated sum of squares type III and probabilities (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are shown

T	G	δ^{13}C (‰) Roots[A]	δ^{13}C (‰) Flag leaf	δ^{13}C (‰) Spike	δ^{18}O (‰) Roots[A]	δ^{18}O (‰) Flag leaf	δ^{18}O (‰) Spike
WW	KS194	–30.22b	–31.72b	–29.71b	28.45a	29.82bc	30.20a
	KS230	–30.36ab	–31.69b	–29.55b	28.23a	30.56c	29.82a
	RIL2108	–30.16b	–32.52ab	–29.81b	27.55a	28.98ab	30.29b
	RIL2510	–30.64a	–32.83a	–30.21a	28.38a	28.59a	31.04b
	Mean	–30.34b	–32.19a	–29.82c	28.15a	29.44b	30.34c
WS	KS194	–30.02a	–31.26a	–28.81a	28.15a	29.65a	30.84ab
	KS230	–30.25a	–30.76a	–28.73a	28.57a	31.56b	30.11a
	RIL2108	–29.54a	–30.86a	–27.06c	28.28a	31.35b	31.77b
	RIL2510	–30.16a	–31.27a	–27.79b	28.49a	31.03b	31.20b
	Mean	–29.99b	–31.04a	–28.10c	28.37a	30.90b	30.98c
ANOVA	G	0.66*	2.94***	3.13****	3.19****	10.18**	6.30****
	T	0.97***	10.45****	24.24****	0.05ns	26.62****	2.80***
	G × T	0.07ns	1.85**	5.79***	2.33***	11.02**	1.54*

[A] From the lower soil section.

Table 5. Effect of water regime and genotype on the root weight density (RWD), specific root length (SRL) and root length (RLD) in each of the three different soil sections

For each trait, the number following the acronym of the trait refers to the soil section where the trait was estimated: 1 refers to soil upper section (0.00–0.26 m); 2 refers to soil middle section (0.26–0.52 m); and 3 refers to soil bottom section (0.52–0.78 m). Data shown is the mean of the four replications of each genotype in each treatment. Means followed by different letters were significantly different ($P < 0.05$) by Tukey's b test. WW, well watered plants; WS, water stressed plants; G, genotype; T, treatment; G × T, genotype by treatment interaction. The associated sum of squares type III and probabilities (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are shown

T	G	RWD1 ($g\,m^{-3}$)	RWD2 ($g\,m^{-3}$)	RWD3 ($g\,m^{-3}$)	SRL1 ($m\,g^{-1}$)	SRL2 ($m\,g^{-1}$)	SRL3 ($m\,g^{-1}$)	RLD1 ($m\,m^{-3}\,10^{-4}$)	RLD2 ($m\,m^{-3}\,10^{-4}$)	RLD3 ($m\,m^{-3}\,10^{-4}$)
WW	KS194	262.0a	107.4a	96.3a	22.9a	29.7a	27.0a	0.60a	0.32a	0.26a
	KS230	213.8a	106.5a	147.2b	29.0b	43.1bc	42.4b	0.62a	0.45a	0.63c
	RIL2108	447.1b	125.0a	134.2b	24.3a	37.4b	33.0ab	1.09b	0.47a	0.43b
	RIL2510	378.6b	160.4b	191.7c	26.1ab	47.5c	38.3ab	0.99b	0.76b	0.73c
	GMean	325.4	124.8	142.4	25.6	39.4	35.2	0.82	0.50	0.51
WS	KS194	272.2a	132.4a	71.3a	16.3a	22.5ab	25.5a	0.45a	0.30a	0.18a
	KS230	323.1ab	161.1b	134.2b	23.4b	28.5b	26.0a	0.76b	0.46b	0.35b
	RIL2108	372.1ab	150.9ab	125.9b	17.5ab	22.0a	21.6a	0.63ab	0.33a	0.28ab
	RIL2510	472.1b	194.5c	148.1b	21.0ab	24.8ab	25.4a	0.97c	0.48b	0.38b
	GMean	360.0	159.7	119.9	19.6	24.5	24.6	0.70	0.39	0.29
ANOVA	G	180365***	14640***	30710***	204.4***	575.9***	339.2**	0.97***	0.42***	0.53***
	T	9513ns	9740***	4038**	289.7***	1790.9***	890.2***	0.12*	0.10***	0.38***
	G × T	43288*	1137ns	1485ns	3.7ns	242.4**	242.2*	0.38**	0.10***	0.09*

water regimes together respectively; Fig. 2*a*), whereas it was negatively correlated with spike δ^{13}C ($r^2 = 0.41$, $P < 0.01$ and $r^2 = 0.75$, $P < 0.0001$ for WS plants and both water regimes together respectively; Fig. 2*b*) and spike δ^{18}O ($r^2 = 0.41$, $P < 0.01$ and $r^2 = 0.27$, $P < 0.01$ for WS plants and both water regimes together respectively; Fig. 2*c*). $WUE_{AerialDM}$ was positively correlated with the δ^{13}C and δ^{18}O of the spike (see Fig. S2), whereas the correlations with δ^{13}C and δ^{18}O of the other two organs were weaker.

Flag leaf g_s was negatively correlated with flag leaf δ^{13}C ($r^2 = 0.30$, $P < 0.05$, $r^2 = 0.39$, $P < 0.01$ and $r^2 = 0.70$, $P < 0.001$ for WW, WS and both water regimes respectively; Fig. 3*a*), as well as with flag leaf δ^{18}O ($r^2 = 0.62$, $P < 0.001$ and $r^2 = 0.60$, $P < 0.0001$ for WW and both water regimes respectively; Fig. 3*b*). Moreover, flag leaf δ^{18}O was negatively correlated with *E* for WW and both water regimes together (data not shown). However, correlations of both δ^{13}C and δ^{18}O with $WUE_{Instant}$ were weak or inconsistent (data not shown).

Relationships of stable isotopes with aerial biomass

Negative significant correlations were found between Aerial DM with δ^{13}C of the flag leaf ($r^2 = 0.37$, $P < 0.01$ and $r^2 = 0.66$, $P < 0.0001$ for WW and both water regimes together

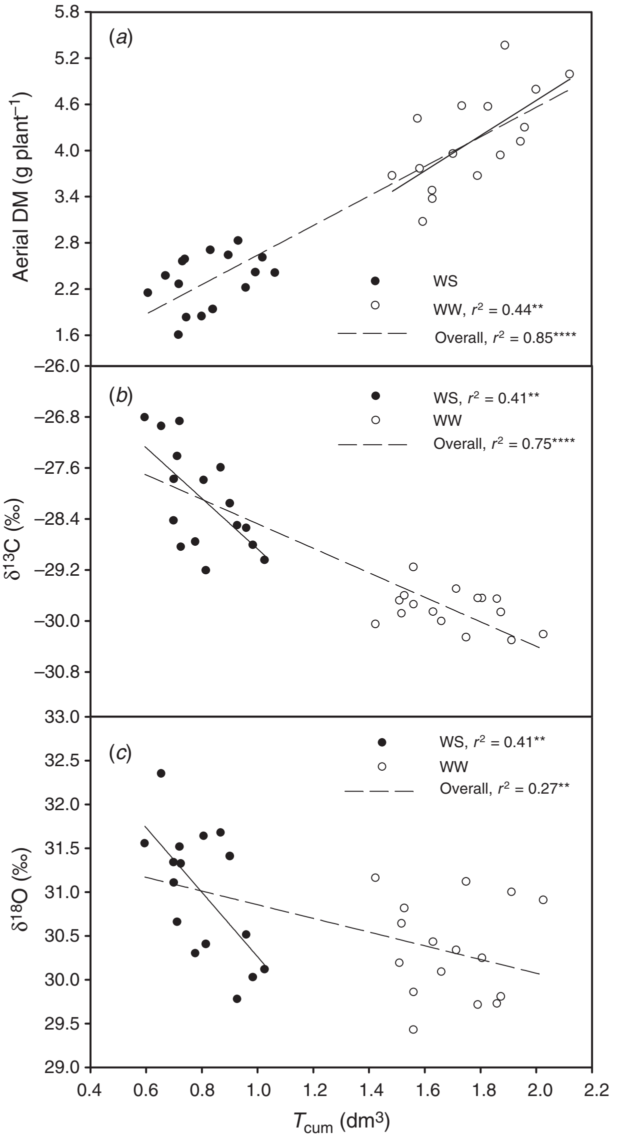

Fig. 2. Relationships between cumulative plant transpiration (T_{cum}) versus (*a*) aerial dry matter (Aerial DM), (*b*) $\delta^{13}C$ of the spike and (*c*) $\delta^{18}O$ of the spike. Data include both well watered (WW, open circles) and water stressed (WS, closed circles) plants. The fitting line is only included for the significant relationships: r^2 and probabilities are shown: **, $P < 0.01$; ****, $P < 0.0001$.

respectively; Fig. 4*a*) and $\delta^{18}O$ of the flag leaf ($r^2 = 0.40$, $P < 0.01$ and $r^2 = 0.41$, $P < 0.0001$ for WW and both water regimes respectively; Fig. 4*b*). Relationships of aerial biomass with the $\delta^{13}C$ and $\delta^{18}O$ of other plant organs were weaker or absent.

Relationship between $\delta^{18}O$ and $\delta^{13}C$

Flag leaf $\delta^{18}O$ was positively correlated with flag leaf $\delta^{13}C$ ($r^2 = 0.41$, $P < 0.01$, $r^2 = 0.63$, $P < 0.001$ and $r^2 = 0.73$, $P < 0.0001$ for WW, WS and both water regimes respectively; (Fig. S3).

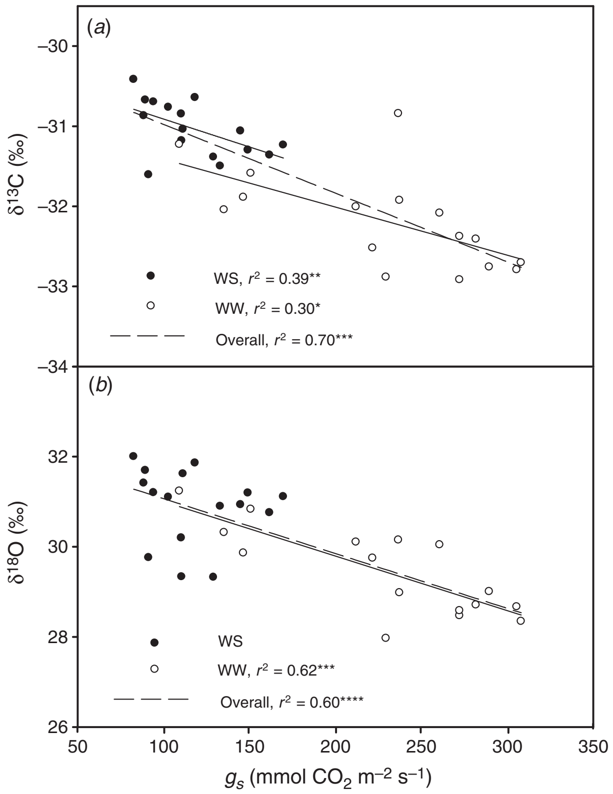

Fig. 3. Relationships between stomatal conductance (g_s) versus (*a*) $\delta^{13}C$ of the flag leaf and (*b*) $\delta^{18}O$ of the flag leaf. Data include both well watered (WW, open circles) and water stressed (WS, closed circles) plants. The fitting line is only included in the significant relationships: r^2 and probabilities are shown: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

Relationships of root traits with aerial biomass

Aerial DM was positively correlated with both RWDa ($r^2 = 0.64$, $P < 0.001$ for plants under WS; Fig. 5*a*) and RLDa ($r^2 = 0.24$, $P < 0.05$ and $r^2 = 0.53$, $P < 0.001$ for WW and WS plants respectively; Fig. 5*b*). A positive correlation between SRLa and Aerial DM was also found over both water regimes but not within each water regime independently ($r^2 = 0.50$, $P < 0.0001$; Fig. 5*c*). Moreover, the RWDs in the different studied soil sections were positively correlated with Aerial DM but only under WS conditions, whereas under WW only the RWD in the bottom soil section was positively correlated with the Aerial DM (Fig. S4*a*–*c*). In the same sense the RLDs of the different studied soil sections were positively correlated with Aerial DM only under the WS regime (Fig. S4*d*–*f*). The SRL of each soil section was positively correlated over both water regimes but not within each water regime (Fig. S4*g*–*i*).

Relationships between stable isotopes and root traits

Negative correlations were found between flag leaf $\delta^{13}C$ with RWDa ($r^2 = 0.44$, $P < 0.01$ for WW plants; Fig. 6*a*), RLDa ($r^2 = 0.53$, $P < 0.001$ for WW; Fig. 6*b*) and SRLa ($r^2 = 0.40$, $P < 0.001$; for both water regimes together; Fig. 6*c*). Negative correlations were also found between flag leaf $\delta^{18}O$ with RWDa

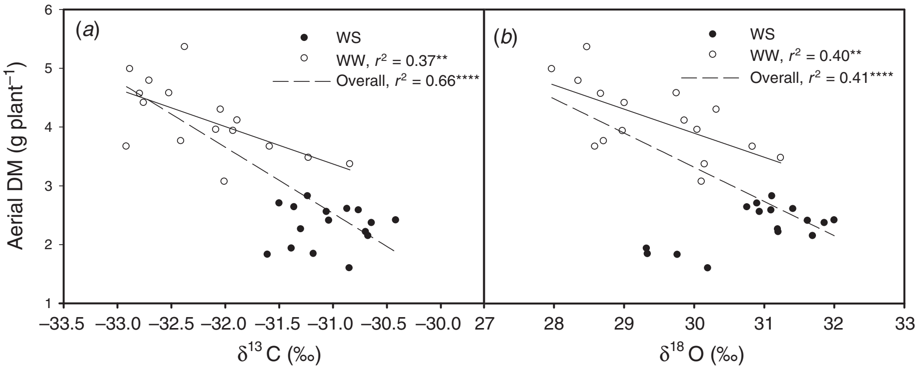

Fig. 4. Relationships of (*a*) δ^{13}C and (*b*) δ^{18}O of the flag leaf versus aerial dry matter (Aerial DM). Data include both well watered (WW, open circles) and water stressed (WS, closed circles) plants. The fitting line is only included in the significant relationships: r^2 and probabilities are shown: **, $P < 0.01$; ****, $P < 0.0001$.

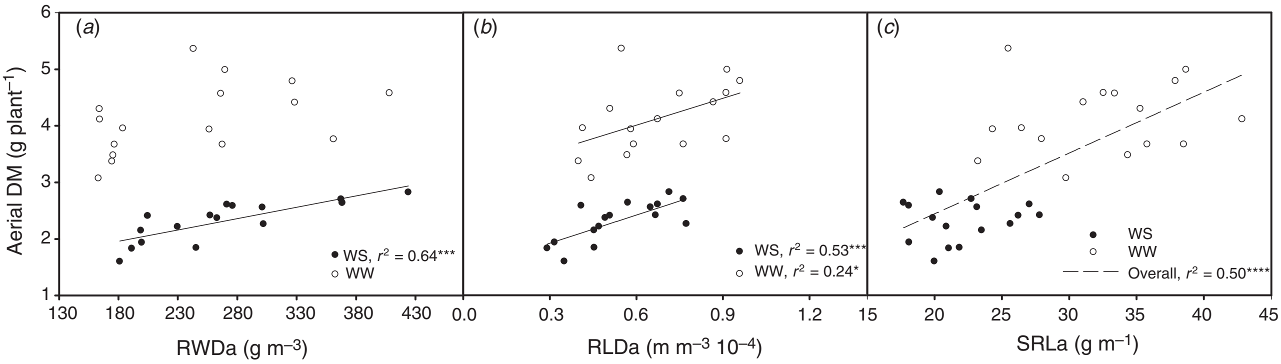

Fig. 5. Relationships of averaged (*a*) root weight density (RWDa), (*b*) root length density (RLDa) and (*c*) specific root length (SRLa) versus aerial dry matter (Aerial DM). Data include both well watered (WW, open circles) and water stressed (WS, closed circles) plants. The fitting line is only included in the significant relationships: r^2 and probabilities are shown: *, $P < 0.05$; ***, $P < 0.001$; and ****, $P < 0.0001$.

($r^2 = 0$ 0.37, $P < 0.01$ for WW plants; Fig. 6*d*), RLDa ($r^2 = 0.39$, $P < 0.01$ for WW plants; Fig. 6*e*) and SRLa ($r^2 = 0.15$, $P < 0.05$; for both water regimes together; Fig. 6*f*). Moreover, under WW conditions the RWD and RLD of the upper and middle soil sections were better correlated with flag leaf δ^{13}C and δ^{18}O than the lower soil section (data not shown). However, under WS conditions no relationships between root traits and δ^{13}C of the flag leaf emerged, whereas δ^{18}O of the flag leaf was weakly but positively correlated with the RLDa ($r^2 = 0.27$, $P < 0.05$, Fig. 6*e*) as well as with RLD of the three different sections and the SRL of the upper soil section (data not shown).

The plant cumulative transpiration per unit of Root DM (T_{cum}/TRoot DM) was negatively correlated with the δ^{13}C of spikes ($r^2 = 0.57$, $P < 0.001$ and $r^2 = 0.54$, $P < 0.0001$ for WS and both water regimes, respectively, Fig. S5*a*). Also, T_{cum}/TRoot DM was negatively correlated with the δ^{18}O of spikes ($r^2 = 0.26$, $P < 0.05$, $r^2 = 0.31$, $P < 0.05$ and $r^2 = 0.36$, $P < 0.001$ for WW, WS and both water regimes, respectively, Fig. S5*b*).

Discussion

Decreases in Aerial DM as well as in the ratio of Aerial DM/TRoot DM and increases in WUE are well known responses of plants to water stress (Zhang 1995; Gregory *et al*. 1997; Tambussi *et al*. 2007). Root growth was less affected (or is even enhanced) by water stress, which is also a well known response (Sharp and Davies 1979; Sacks *et al*. 1997). The decrease in tillers per plant as response to water limitation has been also frequently reported (Carvalho 2009; Yousfi *et al*. 2009).

Although WS plants strongly decreased their transpiring shoot area and transpiration rate on a leaf area basis compared with WW plants, the RWC remained unaffected by water regime, always exhibiting high values. In fact, plants exposed to progressive water stress (the usual situation in real field conditions) may avoid cell dehydration by reducing the transpiring area and the rate of transpiration per unit plant area (Kramer 1983; Cabrera-Bosquet *et al*. 2009*a*).

Stable isotope signatures

Water stress increased δ^{13}C regardless of the plant part considered (Table 4). The spikes showed higher δ^{13}C values than the flag leaf, no matter what water regime was considered, which also agrees with previous studies in wheat (Araus *et al*. 1993; Merah *et al*. 2002). Roots showed intermediate δ^{13}C values, which agrees with evidence from a different C_3 species that roots are ~1‰ ^{13}C

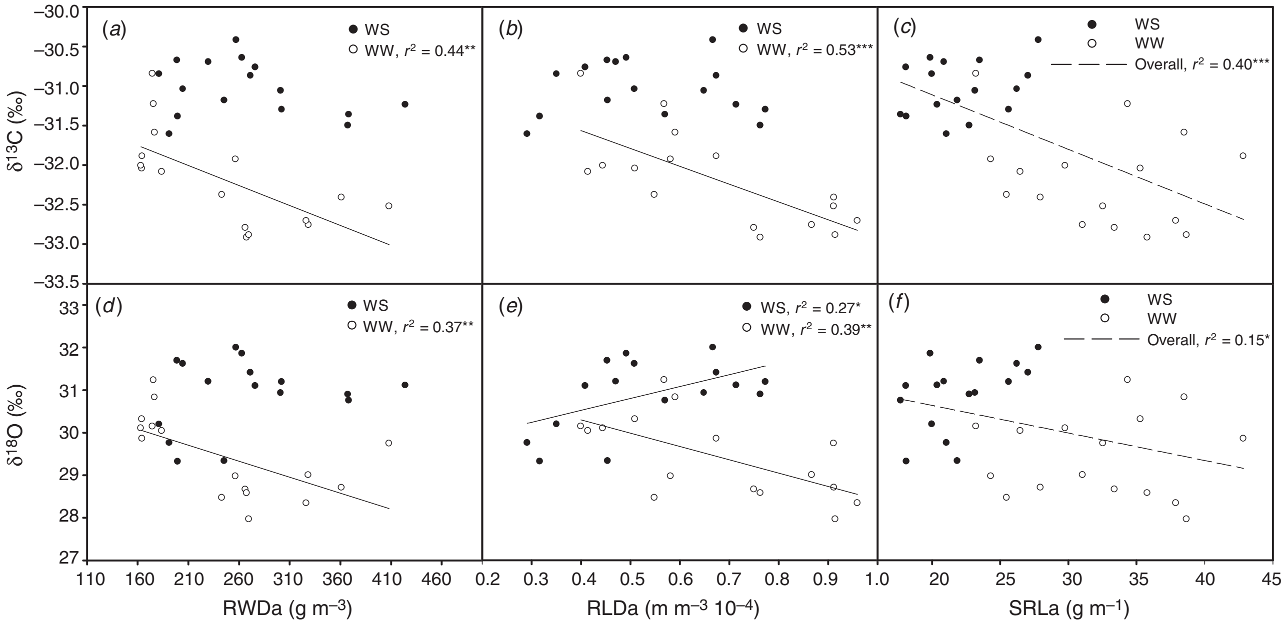

Fig. 6. Relationship between average root weight density (RWDa, left *a*, *d*), average root length density (RLDa, middle *b*, *e*) and average specific root length (SRLa, right *c*, *f*) versus δ^{13}C of the flag leaf. (upper: *a–c*) and δ^{18}O of the flag leaf (lower: *d–f*). Data include both well watered (WW, open circles) and water stressed (WS, closed circles) plants. The fitting line is only included in the significant relationships: r^2 and probabilities are shown: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

enriched compared with leaves (Badeck *et al.* 2005). The provenance of the photosynthetic assimilates or the post-photosynthetic fractionation of stable carbon isotopes during translocation of assimilates to the sink organs may be involved in this process (Badeck *et al.* 2005; Cernusak *et al.* 2009*a*). According to Hobbie and Werner (2004), the sink organs (e.g. roots) are more ^{13}C enriched because the light ^{12}C isotope produced in the source organs (leaves) will be used in synthesis of lignin and lipids and consequently the remaining carbohydrates (sugars) that are already enriched with the heavy ^{13}C isotope will be exported to the sink organs. Nevertheless, other hypotheses aimed at explaining why heterotrophic tissues in C_3 plants tend to be enriched in ^{13}C seem to be more plausible. Among them the isotopic fractionation during dark respiration, the carbon fixation by PEP carboxylase and particularly the differential use of day versus night sucrose between leaves and sink tissues, with day sucrose being relatively ^{13}C-depleted and night sucrose ^{13}C-enriched, have to be considered (Cernusak *et al.* 2009*a*).

The enrichment of ^{13}C in spikes compared with the flag leaf may reflect changes in soil water availability, as well as the increase in evaporative demand occurring during the final stages of crop growth (Condon and Richards 1992; Cabrera-Bosquet *et al.* 2009*a*). Also, the very different photosynthetic characteristics of the spikes, which are characterised by lower permeability than leaves to CO_2 diffusion, could be involved (Araus *et al.* 1993; Tambussi *et al.* 2007).

Water stress also increased the δ^{18}O in the different plant organs compared with WW plants. Similar responses have been reported for bread wheat (Ferrio *et al.* 2007) and durum wheat (Cabrera-Bosquet *et al.* 2009*a*). Moreover, a gradient of increase in δ^{18}O was observed from the roots to the spikes under both water regimes. The increase in evaporative demand later in the crop cycle, the constitutively low g_s of the spike and its uppermost position in the plant may cause the spike to be at a higher temperature and, therefore, more ^{18}O enriched than the leaves (Cabrera-Bosquet *et al.* 2009*b*, 2011). However, other factors may also account for the increase in δ^{18}O from the roots to the spikes. Thus, a progressive enrichment in ^{18}O by evaporation of water as it ascends the plant (Cabrera-Bosquet *et al.* 2009*a*) may also be involved (Wang and Yakir 1995; Gan *et al.* 2003).

Genotypic differences existed for both δ^{13}C and δ^{18}O in each of the three organs studied (particularly in the shoot plant parts). Genotypic variability in durum wheat for both δ^{13}C (Araus *et al.* 1998, 2003) and δ^{18}O (Cabrera-Bosquet *et al.* 2009*a*; Yousfi *et al.* 2012) has been reported already. As expected, δ^{13}C (Farquhar and Richards 1984) and to a lesser extent δ^{18}O (Farquhar *et al.* 2007), were positively correlated with time-integrated WUE (Fig. S1). Genotypic variability in δ^{13}C and δ^{18}O was sustained by differences in leaf permeability. Thus, the negative significant correlation observed between g_s with both δ^{13}C and δ^{18}O in the flag leaf (Fig. 3) and of instantaneous transpiration against the same isotope compositions (data not shown) is in accordance with that reported by Barbour *et al.* (2000) in bread wheat, Barbour and Farquhar (2000) in cotton and Cabrera-Bosquet *et al.* (2009*a*) in durum wheat. Also, the positive correlation between δ^{13}C and δ^{18}O (Fig. S3) indicates that the differences in δ^{13}C within WS and WW were due in large extent to differences in g_s (Barbour and Farquhar 2000; Farquhar *et al.* 2007). Moreover, T_{cum} was also negatively correlated with δ^{13}C and δ^{18}O, at least under WS conditions (Fig. 2), which means that plants with lower δ^{13}C and δ^{18}O exhibited higher transpiration.

However, our results contradict the study by Sheshshayee *et al.* (2005) in groundnut and rice and Cernusak *et al.* (2007) in the tropical tree, *Ficus insipidia*, in which a positive relationship between leaf $\delta^{18}O$ and mean transpiration rate were found. A positive relationship between E and $\delta^{18}O$ enrichment can be expected when the source of variation in E is the evaporative demand (Farquhar *et al.* 2007). A negative relationship between E and $\delta^{18}O$ enrichment is expected if the source of variation in E is g_s (Cabrera-Bosquet *et al.* 2009*a*) and this was the case in our study. In agreement with our results, negative relationships have been reported between $\delta^{18}O$ and both total plant biomass and grain yield in wheat (Barbour *et al.* 2000) and maize (Cabrera-Bosquet *et al.* 2009*b*) under well watered conditions, a relationship that was weaker or absent under water stress (Ferrio *et al.* 2007; Cabrera-Bosquet *et al.* 2009*b*).

Our results show that under WW conditions plants with lower $\delta^{13}C$ and $\delta^{18}O$ exhibited higher biomass (Fig. 4), which is probably associated with a constitutively higher g_s (Fig. 3); that is, more water is used by the plants when they grow faster. However and despite $\delta^{13}C$ and $\delta^{18}O$ not being significantly correlated with cumulative transpiration under WW conditions, biomass was positively correlated with cumulative transpiration (Fig. 2), which supports the concept that, for a given evaporative conditions, those plants with a higher water use will exhibit a higher potential growth (Araus *et al.* 2008; Blum 2009). Differences in water uptake and use and biomass accumulation across plants under WW conditions may be based on differences in root characteristics. The genotypes (RIL2108 and RIL2510) exhibiting lower $\delta^{13}C$ and $\delta^{18}O$ were also those with higher flag leaf N content (Table S1), which would explain their higher gas exchange and aboveground WUE.

Root traits

A decrease in RLD and RWD down through the soil column is a common pattern in wheat and other cereals (Gregory *et al.* 1978; Baburai Nagesh 2006; Carvalho 2009). Kang *et al.* (1994) related this gradient distribution of RLD and RWD in rice to the fact that the adventitious roots, which occupy the upper soil sections, constitute 86–99% of the entire root system and contrary to the seminal roots, they cannot grow deeper in the soil sections (Manske and Vlek 2002). In contrast to RWD and RLD, SRL increased from the upper to the middle of the soil column (Table 5). Kang *et al.* (1994) reported that the diameter of the adventitious roots is relatively large close to their origin point in the upper soil profile and decreases along the main axes and branch roots.

Water regime did not affect TRoot DM, in agreement with the fact that biomass allocated to roots was unchanged by water regime. However, TRoot L was higher under WW than WS conditions (Table S2), which agrees with previous studies in wheat (Barraclough 1989; Baburai Nagesh 2006). This difference in response may be related to the ability of the plants under WW conditions to produce a larger number of adventitious roots. Gregory (1994) and Blum (2005) reported that drying of the soil surface may inhibit the development of the adventitious root system. In addition, the number of adventitious roots depends mainly on the tillering ability of the plant (Manske and Vlek 2002) and the WS regime strongly decreased the number of tillers plant^{-1} (Table 2). In our study, WS plants showed a clear decrease in RWD and RLD down in the soil profile compared with the WW plants, where even a slight increase in RWD and RLD at the lower compared with the middle soil section was observed (Table 5). Gregory *et al.* (1978) also found a slight increase in the RWD in deeper soil sections in winter wheat under field water stress conditions. Dwyer *et al.* (1988) reported in barley that after anthesis root growth could still happen due to water and nutrient availability in deep soil layers.

WS also decreased SRL, which means roots were thicker compared with WW plants. This could be explained as a plant response to soil drying, since the increase in root diameter may enhance the ability to penetrate the soil, resisting the mechanical impedance that increases when drying and also it could improve the water conductance under dry conditions (Ryser 1998; Davies and Bacon 2003; Kage *et al.* 2004). Moreover, even when thinner roots (high SRL) are advantageous for resources uptake (Eissenstat 1992) they tend to be more susceptible to drought (Ryser 2006; Carvalho 2009). However, results reported in the literature concerning the effect of low resource availability on SLR are contradictory (Ryser 1998). Further, SRL itself is a complex trait that depends on both root length (Root L) and Root DM. Since Root DM was not significantly affected by the water regime, the decrease in SRL under the WS regime was mainly due to the decrease in Root L (Table S2). Song *et al.* (2010) and Baburai Nagesh (2006) found increases in wheat SRL as a response to water stress in pot plant and lysimeter experiments, respectively, whereas Carvalho (2009), also in an experiment with lysimeters, found that SRL increased under drought in some years but that it decreased in other years.

Our results agree with a positive effect of a higher RWD and RLD (and to a lesser extent of SRL) on the Aerial DM. This was especially evident under WS conditions where the RWD and RLD of each soil section were positively correlated with Aerial DM (Fig. S4). Moreover, genotypic differences existed for the three root traits under both water regimes as reported in previous studies on wheat and barley by Song *et al.* (2010), Baburai Nagesh (2006) and Carvalho (2009). Our results also agree with previous reports (Passioura 1983; Gregory *et al.* 1992) where a higher RLD is associated with higher water absorption from the soil and consequently improved the final performance of cereal crops (Fig. 5). Further, aboveground vigour has been shown to influence root system growth and may be used to indirectly select for more extensive root systems (Watt *et al.* 2005; Richards *et al.* 2007). Another important trait for deeper root systems in wheat is strong geotropism that favours more deep penetration rather than shallow penetration of roots in the soil profile (Ho *et al.* 2005). Variation for seminal and nodal axile root geotropism exists in wheat (Araki and Iijima 2001; Manschadi *et al.* 2008) and may be used, along with root vigour, in future breeding programs (Richards *et al.* 2010). For other crops the importance of morphological root traits on genotypic performance may be different. This is the case in a recent study with chickpeas in lysimeters, where the magnitude of the variation in root growth components (root depth, RWD and RLD) did not distinguish tolerant from sensitive genotypes (Zaman-Allah *et al.* 2011). Indeed, seed yield was not correlated with RLD in any soil layers. Under these conditions of terminal drought, the most critical component of tolerance in chickpea was the conservative use of water early in the cropping cycle (Zaman-Allah *et al.* 2011).

Relationship of $\delta^{13}C$ and $\delta^{18}O$ to root traits

RWDa and RLDa were negatively related to both $\delta^{13}C$ and $\delta^{18}O$ under WW regime (Fig. 6), meaning that the higher the RWD or RLD, the greater the amount of water that is captured from the soil and finally transpired by the plant. In the same sense, SRLa showed a negative relationship with $\delta^{13}C$ and $\delta^{18}O$, but over both water regimes (Fig. 6). Moreover, under the WW regime, the RWD and RLD in the upper and middle soil sections were better correlated with flag leaf $\delta^{13}C$ and $\delta^{18}O$ than in the lower soil section (data not shown). These results suggest the role of adventitious roots rather than deeper seminal roots under well watered conditions (Manske and Vlek 2002). A higher density of adventitious roots improves water uptake under conditions where water availability is not limiting and the upper part of the soil profile remains wet (Gregory 1994). However, under the WS regime no clear pattern of root distribution in relation to stable plant carbon and oxygen signatures emerged. Thus RWD, despite its strong positive correlation with Aerial DM (e.g. RWD1 explained 64% of the variation in Aerial DM under WS conditions) was not negatively correlated with either $\delta^{13}C$ or $\delta^{18}O$. Therefore, at least under the WS conditions of our study, differences in $\delta^{13}C$ or $\delta^{18}O$ are not necessarily accounted for by the amount and distribution of roots. Lopes and Reynolds (2010) working with wheat under water stress in the field failed to find any relationship between root traits and carbon isotope discrimination. Moreover, the negative correlations of the plant cumulative transpiration per unit root dry matter with $\delta^{13}C$ and $\delta^{18}O$ (Fig. S5) may reveal that besides the size and distribution of the root system, other plant characteristics may account for genotypic differences in water uptake, $\delta^{13}C$ and $\delta^{18}O$. For example, shoot or root hydraulic conductivity (e.g. aquaporins) may be involved in the genotypic differences in water uptake (Galmés *et al.* 2007; Lovisolo *et al.* 2008).

Although the question of whether bigger plants have bigger roots (driven by aerial parts) or *vice versa* remains open, it may provide some insights on the lack of relationship between isotopic composition and root size under water stress. Hence, the gas-exchange properties may affect $\delta^{13}C$ and $\delta^{18}O$ directly, but they also influence assimilation and water loss and via that route indirectly affect root development mediated by hormones or some other signalling (Sharp and Davies 1979; Sharp and LeNoble 2002; Schraut *et al.* 2004). Water stress may increase $\delta^{13}C$ and $\delta^{18}O$, but its effect on root biomass is less clear since on one hand the absolute plant biomass may decrease in response to water stress, but the relative portion of biomass allocated to roots will increase. This may explain the lack of a (negative) relationship between $\delta^{13}C$ and $\delta^{18}O$ and root development under water stress. In contrast, under well watered conditions genotypic differences in biomass and isotope composition may be the consequence of constitutive differences in root traits. This would explain the negative relationship between isotope signatures and root development. The analysis of (the carbon) isotope composition of the soluble fraction (i.e. recently assimilated) of leaves in future studies might help to elucidate the relationships between root architecture and isotopic signature.

Conclusions

This study highlights the role of a stronger root system increasing durum wheat growth under well watered conditions as well as progressive water stress conditions. Both $\delta^{13}C$ and $\delta^{18}O$ adequately recorded the water status that the plant is experiencing, increasing in response to water stress. Moreover, there were genotypic differences observed for the stable isotopes and root traits studied. RLD was positively associated with Aerial DM regardless of the water regime, meaning that the higher the RLD, the greater the amount of water that is taken up and transpired by the plant. However, the negative relationships of $\delta^{13}C$ and $\delta^{18}O$ with root traits and shoot growth were only evident under WW conditions, whereas no clear relationship between root traits and stable isotope signatures emerged under WS. Our study supports the use of the stable carbon and oxygen isotopes as proxies to select better root traits and higher growth potential in the absence of water stress. Moreover, Richards *et al.* (2010) concluded that for most traits of importance in dry environments, selection is generally conducted most effectively in favourable moisture environments.

Isotopic compositions do not appear a priory to be good proxies under the water stress conditions of our experiment. Beside their lack of relationships with aboveground dry matter and root traits, both compositions exhibited significant genotype × treatment interactions. Nevertheless, further studies should broad the environments and plant parts to analyse; for example in cases where soil moisture is stored at depth. The isotope composition of mature grains might reveal information about access to water after anthesis – a crucial period when water stress normally develops under Mediterranean conditions. To that end, deeper lysimeters or even better experiments under real field conditions over the full lifecycle of the plant should be performed (Lopes and Reynolds (2010).

Acknowledgements

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Supplementary Tables and Figures

Supplemental Table S1.

T	G	Spike DM	Culm DM	Leaf DM	Root N	Flag leaf N	Spike N
		g plant^{-1}	g plant^{-1}	g plant^{-1}	(%)	(%)	(%)
WW	KS194	0.78 a	2.20 ab	0.61 a	1.48 b	3.58 a	3.13 b
	KS230	1.07 a	2.10 a	0.72 a	1.22 ab	3.28 a	3.55 c
	RIL2108	1.05 a	2.78 ab	0.70 a	1.34 ab	4.36 b	2.57 a
	RIL2510	0.95 a	2.88 b	0.67 a	1.09 a	4.55 b	2.53 a
	Mean	0.96	2.49	0.67	1.28	3.94	2.95
WS	KS194	0.30 a	1.27 a	0.23 a	0.81 a	3.17 bc	2.58 b
	KS230	0.39 b	1.62 b	0.40 c	0.80 a	2.44 a	3.15 c
	RIL2108	0.68 d	1.51 ab	0.24 ab	0.67 a	2.99 b	2.18 a
	RIL2510	0.58 c	1.69 b	0.32 b	0.66 a	3.58 c	2.01 a
	Mean	0.49	1.52	0.30	0.73	3.05	2.48
Anova	G	0.45***	1.54***	0.08**	0.28*	6.21***	5.90***
	T	1.81***	7.44***	1.14***	2.40***	6.44***	1.74***
	G x T	0.13ns	0.75*	0.02ns	0.12 ns	0.94*	0.04 ns

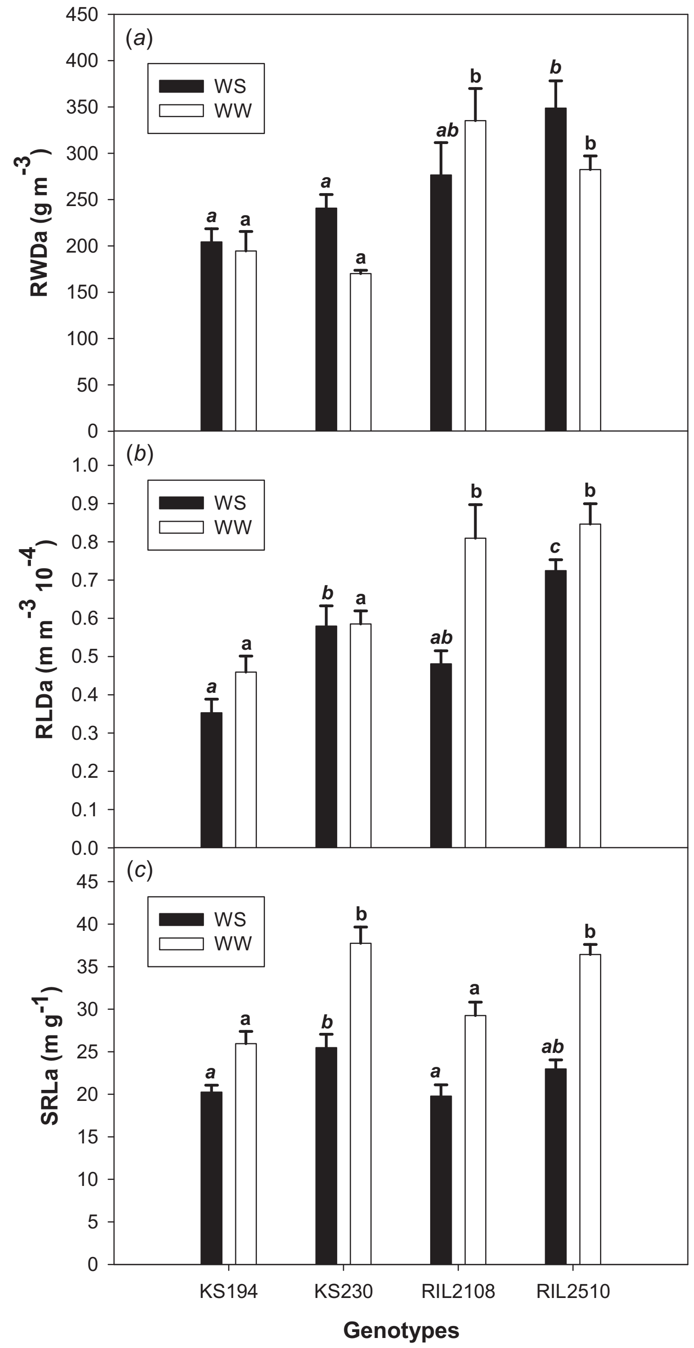
Effect of water regime and genotype on different growth parameters: spike dry matter (Spike DM), culm dry matter (Culm DM), leaf dry matter (Leaf DM), root nitrogen content (Root N), flag leaf nitrogen content (Flag leaf N) and spike nitrogen content (Spike N). For each genotype and treatment data shown are the means of the four replications. Means followed by different letters were significantly different (P <0.05) by Tukey's b test. WW, well watered plants; WS water stressed plants; G: Genotype; T: Treatment; G x T: genotype by treatment interaction. The associated sum of squares type III and probabilities (ns, not significant; $^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ < 0.001) are shown.

Supplemental Table S2.

T	G	Root DM 1 g	Root DM 2 g	Root DM 3 g	Total Root DM g	Root L 1 m	Root L 2 m	Root L 3 m	Total Root L m
WW	KS194	0.18 a	0.07 a	0.07 a	0.31 a	4.04 a	2.16 a	1.75 a	7.95 a
	KS230	0.14 a	0.07 a	0.10 b	0.32 a	4.18 a	3.06 a	4.23 c	11.47 b
	RIL2108	0.30 b	0.08 a	0.09 b	0.48 b	7.36 b	3.16 a	2.92 b	13.44 b
	RIL2510	0.26 b	0.11 b	0.13 c	0.49 b	6.67 b	5.15 b	4.94 c	16.75 c
	Mean	0.22	0.08	0.10	0.40	5.56	3.38	3.46	12.40
WS	KS194	0.18 a	0.09 a	0.05 a	0.32 a	3.01 a	2.02 a	1.20 a	6.22 a
	KS230	0.22 ab	0.11 b	0.09 b	0.42 ab	5.11 b	3.09 b	2.35 b	10.55 bc
	RIL2108	0.25 ab	0.10 ab	0.09 b	0.44 ab	4.28 ab	2.22 a	1.86 ab	8.36 ab
	RIL2510	0.32 b	0.13 c	0.10 b	0.55 b	6.57 c	3.25 b	2.54 b	12.36 c
	Mean	0.24	0.11	0.08	0.43	4.74	2.65	1.99	9.38
ANOVA	**G**	0.08***	0.01***	0.014***	0.20***	44.00***	18.99***	24.17***	223.46***
	T	0.00ns	0.00***	0.00**	0.01ns	5.37*	4.31***	17.39***	73.33***
	G x T	0.020*	0.001ns	0.001ns	0.02ns	17.44**	4.64***	4.07*	24.33*

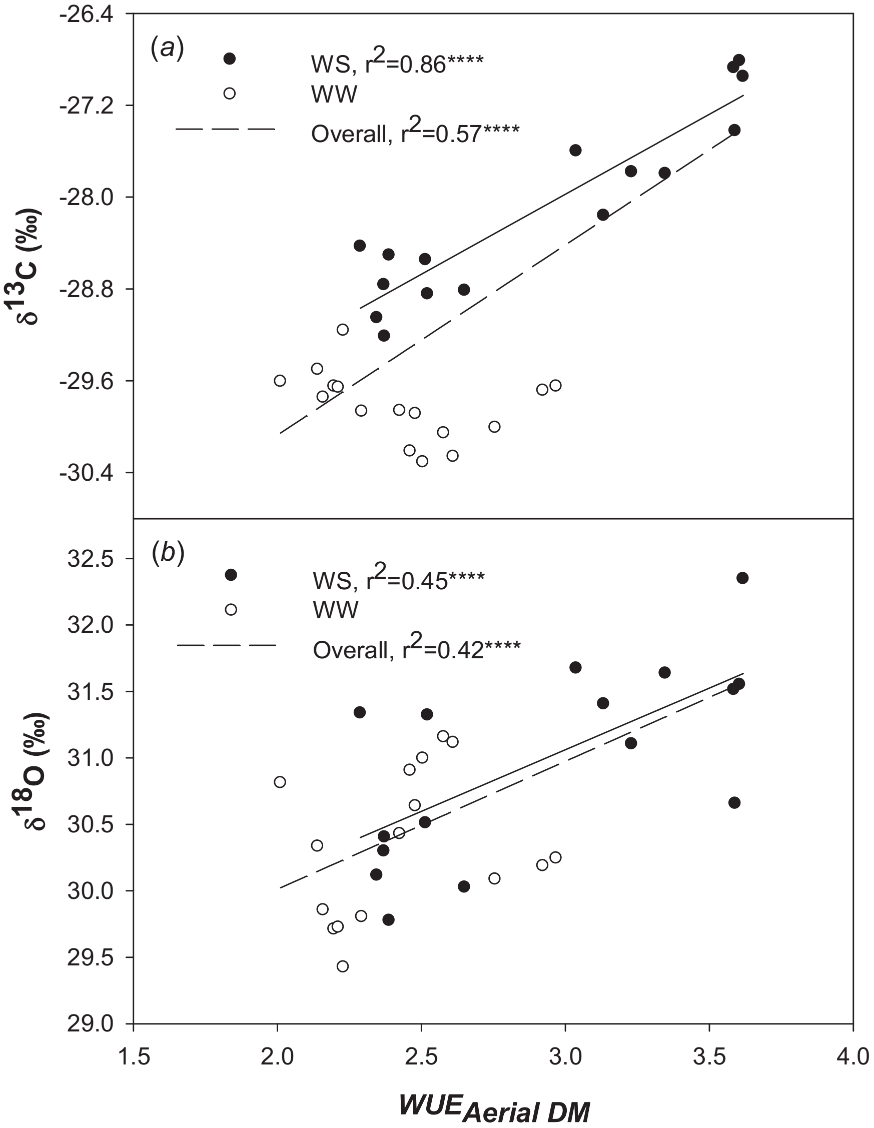
Effect of water regime and genotype on the root dry matter weight (Root DM), and the root length (Root L) in each of the three different soil sections, as well as the total values for both traits through all the soil sections. For each trait the number following the acronym refers to the soil section where the trait was estimated: 1 refers to soil upper section (0.00-0.26 m); 2 refers to soil middle section (0.26-0.52 m); and 3 refers to soil bottom section (0.52-0.78 m), while “T” before the acronym refers to the total trait value through the three soil sections. Data shown is the mean of the four replications of each genotype in each treatment. Means followed by different letters were significantly different (P <0.05) by the Tukey-b’s test. WW, well watered plants; WS water stressed plants; G: Genotype; T: Treatment; G x T: genotype by treatment interaction. The associated sum of squares type III and probabilities ($^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ < 0.001) are shown.

Supplementary Figure S1.



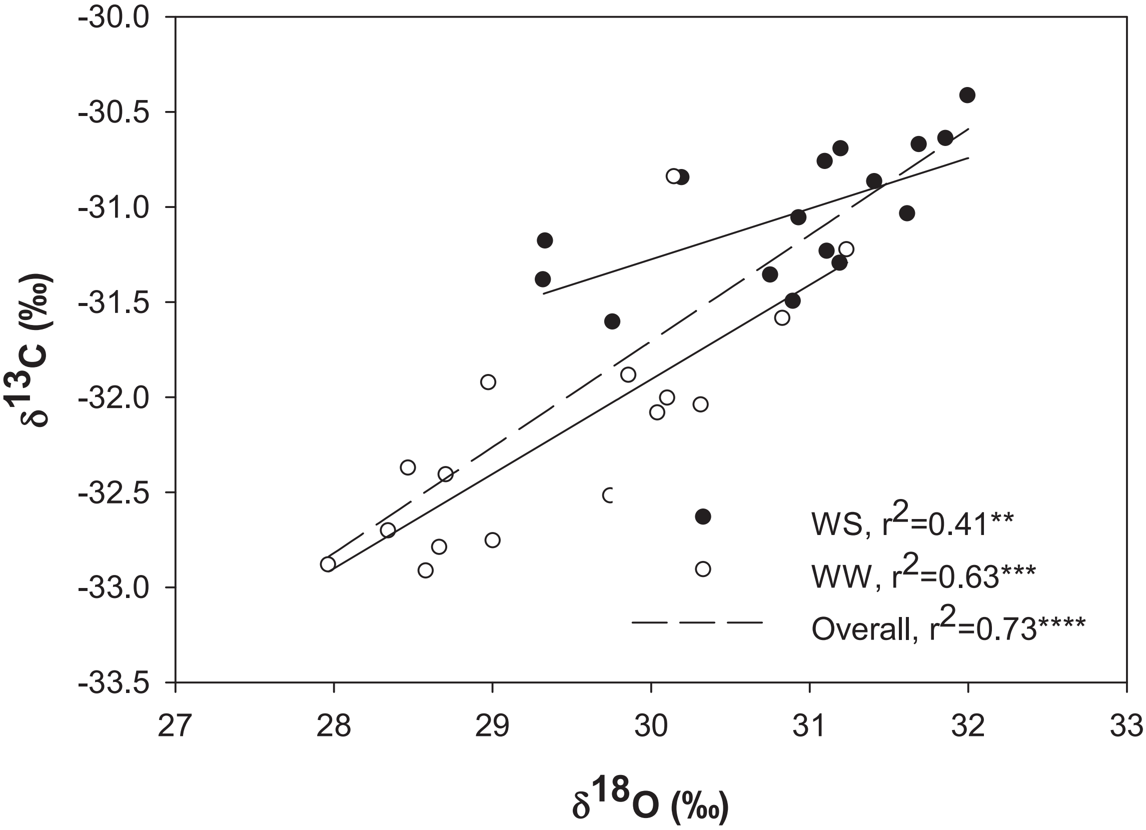
Averaged values through all the soil sections for a) root weight density (RWDa), b) root length density (RLDa) and c) specific root length (SRLa) for genotypes KS194, KS230, RIL2108, and RIL2510. Data include both well-watered (WW, white bars) and water-stressed (WS, black bars) plants. Errors bar represent the standard error of the mean (SEM). Means followed by different letters were significantly different (*P* <0.05) by Tukey's b test. Genotype, treatment, and genotype by treatment interaction were significant for all traits except for RWDa the treatment was not significant.

Supplementary Figure S2.



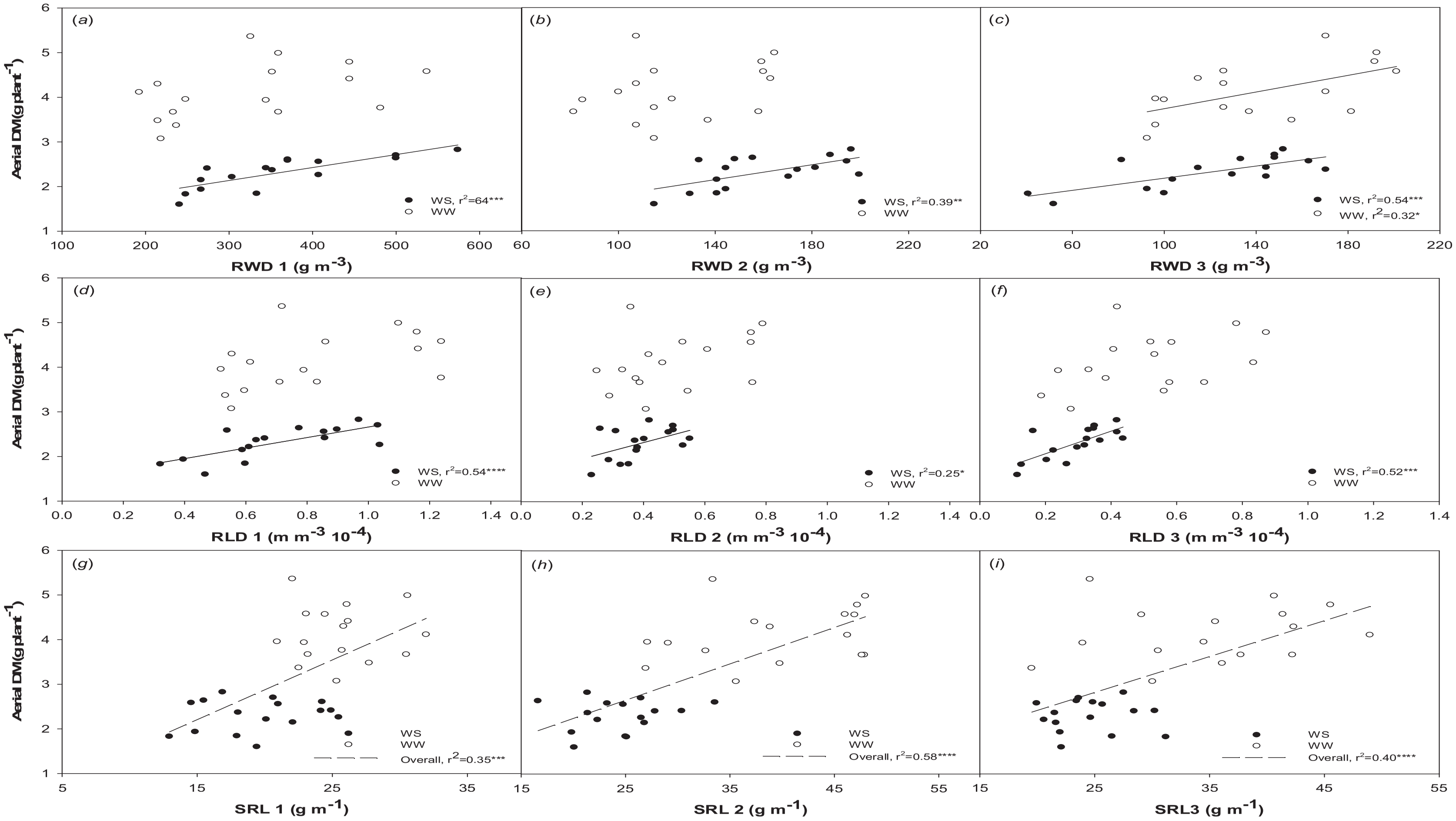
Relationships between plant time-integrated water use efficiency (*$WUE_{Aerial\ DM}$*) vs. a) $\delta^{13}C$ of the spike and b) $\delta^{18}O$ of the spike. Data include both well-watered (WW, open circles) and water-stressed (WS, filled circles) plants. The fitting line is only included for the significant relationships. r^2 and probability is shown: $^{****}P$ <0.0001.

Supplementary Figure S3.



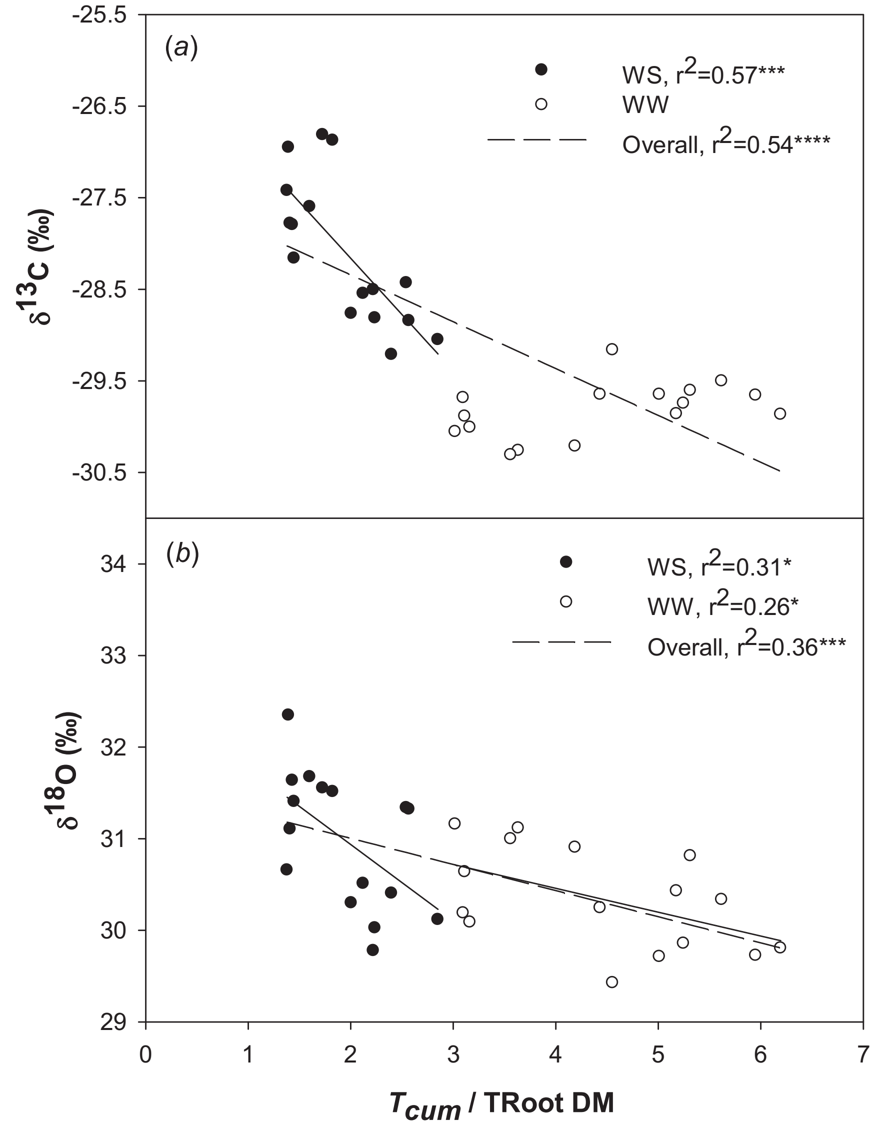
Simple linear regression between flag leaf δ^{18}O and δ^{13}C. Correlation data include both well-watered (WW, open circles) and water-stressed (WS, filled circles) plants. The fitting line is only included in the significant relationships. r^2 and probabilities are shown : $^{**}P$ <0.01; $^{***}P$ < 0.001; and $^{****}P$ <0.0001).

Supplementary Figure S4.



Relationships between: root weight density (RWD, upper Figs. a, b, c), root length density (RLD, middle Figs. d, e, f) and specific root length (SRL, lower Figs. g, h i) vs. aerial DM. The number behind the trait refers to the soil section where the trait was estimated: 1, upper section (0.00-0.26 m); 2, middle section (0.26-0.52 m); and 3, soil bottom section (0.52-0.78 m). Data include both well-watered (WW, open circles) and water-stressed (WS, filled circles) plants. The fitting line is only included in the significant relationships. r^2 and probabilities are shown: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

Supplementary Figure S5.



Relationship between the plant cumulative transpiration per unit of Root DM (T_{cum} / TRoot DM) vs. a) δ^{13}C and b) δ^{18}O of the spike. Data include both well-watered (WW, open circles) and water-stressed (WS, filled circles) plants. The fitting line is only included in the significant relationships. r^2 and probabilities are shown: $^{*}P < 0.05$; $^{***}P < 0.001$; and $^{****}P < 0.001$.

Chapter 3

Interactive effect of water and nitrogen regimes on plant growth, root structure and water status of old and modern durum wheat genotypes

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Durum wheat genotypes were grown in lysimeters in the Experimental Field Facilities of the Faculty of Biology, University of Barcelona. 2011/2012 cycle.

Resumen

La escasez de agua y la falta de nitrógeno son los principales factores limitantes de la productividad del trigo duro (*Triticum turgidum* L. var *durum*) en las regiones mediterráneas. En este experimento se cultivaron ecotipos junto con genotipos modernos de trigo duro en lisímetros de 150 cm de alto, con dos regímenes de agua y nitrógeno contrastantes, y se estudió el efecto de estos tratamientos sobre la biomasa aérea (AB, por sus siglas en inglés), el crecimiento, la distribución y la morfología de las raíces, las tasas de fotosíntesis y transpiración y la eficiencia en el uso del agua. Los tratamientos con un nivel alto de agua y nitrógeno aumentaron de forma significativa la AB. Por el contrario el peso y longitud de la raíz y la densidad de longitud de la raíz aumentaron y disminuyeron, respectivamente, en respuesta a un mayor aporte de agua y nitrógeno. Un nivel elevado de riego no afectó a la densidad de peso de la raíz, mientras que un nivel alto de nitrógeno disminuyó este parámetro. El régimen de nivel alto de nitrógeno aumentó de forma significativa la composición isotópica de carbono de la hoja bandera ($\delta^{13}C_{FL}$), mientras que disminuyó la fotosíntesis neta, de la conductancia estomática y de la transpiración en hojas, mientras que se observó el efecto opuesto al aumentar el aporte de agua. Además, el régimen de riego elevado disminuyó significativamente la composición isotópica de oxígeno de la hoja bandera ($\delta^{18}O_{FL}$) e incrementó la transpiración acumulada de la planta, mientras que un aumento en el abonado nitrogenado no afectó de forma significativa a ninguno de esos parámetros. Independientemente del régimen hídrico, la AB se correlacionó positivamente tanto con el $\delta^{13}C_{FL}$ como con la eficiencia del uso del agua integrado en el tiempo (WUE_{AB}, por sus siglas en inglés) y negativamente con los parámetros de intercambio de gases. El estudio reveló claramente: (i) la importancia de las características de las raíces para conseguir mayor biomasa aérea y mejor WUE_{AB} bajo condiciones de escasez de agua; (ii) que la interacción entre el nitrógeno y el régimen de agua puede afectar a la naturaleza predictiva del $\delta^{13}C$ en programas de mejora por resistencia a sequía; y (iii) la selección de la estructura ideal del sistema radicular debería tener en cuenta el gasto metabólico (la producción y el mantenimiento de los tejidos de la raíz, a menudo medibles en unidades de carbono) junto con la capacidad para capturar recursos.

Interactive effect of water and nitrogen regimes on plant growth, root structure and water status of old and modern durum wheat genotypes

Running title: Shoots and roots growth under different growth conditions

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Abstract

Ten durum wheat (*Triticum turgidum* L. var *durum*) genotypes were grown in lysimeters under two contrasting water and nitrogen regimes and the effect of such growth conditions on the aerial biomass (AB), the growth, distribution and morphology of the roots, different photosynthetic and transpiratory traits and water use efficiency were studied. Both high water and nitrogen regimes significantly increased AB. Root dry biomass, root length and root length density increased and decreased, as response to improved water supply and nitrogen regimes, respectively. The high water regime did not affect root weight density, whereas the high nitrogen regime decreased this trait. The high nitrogen regime significantly increased the carbon isotope composition of the flag leaf ($\delta^{13}C_{FL}$) while the opposite effect was observed for high water regime. The study clearly revealed: (i) the importance of root traits for higher AB and water use efficiency under the low water regime; (ii) that the interaction between nitrogen and the water regime may affect the predictive nature of the $\delta^{13}C$ in drought breeding programs; and (iii) the selection of the ideal root system structure should take in account the metabolic costs (the production and maintenance of root tissues) alongside the capacity to capture resources.

Key words: Lysimeters, *Triticum turgidum* ssp. *durum*, root traits, $\delta^{13}C$, $\delta^{18}O$.

Introduction

Durum wheat (*Triticum turgidum* L. var *durum*) is the main cereal crop grown in the Mediterranean basin, constituting 54% of the total cereal area (FAO 2012). Water deficit, which is frequently accompanied by low nitrogen (N) availability, is the main constraint limiting productivity (Loss and Siddique 1994; Oweis et al. 2000; Araus et al. 2002; Passioura 2002; Cabrera-Bosquet et al. 2009a). Water deficits are predicted to increase in the future because climatic change is expected to decrease precipitation and increase evapotranspiration in the Mediterranean region (Lobell et al. 2008; Carvalho et al. 2014). On the other hand, the cost of energy together with environmental concerns will restrict the indiscriminate used of nitrogen fertilizers (Renard et al. 2004).

Improving water use efficiency (*WUE*) is an important goal of wheat research for Mediterranean environments (Cabrera-Bosquet et al. 2007). A better management of N fertilization may affect the *WUE* greatly, even under terminal water deficits of the Mediterranean regions (Ferrante et al. 2010) and this in turn would affect yield (Oweis et al. 2000). In addition, selecting more drought tolerant genotypes is a key factor for improving durum wheat adaptation to Mediterranean field conditions (Araus et al. 2002). However, breeding for adaptation to abiotic stresses is often limited by: (1) the lack of proper phenotyping traits that could address the stress (Elazab et al. 2015); (2) the fact that the relationship of the trait with biomass and grain yield (the usual target in crop breeding) frequently changes according to the type and level of stress (Voltas et al. 1999; Araus et al. 2003); and (3) phenotyping traits usually address each stress individually, while drought and low N deficiency often occur together (Araus et al. 2013).

In C_3 plants the carbon isotope composition ($\delta^{13}C$) measured in plant tissues is considered as one of the most promising secondary traits in wheat (and other C_3 cereals) when breeding for drought resilience (Rebetzke et al. 2002; Condon et al. 2004; Richards et al. 2011). The $\delta^{13}C$ has been reported to negatively correlate with C_i/C_a (the ratio of leaf intercellular to ambient CO_2 concentration) and positively correlated with *A/E* (the ratio of net assimilation to water evaporated from the transpiring organs). Therefore, the $\delta^{13}C$ is positively related to *WUE*, which is considered as the biomass produced per unit of water transpired (Farquhar and Richards 1984; Monneveux et al. 2006; Elazab et al. 2012). Correlations between $\delta^{13}C$ and grain yield (GY) and/or aerial biomass (AB) may be either negative or positive according to the plant tissue sampled and environmental conditions tested (Condon and Richards 1992; Voltas et al. 1999; Araus et al. 2003). High $\delta^{13}C$ has been used as a selection trait for high *WUE* in commercial wheat breeding for the summer-dominant rainfall growing conditions of Australia where crop yield relies on the water accumulated before planting (Rebetzke et al. 2002; Condon et al. 2004). However, the $\delta^{13}C$ in some cases may reflect the effective water used (*EWU*) by the crop (Blum 2009), and thus the relationship between $\delta^{13}C$ and either AB or GY may be negative (Araus et al. 2003, 2013; Monneveux et al. 2006), meaning that genotypes that are able to maintain a higher *EWU* (even if at the expense of a lower *WUE*) are the most productive (Blum 2009; Araus et al. 2013). Furthermore, the high $\delta^{13}C$ genotypes, which were selected for the summer-dominant rainfall environments of Australia have almost no increase in GY in either the winter dominant rainfall environments (Mediterranean type environments) or in the environments with highly variable rainfall of Australia (Condon et al. 2004). Overall, high $\delta^{13}C$ (and therefore *WUE*) is a 'conservative' trait in terms of water use and crop growth rate, and thus in the absence of soil water deficit, high $\delta^{13}C$ genotypes tend to grow more slowly than low $\delta^{13}C$ genotypes, resulting in lower AB and GY (Condon and Richards, 1992; Condon et al. 2004).

The effect of the N amount on *WUE* and its interaction with water availability remains unclear and contradictory results have been reported. Cabrera et al. (2007) reported in a durum wheat pot trial under different water and nitrogen combinations that regardless of the water regime a high N supply increased *WUE* when measured either instantaneously (through gas exchange as *A/E*) or agronomically (as biomass/water used) as well as the $\delta^{13}C$ of the AB. In the same sense, the $\delta^{13}C$ of mature grains (and therefore *WUE*) slightly increased in a durum wheat field trial under high N application regardless of the water regime (Araus et al. 2013). However, Shen et al. (2013) reported in a winter wheat lysimeter experiment that the high N supply increased the *WUE* (measured agronomically) under low water conditions, while no effect was reported under well-watered conditions.

The oxygen isotope composition ($\delta^{18}O$) when measured in plant dry matter is known to integrate the evaporative conditions throughout the crop cycle (Barbour et al. 2000; Cabrera-Bosquet et al. 2009a, b; Araus et al. 2013), and thus it has been proposed as a proxy method for measuring transpiration and water use in different crop species including wheat (Barbour et al. 2000; Ferrio et al. 2007; Cabrera-Bosquet et al. 2009a; Elazab et al. 2012; Araus et al. 2013). Moreover, the combined measurements of $\delta^{13}C$ and $\delta^{18}O$ in the same plant tissue may help in separating the independent effects of photosynthetic capacity (A) and stomatal conductance (g_s) on $\delta^{13}C$ because the $\delta^{18}O$ is not affected by photosynthesis (Barbour and Farquhar, 2000; Elazab et al. 2012). However, the use of the $\delta^{18}O$ signature for breeding is complicated because it is not only affected by air humidity and temperature (Barbour and Farquhar 2000; Barbour et al. 2000) and soil moisture (Ferrio et al. 2007) but also is potentially affected by a set of miscellaneous factors such as responses to source water (Yakir and Deniro 1990) and N fertilization (Cernusak et al. 2007; Cabrera-Bosquet et al. 2009a; Araus et al. 2013) as well as its lower analytical precision compared with $\delta^{13}C$ (Araus et al. 2013). Thus, there are few studies reporting the use of $\delta^{18}O$ in cereals grown under low water conditions and the results are contradictory. Under well-watered conditions in wheat the $\delta^{18}O$ of flag leaves correlated negatively with GY and g_s (Barbour et al. 2000) while Ferrio et al. (2007) failed to find significant relationships between the $\delta^{18}O$ of mature grains and GY using a set of wheat genotypes grown under three different water regimes. Also, Cabrera-Bosquet et al. (2009b) reported a negative relationship in maize between the $\delta^{18}O$ of mature grains and GY under well-watered and moderate water stress conditions, while under severe water stress conditions the relationship was positive.

The plant functions as a complete system with a balance between the shoot and root in the capture of resources and the exchange of assimilates over the long term (Lawlor et al. 2001). Plant roots are the key organs in the plant responsible for the absorption of water and nutrients (Shen et al. 2013). Root growth is known to vary according to soil conditions such as water supply (Elazab et al. 2012) and N supply (Comfort et al. 1988; Carvalho 2009; Shen et al. 2013; Wang et al. 2014). The responses of root growth to water deficits are important factors in drought tolerance. However, root drought adaptive traits have received much less attention in breeding programs in comparison to the drought adaptive traits of shoots (Zhang et al. 2009). According to several authors (Zhang et al. 2009; Richards et al. 2010; Shen et al. 2013; Carvalho et al. 2014; Wang et al. 2014) the limited knowledge of root growth and functioning is due, among other factors, to: (i) lack of efficient and easy root screening methods and difficulties in evaluating root traits on a large number of plants and to depths that may exceed 100 cm (and even reaching in some cases up to 200 cm); (ii) the large number of factors that affect roots such as, soil type, planting density, water and fertilizer regime, and tillage method; and (ii) studies under controlled conditions frequently examining the effects of a single environmental condition on the root system, (usually water regime) and obviating the interaction effects with other major environmental conditions at the soil level (e.g. N fertilization).

According to several authors (Siddique et al. 1990; Richards et al. 2007; Lilley and Kirkegaard 2011) genotypic variation exists in wheat root systems and some traits have been suggested to improve yield under water limited conditions, including: increased root elongation rate, depth of rooting; root distribution at depth; xylem vessel diameter; angle of seminal roots; and root to shoot dry matter ratio (Siddique et al. 1990; Manske and Vlek 2002; Lopes and Reynolds 2010; Lilley and Kirkegaard 2011). Generally, the capacity of roots to extract water and nutrients from a given volume of soil is dependent on root architectural traits such as length and weight distribution of the roots with depth (King et al. 2003; Carvalho et al. 2014). Thus, root weight density (RWD) and root length density (RLD) are frequently used in root studies (Zhang et al. 2009; Ayad et al.

2010; Elazab et al. 2012; Carvalho et al. 2014; Wang et al. 2014) to describe the root weight and root length, respectively, within a soil volume. Currently, RLD is the most commonly used trait in describing root distribution (Ayad et al. 2010; Elazab et al. 2012; Carvalho et al. 2014) and it is an important parameter to model water and nutrient uptake (Barraclough 1989; King et al. 2003; Ayad et al. 2010; Carvalho et al. 2014). Overall, the results of wheat and barley field experiments indicate that a RLD of 1 cm cm^{-3} is a critical value for extraction of 90% of the available soil water (the amount of water in the soil between field capacity and the permanent wilting point), whereas RLD values above 1 cm cm^{-3} are excessive and inter-root competition occurs (Barraclough 1989; King et al. 2003; Carvalho et al. 2014).

Beside architectural root traits (RWD and RLD) the specific root length (SRL) is considered one of the most important and commonly measured morphological traits, which has a large influence on the rates of resource uptake, root respiration and rhizodeposition (Hajek et al. 2013). SRL is the ratio of root length to root biomass (Eissenstat 1991; Cornelissen et al. 2003) and it characterises the pattern of biomass allocation into root length (Ryser 2006; Song et al. 2010).

According to Ryser (1998) and Grossman and Rice (2012) a high SRL facilitates nutrient uptake in low-nutrient environments and makes plants more competitive for soil nutrient uptake (Bonifas and Lindquist 2009). This is because plants with higher SRL have a larger root surface area within a given soil volume, and therefore a greater absorption area than plants with low SRL (Eissenstat 1991; Bonifas and Lindquist 2009). Moreover, the metabolic costs (the production and maintenance of root tissues, often measurable in units of carbon) for high SRL plants are less expensive than for low SRL plants (Eissenstat 1991; Cornelissen et al. 2003; Bonifas and Lindquist 2009; Hajek et al. 2013; Lynch et al. 2014). However, roots with high SRL may have limited transport, storage and support capacity and a shorter life span than low SRL roots (Fitter 1996; Cornelissen et al. 2003). Also, the SRL is a complex trait because it combines information for root diameter and/or root tissue density (Carvalho et al. 2009), and thus a high SRL could be due to a decreasing root diameter and /or decreasing root tissue density (Ryser 1998; Hajek et al. 2013). The value of SRL in breeding programs is still difficult to assess (Løes and Gahoonia 2004) because unlike other root traits (i.e. total root biomass and length, RLD and RWD) the previous studies on genetic variation in cereal species under different resources availability (water and nutrient) for the SRL are contradictory and often based on studies with a few number of lines or varieties (Ryser 1998; Manske and Vlek 2002; Løes and Gahoonia 2004; Carvalho et al. 2009, 2014).

Due to the difficulty of measuring root traits, the indirect screening for specific shoot traits has been used in field trials for selecting thousands of genotypes, especially during the grain filling period (Richards et al. 2010). For example, selection can be made for genotypes that maintain greener leaf area, higher g_s, or lower canopy temperature, lower $\delta^{13}C$ or lower $\delta^{18}O$ since these traits are expected to be good indicators of which genotypes have deeper roots and are able to access more soil water in the deep soil layers (Lopes and Reynolds 2010; Richards et al. 2010; Elazab et al. 2012). However, the usefulness of these traits is countered by their low heritability in drying soils (Richards et al. 2010). Moreover, studies that relate genotypic variability of $\delta^{13}C$ and $\delta^{18}O$ to root characteristics are scarce and the results contradictory (White et al. 1990; Lopes and Reynolds 2010; Elazab et al. 2012). Thus, White et al. (1990) found in common bean grown under rainfed conditions that genotypes with higher $\delta^{13}C$ exhibited lower RLD, but under well-watered conditions the relationship was absent. Lopes and Reynolds (2010) did not find a significant correlation of root traits (root biomass at depth) with $\delta^{13}C$ in a wheat field experiment, despite the fact that they found the canopy temperature at grain filling and the $\delta^{13}C$ of grains to be negatively correlated with GY, and also observed that the $\delta^{13}C$ of grains correlated negatively with total root dry biomass. Elazab et al. (2012) reported in durum wheat that when both $\delta^{13}C$ and $\delta^{18}O$ were analysed in flag leaf blades there was a negative correlation with the RWD, RLD and AB under well-watered conditions, whereas these correlations were absent under water-stressed conditions.

For durum wheat grown under Mediterranean environments, the interaction between water and N application on yield, water use and root growth are crucial. Studies on the combined effect of water and nitrogen regimes on durum wheat growth, productivity and both $\delta^{13}C$ and $\delta^{18}O$ have been reported in greenhouse (Cabrera et al. 2007, 2009a) and field conditions (Araus et al. 2013; Elazab et al. 2015). Also, apart from a few studies (Ayad et al. 2010; Shen et al. 2013; Wang et al. 2014), there is little known about the combined effect of different water and nitrogen regimes on wheat root characteristics, and to date there have been no known investigations of the relationship of root traits to plant growth and stable isotope signatures under the combined growing conditions of water and nitrogen. Moreover, earlier studies have not investigated the performance of $\delta^{13}C$ and $\delta^{18}O$ or other secondary traits to assess the interaction of these different growing conditions on root characteristics.

The present study evaluated the performance of old and modern durum wheat genotypes grown in lysimeters under two different water and nitrogen regimes to assess the combined effect of such growth conditions on: (i) aerial biomass, (ii) total root growth, morphology and distribution through the soil profile and (iii) the relationships of these root traits with AB, photosynthetic and transpirative characteristics and *WUE*.

Results

Plant growth

The analysis of variance (ANOVA) results (Table 1) showed that the above and belowground biomasses were affected significantly by the water and nitrogen regimes. The water regime had a greater effect on the aboveground biomass than the nitrogen regime, which had a greater effect on the belowground biomass. Compared with the low-watered (LW) plants, the high-watered (HW) plants had significantly higher aerial biomass (AB) including the spike, culm and leaf blade biomass, whereas the number of spikes per plant and plant height also increased by 45, 33, 48, 53, 33 and 11% respectively. Also, days to heading were delayed on average by 3.4 days. The low nitrogen (LN) regime significantly increased the root dry biomass and root length and decreased the ratio of aerial to root dry biomass by 35, 27, and 47%, respectively, compared with the high nitrogen (HN) regime. Also, the LN regime decreased the flag leaf nitrogen content (N_{FL}) and the specific leaf nitrogen (SLN) by 19 and 26%, respectively. In addition, significant differences in plant growth were detected between the genotypes for all studied traits except for the number of spikes per plant. The specific leaf area (SLA) was affected only by the genotypes. Overall, the old genotypes exhibited higher values of AB as well as culm, leaf and root biomass, root length, plant height, SLA, the ratio of aerial to root biomass, N_{FL} and SLN, with general means being 37, 60, 43, 55, 51, 43, 20, 39, 16 and 36% higher than the modern genotypes, respectively. By contrast, spike biomass was 26% lower in old compared to modern genotypes. Also, days to heading for old genotypes was delayed about 7 days compared with modern genotypes. Whatever the water or nitrogen combination, old genotypes had higher AB and root biomass, as well as increased root length and a decreased ratio of aerial to root biomass than the modern varieties (Figure S1). Figure S2A-D shows clearly that regardless of the water and the nitrogen regime combination, the modern genotype Sula tended to have the lowest AB (although only a tendency with no significant difference from other modern genotypes), while the old genotypes Negro, Jerez 37 and Forment de Artes tended to have the highest AB (again with no significant difference between the three genotypes).

Leaf gas exchange

Both water and N regimes had significant effects on all flag leaf gas exchange parameters (Table 2). The HW regime significantly increased the net CO_2 assimilation rate (A_{sat}), stomatal conductance (g_s), transpiration rate (E) and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) while it decreased the instantaneous water use efficiency (WUE_{Inst}) compared with LW regime. The HN regime significantly decreased the A_{Sat}, g_s, E, C_i/C_a and increased the WUE_{Inst} more than the LW regime. Significant genotypic differences existed for all gas exchange parameters except for both g_s and E. Old genotypes had significantly higher A_{Sat}, and WUE_{Inst} (11 and 9%, respectively) and lower C_i/C_a (7%) than modern genotypes.

Stable carbon and oxygen isotopes, *WUE* and cumulative plant transpiration

The HW regime significantly increased the cumulative plant transpiration (T_{cum}) by 48% compared with LW regime plants (Table 2). Also, the genotypic effect was significant for the T_{cum}, with the old genotypes having on average around 16% higher T_{cum} than the modern genotypes. The HN regime significantly increased the time-integrated water use efficiency (WUE_{AB}) compared with the LN regime and also the genotypic effect was significant, with the old genotypes exhibiting values 25% higher than the modern genotypes. However, the LW regime did not significantly increase the WUE_{AB} compared with the HW. The LW and HN regimes significantly increased the carbon isotope composition of the flag leaf blades ($\delta^{13}C_{FL}$) with the N regime having a greater effect than the water regime. Also, significant genotypic differences exist and in general the old genotypes had a higher $\delta^{13}C_{FL}$ than the modern genotypes. The HW regime significantly decreased the oxygen isotope composition of the flag leaf blades ($\delta^{18}O_{FL}$), and also the genotypic effects were highly significant, while the N regime had no significant effects on $\delta^{18}O_{FL}$ (Table 2). Whatever the water and nitrogen regime combination the old genotypes tended to show higher T_{cum}, WUE_{AB} and $\delta^{13}C_{FL}$ and lower $\delta^{18}O_{FL}$ than the modern genotypes (Figure S1).

Root distribution and morphology

The ANOVA results (Table 3) showed that water regimes did not significantly affect the root weight density (RWD) in any of the soil layers, while the LN regime compared with the HN regime significantly increased the RWD in all soil layers except for the upper one (0-30 cm). The genotypic differences for the RWD were significant, with the old genotypes being 48, 49, 59, 69 and 75% higher than the modern genotypes at the 0-30, 30-60, 60-90, 90-120 and 120-150 cm layers, respectively. Whatever the water and nitrogen regime combination the old genotypes showed higher RWD in the whole soil layers than the modern genotypes (Figure 1A and C). Figure S4A-D shows that regardless of the water and nitrogen regime combination there were significant genotypic differences between the studied genotypes in all soil layers, with the modern genotype Sula tending to have the lowest RWD in almost all soil layers, while the opposite occurred for the old genotype, Forment de Artes.

The HW regime significantly increased the root length density (RLD) in all soil layers except for the 30-60 cm layer, while the HN regime significantly decreased the RLD in all soil layers except for the upper soil layer (0-30 cm) (Table 3). The genotypic effect was significant for all studied soil layers with the old genotypes having higher RLD than the modern genotypes and differences increasing from the upper to the lowest layer: 37, 44, 53, 60, and 70% (Table 3). In fact, irrespective of the water and nitrogen regime combination the old genotypes had higher RLD in all soil layers than the modern genotypes (Figure 1B and D), with Sula tending to show the lowest RLD in almost all soil layers (Figure S4E-H).

The HW regime significantly increased the specific root length (SRL) in the upper soil layers (0-30 and 30-60 cm), while the HN regime increased the SRL significantly in the middle and lower soil layers (from 60-90, 90-120 and 120-150 cm). The genotypic effect was significant for all studied soil layers where the modern genotypes had, on average, SRL values that were 22, 16, 19, 26 and 28% higher in the 0-30, 30-60, 60-90, 90-120 and 120-150 cm soil layers, respectively, than the old genotypes (Table 3).

Under the LW regime and regardless the nitrogen regimes Sula produced no root biomass in the lower soil layer (120-150 cm), and thus no distributional data (RWD and RLD) was recorded at that depth of the soil column (Figure S4A, B, E and F).

Relationships between traits

For both water regimes and for both old and modern genotypes, AB showed significant positive correlations with WUE_{Inst}, WUE_{AB} and $\delta^{13}C_{FL}$ and significant negative correlations with A_{Sat} (except for modern genotypes under LW and old genotypes at HW), g_s, E and C_i/C_a (Table 4).

Likewise, for both the water regimes and for both old and modern genotypes, g_s showed significant positive correlations with A_{Sat}, E and C_i/C_a and significant negative correlations with WUE_{Inst} (except for modern genotypes under LW), WUE_{AB} and $\delta^{13}C_{FL}$.

The AB under the LW regime correlated negatively with: (i) both root biomass and root length in the old genotypes (Table 4); (ii) RWD of the soil layer (30-60 cm) and the upper and middle soil layers (0-30, 30-60 and 60-90 cm) in the modern and old genotypes, respectively (Table 5); and (iii) the RLD in the middle soil layer (60-90 cm) of old genotypes (Table 5). On the other hand, the AB under the LW regime correlated positively with the SRL of the lower soil layers (90-120 and 120-150 cm) and (120-150 m) in the modern and old genotypes, respectively (Table 5). In contrast, under the HW regime the AB did not show any significant correlation with either root biomass or root length across the whole profile (Table 4) or with RWD, RLD and SRL within any specific soil layer (Table 5).

For both old and modern genotypes under the LW regime, root biomass and root length showed significant negative correlations with the ratio of aerial to root biomass and WUE_{AB}, whereas these two traits were positively correlated with each other (Table 4). Moreover, the RWD of different soil layers for the modern (except 0-30 and 120-150 cm) and old genotypes (except 0-30 cm) showed significant negative correlations with the WUE_{AB} (Table 5). Furthermore, the RLD of the lower soil layers (120-150 cm) of the modern genotypes and of almost all soil layers (except 0-30 cm) of old genotypes (except 0-30 cm) showed significant negative correlations with WUE_{AB} (Table 5). In contrast, the SRL for almost all soil layers (except 30-60 cm) of the modern genotypes and the lower soil layer (120-150 cm) of the old genotypes showed significant positive correlations with WUE_{AB} (Table 5).

Under the HW regime the root biomass and root length of both old and modern genotypes correlated negatively with the ratio of aerial to root biomass and this trait was positively correlated with WUE_{AB}, whatever the water regime (Table 4). However, under the HW regime the RWD and RLD of the different soil layers did not show any significant correlations with the WUE_{AB} (Table 5). Also, the SRL of the different soil layers under the HW regime did not show any significant correlations with the WUE_{AB} (Table 5). Under the LW regime, root biomass and root length of old genotypes showed significant negative correlations with the $\delta^{13}C_{FL}$, while only the root biomass of modern genotypes correlated negatively with $\delta^{13}C_{FL}$ (Table 4). Moreover, for both old and modern genotypes the RWD of several layers (30-60, 60-90 and 120-150 cm) showed significant negative correlations with the $\delta^{13}C_{FL}$ (Table 5), whereas the RLD of the lower soil layer (120-150 cm) and the central soil layers (30-60 and 60-90 cm) of modern and old genotypes, respectively, correlated negatively with the $\delta^{13}C_{FL}$ (Table 5). Only in the case of old genotypes was there a significant correlation of the RWD in the lower soil layer (90-120 cm) with $\delta^{18}O_{FL}$ (Table 5). Contrary to the RWD and RLD, the SRL under the LW regime correlated positively with the $\delta^{13}C_{FL}$ in almost all soil layers of the modern genotypes (except 120-150 cm) and the lower soil layer (120-150 cm) of the old genotypes. Under the HW regime, no correlations were detected between both $\delta^{13}C_{FL}$ and $\delta^{18}O_{FL}$ with root biomass, root length, RWD, RLD and SRL (Tables 4 and 5).

Irrespective of the water regime, the root biomass and root length of modern genotypes showed significant positive correlations with T_{cum} (Table 4). In the case of old genotypes root biomass and root length only correlated with T_{cum} under the HW regime (Table 4). Moreover, root biomass only correlated positively with g_s, E and C_i/C_a for the modern genotypes under the LW regime (Table 4).

For modern genotypes and irrespective of the water regime the RWD and RLD correlated positively with T_{cum} in all of the soil layers except under the LW regime for RWD in the central soil layer (30-60 cm) and the RLD of the upper soil layers (0-30 and 30-60 cm) (Table 5). For old genotypes, the RWD not significantly correlated with T_{cum} in any of whole soil layers under the LW regime, whereas under the HW regime positive correlations were detected in the 0-30, 60-90 and 90-120 cm soil layers (Table 5). Also, the RLD of old genotypes showed significant positive correlations with T_{cum} in the lower soil layers (90-120 and 120-150 cm) and the upper and lower soil layers (0-30 and 90-120 cm) under the LW and HW regimes, respectively (Table 5).

Discussion

The effect of nitrogen and water status on aerial biomass, gas exchange and water status

As expected, the decrease in the amount of irrigation diminished the aerial biomass (AB), the gas exchange traits of stomatal conductance (g_s), transpiration (E), the CO_2 assimilation rate (A_{Sat}), and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a), as well as the stable carbon and oxygen signatures of the flag leaf ($\delta^{13}C_{FL}$ and $\delta^{18}O_{FL}$, respectively), while the instantaneous water use efficiency (WUE_{Inst}) increased (Cabrera-Bosquet et al. 2009b; Elazab et al. 2012) and differences in the time-integrated water use efficiency (WUE_{AB}) did not reach significance (Table 2).

The high nitrogen (HN) regime increased the AB but decreased the root biomass and root length (Table 1). This resulted in a negative effect on the water status, decreasing the g_s, the E and to a lesser extent the C_i/C_a. As a consequence the WUE_{Inst}, the WUE_{AB} and $\delta^{13}C_{FL}$ increased (Table 2). In the same sense, no matter which water regime or group of genotypes was studied, when the two N regimes were combined the AB correlated positively with $\delta^{13}C_{FL,}$ WUE_{AB} and WUE_{Inst} and all these parameters correlated negatively with g_s (Table 4). It has been report by Cabrera-Bosquet et al. (2007) in a durum wheat pot experiment and by Kondo et al. (2004) in field grown rice that although high nitrogen fertilization increases the AB it also induces water deficit, leading to a decrease in g_s whereas the $\delta^{13}C$ increased. The positive relationship between $\delta^{13}C$ and WUE has been reported extensively and agrees with the theoretical basis of carbon isotope fractionation in plants (Farquhar and Richards 1984; Farquhar et al. 1989).

A negative relationship between $\delta^{13}C$ with AB or/and GY across water regimes has been frequently reported for durum wheat plants grown under different water regimes with a similar nitrogen level (Araus et al. 1998; 2003; Monneveux et al. 2006; Elazab et al. 2012). In contrast, the positive relationship obtained in this study between $\delta^{13}C_{FL}$ and AB within each water regime agrees with the results of Cabrera-Bosquet et al. (2007) who reported that the nitrogen regime, in addition to the water regime, affected the predictive nature of $\delta^{13}C_{FL}$ as an indirect trait for breeding under drought.

An increase in the $\delta^{13}C_{FL}$ (and therefore in WUE) may be due to either greater photosynthetic capacity or lower g_s (and therefore C_i/C_a), or both factors (Farquhar and Richards 1984; Farquhar et al. 1989; Condon et al. 2004; Cabrera-Bosquet et al. 2007, 2009a). The correlation results (Table 4) for both old and modern genotypes supported the idea of lower g_s being responsible for higher $\delta^{13}C_{FL}$ as both g_s and A_{Sat} showed significant negative correlations with $\delta^{13}C_{FL}$, WUE_{AB} and WUE_{Inst} in almost all cases, and A_{Sat} showed significant positive correlations with C_i/C_a.

Although the HN regime had a clear effect on increasing the AB, the HN regime did not significantly affect either the cumulative plant transpiration (T_{cum}) or the $\delta^{18}O_{FL}$ (Table 2 and Figure S1A, E and H). According to Cabrera-Bosquet et al. (2009a) a higher T_{cum} is not only associated with higher gas exchange (higher g_s) per unit leaf area but also with the increase in the total transpiring leaf area. The oxygen isotope composition of the flag leaf ($\delta^{18}O_{FL}$) decreased in response to increased watering (Table 2), which agrees with previous reports in durum wheat (Cabrera-Bosquet et al. 2009b; Elazab et al. 2012). However, within each genotype (old or modern) and watering regime (LW and HW) group the $\delta^{18}O_{FL}$ was not the best physiological trait to inform on the effect of N fertilization on plant water status because it lacked the ability to show any significant correlation with the time-integrated measures of the total AB, T_{cum}, $\delta^{13}C_{FL}$ or the instantaneous g_s (Table 4). The only exception was the old genotypes under the LW regime, where a significant negative correlation was detected between $\delta^{18}O_{FL}$ and T_{cum}, which may reveal the negative effect of a HN regime on the plant water status (decreasing transpiration). Previous studies (Ferrio et al. 2007; Cabrera-Bosquet et al. 2009b; Elazab et al. 2012; Araus et al. 2013) have already reported doubts about the use of $\delta^{18}O$ to evaluate wheat genotypic performance under different water regimes due to a number of miscellaneous factors (e.g. the $\delta^{18}O$ of the source water, exchange with water during assimilate formation and metabolic fractionation during assimilate transport) which can distort the usefulness of $\delta^{18}O$ as an environmental indicator.

The effect of water and nitrogen on root growth and morphology

Root dry biomass slightly increased under the HW regime (Table 1) whereas the root weight density (RWD) was not affected (Table 3). A previous study by Gajri et al. (1989) reported that water stress restricted root growth, while Elazab et al. (2012) reported that root growth was not affected or even slightly enhanced by the water deficit and that the RWD increased in almost all soil layers. According to several authors (Sharp et al. 1990; Elazab et al. 2012; Wang et al. 2014) root growth is less affected or even enhanced by water deficits in comparison with the shoot. The osmotic adjustment occurs in root tips and thus prolongs root cell expansion, which has been deduced as the mechanism that allows the root size to grow even further under moderate water deficits

(Sharp et al. 1990), while severe water deficits are reported to inhibit root growth (Sharp et al. 2004).

Both root length and root length density (RLD) in almost all soil layers responded positively to the HW regime (Tables 1 and 3). This is in agreement with previous studies in wheat (Barraclough 1989; Nagesh 2006; Elazab et al. 2012). The increase in root length could be due to the fact that plants under well-watered conditions produce a larger number of adventitious roots (Elazab et al. 2012). The adventitious roots are known to constitute 86–99% of the entire root system, occupy the upper soil layer (0-40 cm) and are unable to grow in deep soil layers like seminal roots (Manske and Vlek 2002; Elazab et al. 2012). In fact, the HW regime increased the RLD of the upper layer (0-30 cm) by 30% compared with the LW regime (Table 3). Drying of the soil surface may inhibit development of the adventitious root system (Blum 2005). In addition, the number of adventitious roots depends mainly on the tillering ability of the plant (Manske and Vlek 2002) and the LW regime significantly decreased the number of tillers and subsequently the number of spikes per plant (Table 1).

Besides water availability, root growth can also vary according to the availability of N (Herrera et al. 2007). Both root biomass and root length increased under the LN regime (Figure S1B and C; Table 1). These results are in agreement with earlier studies (Comfort et al. 1988; Wang et al. 2009, 2014; Shen et al. 2013) reporting high N fertilization as affecting root growth negatively (decreasing both roots biomass and length).

The ratio of aerial to root dry biomass is a parameter that evaluates the distribution of dry matter between shoots and roots (Wang et al. 2014). It is generally reported to decrease under water deficit (Ayad et al. 2010; Elazab et al. 2012; Carvalho et al. 2014) or under nitrogen deficiency (Dreccer et al. 2000; Miller and Cramer 2005). The present study agrees with this previous work because the decrease in this ratio under the LW regime was mainly due to the decrease in AB (decreased by 45%) rather than a decrease in the root biomass (decreased only by 13%), whereas under LN conditions it was the consequence of a decrease in AB (by 27%) together with an increase in the root biomass (by 35%).

Studies on the effect of different water regimes on the specific root length (SRL) have reported contradictory results (Ryser 1998; Manske and Vlek 2002; Carvalho 2009; Elazab et al. 2012; Carvalho et al. 2014). In addition, reports about the effect of N fertilization on the SRL are scarce and the results are inconsistent (Carvalho 2009). In our study, the SRL increased under HW conditions compared to LW conditions, which is in agreement with previous studies done by Elazab et al. (2012) in a lysimeter experiment with durum wheat. Contrary to these findings, an increase of SRL in response to water stress was reported by Nagesh (2006) in a winter wheat lysimeter experiment and Song et al. (2010) in spring wheat in pots. Furthermore, Carvalho et al. (2014) observed an increase in SRL in the water-stressed regime in some years in a lysimeter experiment with durum wheat and barley, but in other years the water regime did not affect the SRL. In our study the higher SRL in the upper soil layers (0-30 and 30-60) under HW conditions (Table 3) could be explained as a response of the plant roots to the low soil water holding capacity of the substrate filling the lysimeters. This encouraged the plants to respond rapidly by increasing their root length and in turn increase the root surface area, and thus facilitate more root interception for water absorption in the upper soil layer particles. On the other hand, the high SRL in the middle and lower soil layers under the HN regime compared to the upper soil layers could be due to the mobility of N into deeper soil layers due to leaching (Herrera et al. 2007). While high N availability is reported to induce reductions in root biomass (Comfort et al. 1988; Wang et al. 2009; Shen et al. 2013), it also increases root branching and thus increases the production of thinner roots with an associated low biomass allocation to roots and an increase in SRL as a consequence (Belford et al. 1987; Herrera et al. 2007; Bonifas and Lindquist 2009).

The effect of water and nitrogen regime on root distribution

Root distribution in the soil profile may have a vital role in resource capture and dry matter portioning, especially under water and nitrogen limited conditions (Ayad et al. 2010). The root distributional traits in the present study were measured at about three weeks after anthesis (mid-grain filling). This was due to the reported increase in root growth from sowing up to the start of grain filling (Gregory et al. 1978; Wang et al. 2014) after which root growth stops because grains becoming the main sinks for photoassimilates and a general reduction in the growth of all other plant parts occur (Begg and Turner 1976). However, root growth may continue during grain filling (Gregory et al. 1978; Herrea et al. 2007) due to the presence of water and nutrients in the deep soil layers (Carvalho 2009; Elazab et al. 2012). Moreover, small differences in resource uptake during flowering and grain filling are extremely important and could greatly increase the GY (Lilley and Kirkegaard 2011).

The RWD and RLD of the old genotypes under the LN regimes and irrespective of the water regimes tended to increase in the middle and deeper soil layers (60-90, 90-120 and 120-150 cm) (Figure 1C and D). Moreover, under the low-watered (LW) and LN regime combination the RLD at the deeper soil layers (90-120 and 120-150 cm) was almost the same or even little higher than the RLD of the upper soil layer (0-30 cm). Similar to the case of SRL (indicated above) this increase in RLD could be due the mobility of N in the soil (Herrera et al. 2007). Although high root density is not considered an advantage in the uptake of available soil N (Herrera et al. 2007), root growth has the highest priority for N when it is deficient (Herrera et al. 2007). In fact, wheat may exhibit high nitrate uptake until the end of grain filling (Barraclough 1986). Unlike the LN treatment, under high N supply and regardless of the water regime, old genotypes exhibited a sharp decrease in RWD in the deeper soil layers (120-150 cm). Such a decrease in RWD could be due to the excessive N availability in the deep soil layers, which is known to inhibit root growth (Comfort et al. 1998). In fact HN conditions significantly decreased the RWD and RLD in almost all soil layers for both old and modern genotypes regardless of the water regime. However, the modern genotypes had increases in RWD and RLD in the upper soil layer (0-30 cm) in response to a combination of HN and HW conditions. These results are in agreement with recent studies by Ayad et al. (2010) and Wang et al. (2014). The increased RWD and RLD in the upper soil may be due to the fact that the early growth phases were accompanied by greater resource availability (water and nutrients) in the top soil layers (due to the frequent surface irrigation with nutrient solution), which encourages root proliferation in the upper soil layer (Barraclough 1989; King et al. 2003). In contrast, later in the growing season the transport of water and nutrients between soil layers may change the RWD and RLD distribution in the soil profile (King et al. 2003).

The HW regime increased the RWD and RLD of old genotypes in almost all soil layers and of the modern genotypes in the upper and lower soil layers (0-30, 90-120 and 120-150 cm) (Figure 1). This is in agreement with several studies (Zhang et al. 2004; Wang et al. 2014) that reported that wheat plants with greater levels of irrigation have a higher RWD and RLD than those with lower irrigation, especially in the deeper soil profiles. However, our results contradict those of Ayad et al. (2010) who reported for one of the durum wheat genotypes in their study that water deficits increased root growth in almost all soil layers, as well as those of Xue et al. (2010) who reported that water deficit increased root growth in the deeper soil layers (60-100 cm).

Relationship of root traits to AB and water status

The ability of a plant to extract water and nutrients has a large effect on the growth of the AB (Ayad et al. 2010). Generally, it is well established that plants with large deep root systems are more tolerant of drought than shallow-rooted plants (Kramer and Boyer 1995) because a larger root system can access more water resources from the deep soil layers (Kramer 1969; Ma et al. 2010; Wang et al. 2014). Therefore, breeding strategies for drought have included selection of progeny with large root systems (Ma et al. 2010). The results of the present study reinforce the importance of a large root system in capturing more resources, where root biomass, length and distribution (RWD and RLD) in the soil profile indicated significant positive correlations with T_{cum} (Tables 4 and 5). However, developing and further maintaining a large root system (i.e. a high root biomass) is very resource consuming for the plant in terms of assimilates required (Passioura 1983) and respiration costs (Ma et al. 2010). Therefore, several studies have proposed that a larger root system might not necessarily be an advantage for higher AB and/or GY production under water stress (Lynch et al. 2014). In actuality, a plant root system that is able to acquire a limiting soil resource at a reduced metabolic cost would increase the agronomical *WUE* (AB or GY per unit of water transpired) (Passioura 1983; Carvalho 2009; Zhang et al. 2009; Ma et al. 2010; Lynch et al. 2014). In fact, the results of the present study support the idea that a large root system might not be necessary to improve the performance under the LW regime because AB correlated negatively with root biomass, length (Table 4) and their distribution (RWD and RLD, respectively) across soil layers (Table 5). Also, root biomass and root length showed significant negative correlations with the ratio of aerial to root biomass (irrespective of the water regime) and with WUE_{AB} (only under the LW regime) and these two traits correlated positively with each other under both water regimes (Table 4). Moreover, under the LW regime, RWD and RLD showed negative correlations with WUE_{AB} throughout the soil profile (Table 5). Indeed, extensive root growth early in the growing season may also be counterproductive by exhausting soil water reserves before the plant is able to complete its life cycle (Richards and Passioura 1989; Lynch et al. 2014). It could be concluded from our study that the HN regime increased the AB but in turn accelerated soil moisture depletion (Ayad et al. 2010) generating some degree of water stress even under what is considered a HW regime with a frequent replenishment of transpired water. In fact, even a large transpiring area under what is considered as a HW regime may contribute to the generation of drought, for example, the occurrence of the typical midday decrease in g_s (Chaves 1991). Consequently, a larger root system is not necessarily a favourable trait under water stress conditions because it could sometimes result in rapid soil moisture consumption (Ma et al. 2009).

Contrary to RWD and RLD, the SRL under the LW regime correlated positively with AB and WUE_{AB} (Table 5). Previous studies (Løes and Gahoonia 2004; Bonifas and Lindquist 2009; Carvalho 2009) reported the importance of a high SRL in increasing the root surface area and thus the access to resources within a given soil volume. However, and as mentioned previously, a high SRL limits transport, storage and support capacity of the roots and it is associated with a shorter root life span than roots with a low SRL (Fitter 1996; Cornelissen et al. 2003). It could be concluded that under LW conditions wheat plants increase their SRL by decreasing the root diameter, and this decreases the soil water uptake earlier in the growth cycle therefore preserving water for the reproduction stages (grain filling). In this sense, the wheat breeding strategies for the summer-dominant rainfall environments of Australia, where crops rely on the water accumulated in the soil prior to emergence have focused on designing wheat plants with a conservative strategy of water consumption, allowing the crop to complete their life cycle before available soil water is exhausted. In these plants the xylem diameter of seminal roots was reduced to increase their axial resistance to water from the root system to the shoot (Richards and Passioura 1989; Rebetzke et al. 2002; Condon et al. 2004). However, we could not confirm whether increasing SRL is related to a decrease in root diameter and/ or reduction in the root tissue density (Ryser 1998; Hajek et al. 2013) because measures of root density and root tissue density were not performed in this study.

On the other hand, under the HW regime the AB did not show any significant correlation with either root biomass or root length across the whole profile (Table 4) or with RWD and RLD and SRL within any specific soil layer (Table 5). In the same sense, Elazab et al. (2012) reported only a medium significant correlation ($r = 0.49$, $P < 0.05$) between RLD and AB in a durum wheat lysimeter experiment under a well-watered regime, while both RWD and RLD showed strong highly significant correlations ($r = 0.80$, $P < 0.001$ and $r = 0.73$, $P < 0.001$) with the AB under the water-stressed regime. It could be concluded that expression of root traits appears more clearly under resource limitation.

Previous studies (White et al. 1990; Lopes and Reynolds 2010; Elazab et al. 2012) using indirect approaches to screen for root performance such as canopy temperature, $\delta^{13}C$ and $\delta^{18}O$ reported negative relationships of these traits with root traits (i.e. RWD, RLD and SRL). These relationships could be explained by a higher AB and/or GY being expected under a favourable water status, where a deep root system (i.e. high RWD and RLD at deeper soil layers) can access more soil water in the deep soil layers, or where roots with a high SRL could increase root resource interception within a given soil layer (Wasson et al. 2012; Lynch et al. 2014). The negative correlations of root biomass and root length, as well as RWD and RLD throughout the soil layers with $\delta^{13}C_{FL}$ (Tables 4 and 5) were not due to a better water status because the AB was positively correlated with $\delta^{13}C_{FL}$ (Table 4). Indeed, the negative correlations of $\delta^{13}C_{FL}$ with different root traits were mainly due to the effect of the HN regime on increasing the AB, WUE_{AB} and $\delta^{13}C_{FL}$, while decreasing root biomass, root length and root distribution (RWD and RLD) in the soil (Tables 1 and 3). On the other hand, the positive correlations between SRL and $\delta^{13}C_{FL}$ were due to the additive effect of the HN regime in decreasing the root biomass (Comfort et al. 1988; Wang et al. 2009, 2014; Shen et al. 2013), while increasing root branching (i.e. producing thinner and longer roots) (Belford et al. 1987; Herrera et al. 2007). Overall, it could concluded that: (i) the interaction of nitrogen and the water regime could affect the predictive nature of the $\delta^{13}C_{FL}$ as a proxy trait for root structure, and (ii) the selection of the optimal root structure should take into account the metabolic costs of root growth and maintenance in addition to the ability of the root system to capture water (Wasson et al. 2012).

Apart from a negative relationship of the $\delta^{18}O_{FL}$ with T_{cum} and the RWD of the lower soil layer (90-120 cm) in old genotypes under LW conditions (Tables 4 and 5) this isotopic signature did not correlate with any transpirative or root traits (Tables 4 and 5). The lack of significant relationships of $\delta^{18}O_{FL}$ with T_{cum} and root traits under both HW and LW regimes raises doubts about the use of $\delta^{18}O_{FL}$ in detecting genotypic performance under different water regimes.

Modern vs. old genotypes

The AB of old genotypes was higher than that of the modern genotypes regardless of the water and nitrogen regime combinations (Figures S1A and S2A-D). This was due to the larger culms and leaf blades and the greater plant height of the older varieties (Table 1). The spike dry biomass was higher in modern genotypes than old ones (Figure S3B and Table 1), and this was associated with the changes in the harvest index (HI) due to the green revolution (Passioura 1983; Waines and Ehdaie 2007).

Overall, the root biomasses of old genotypes were almost double that of the modern genotypes (post green revolution) (Table 1). Similar results have been reported by Mac Key (1973) where the German landrace Brown Schlanstedt had three times the root biomass of the post green revolution dwarf, Mexican Mayo 64, and the dwarf Japanese variety, Kohnosu. Irrespective of the water and the nitrogen regime combination the RWD and RLD in the old genotypes were higher than in the modern genotypes across all the soil profile layers (Figure 1). Siddique et al. (1990) found that the old genotypes had higher root densities (RWD in his case) in the upper soil layers (0-40), while both old and modern (post green revolution) genotypes showed no genotypic differences in the deeper soil layers. Indeed, the improvement in the modern wheat genotypes of the green revolution is associated with reduced root size due to the direct selection of the aboveground organs, which may indirectly select for small root systems (Siddique et al. 1990; Waines and Ehdaie 2007).

Modern genotypes showed lower flag leaf nitrogen content (N_{FL}) and shorter days to heading (DH) than old genotypes (Table 1). Similar results were reported by Araus et al. (2013) for the same old and modern durum wheat genotypes under different combinations of water and nitrogen regimes in the field. No matter which water and nitrogen regime combination was tested, the old genotypes showed higher $\delta^{13}C_{FL}$ and T_{cum} and lower $\delta^{18}O_{FL}$ than modern genotypes (Table 2 and Figure S1E, G and H). A similar pattern in the $\delta^{13}C$ between old and modern durum wheat genotypes has been reported before (Royo et al. 2008; Araus et al. 2013) and can be attributed a lower g_s of old genotypes compared to modern genotypes (Royo et al. 2008; Araus et al. 2013). However, in our study this genotypic difference in the $\delta^{13}C_{FL}$ between old and modern genotypes could not be attributed to constitutive or environmentally-driven differences in g_s but rather to a higher A_{Sat} and lower C_i/C_a of the old genotypes compared to the modern genotypes (Table 2). The higher photosynthetic capacity of the old genotypes may be associated with a lower SLA and higher N_{FL} (Table 1). According to Araus et al. (1997) and Cabrera-Bosquet et al. (2007) a higher N_{FL} and specific leaf nitrogen (SLN) and a lower SLA could result in a higher rate of photosynthesis.

Modern genotypes exhibited higher $\delta^{18}O_{FL}$ than old genotypes (Table 2 and Figure S1H). Such differences could be due to the fact that a large root system being more advantageous to the plant than a small root system for acquiring water (Kramer 1969;

Ma et al. 2010). Thus, the larger root biomass and root length as well as the higher RWD and RLD values across the soil profile (Figure S1B and C; Tables 1 and 3) of the old compared with the modern genotypes were translated into a greater ability to access water in the soil profile layers, and thus higher T_{cum} and lower $\delta^{18}O_{FL}$ than modern genotypes (Table 2). In a durum wheat lysimeter experiment Elazab et al. (2012) reported the $\delta^{18}O$ to reflect genotypic differences in RWD and RLD under well-watered conditions. In fact, the high yielding modern genotypes were reported to have less water capture ability from the soil than old genotypes (Ayad et al. 2010). The results presented in Table 3 confirmed the previous idea as the old genotypes had lower SRL than the modern genotypes and plants with low SRL tend to: (i) exert greater penetration force on soil, and thus can grow deeper into the soil profile (Cornelissen et al. 2003; Carvalho 2009), (ii) have better ability to withstand low soil water content (Cornelissen et al. 2003) and (iii) higher rates of water transport within the root (Fitter 1996; Cornelissen et al. 2003). Thus, the old genotypes were able to extract more soil water, extend the duration of green leaf area (Christopher et al. 2008) and consequently increased the T_{cum} compared with the modern genotypes. Greater SRL generally results in greater RLD (Bonifas and Lindquist 2009; Elazab et al. 2012; Carvalho et al. 2014).

Conclusions and further work

This study highlighted the importance of the $\delta^{13}C$ as a phenotyping tool for informing on the plant water status, plant growth and water use efficiency under the combined effect of different water and nitrogen regimes, and also the performance of root traits under the LW regime. In contrast, the usefulness of $\delta^{18}O$ as a phenotyping tool is less evident. The positive correlations across nitrogen regimes of AB and WUE_{AB} with $\delta^{13}C$ across different water regimes revealed that the nitrogen regime also affected the predictive nature of $\delta^{13}C$ as a phenotyping trait in drought breeding programs. In addition, the study highlights the importance of both root biomass and root length and their distribution (RWD and RLD) in the capture of soil water in the different soil layers under both water regimes. However, the negative relationships under the LW regime of root biomass, root length, RWD and RLD with AB, $\delta^{13}C$ and WUE revealed that consideration of the optimal root system for high AB production also requires attention to the costs of water capture in terms of carbon resources (metabolic costs for root construction and maintenance) alongside the root system’s ability to capture water.

Thus, selecting for root traits like high SRL could be very interesting in breeding programs under resource deficits (water or N or combination of both), as it could enhance the root system's absorption capacity with low metabolic costs. The current study also revealed that using proxy traits ($\delta^{13}C$ and $\delta^{18}O$) as indicators of special root structure (the deeper root system) in drought breeding programs could be misleading. Overall, clear differences were detected for both $\delta^{13}C$ and $\delta^{18}O$ between the old and modern genotypes under all growth conditions tested, and these differences in traits were of a diverse nature (total root size, N_{FL}, phenology, etc).

Methods

Plant materials and growth conditions

Ten durum wheat (*Triticum turgidum* L. var. *durum*) genotypes representing five modern Spanish commercial varieties released after 1990 (Anton, Bolo, Don Pedro, Regallo and Sula) and five old Spanish landraces (Blanqueta, Griego de Baleares, Negro, Jerez 37, and Forment de Artes) were tested. The experiment was carried out in the experimental field facilities of the Faculty of Biology, University of Barcelona, Spain, from the end of December 2011 to the start of May 2012. Grains of the ten genotypes were washed in Petri dishes with sodium hypochlorite (NaOCl 5%) for 1 minute, drained and then washed with distilled water and finally soaked in deionised water and left to imbibe overnight. The imbibed grains were planted into 2L plastic pots filled with fine perlite-sand aggregates and left to vernalise at 4ºC for two weeks in the cold chamber. The pots were watered with deionised water twice a week throughout the whole vernalisation period. After the vernalisation period germinated seedlings of about 2 cm in length were transplanted into the lysimeters, which are built from PVC tubes of 14 cm in diameter and 150 cm long. The lysimeters were filled with a mixture of coarse perlite-sand (A13 commercial sizes; EUROPERL, Passau, Germany) aggregates (95%) and peat moss (5%). Prior to transplanting the lysimeters were longitudinally split into two halves, which were then taped together with parcel tape and the bottom end of the PVC tube was closed with a plastic mesh to enhance water drainage and keeping the substrate from being lost. Within each lysimeter, five seedlings of a given genotype were transplanted to achieve a plant density of 324 plants m^{-2}, within the range of a real field plant density. The experiment was arranged as a factorial randomized complete block design in three replicates with a total number of 120 lysimeters (ten genotypes ×

two water regimes × two nitrogen regimes x three replicates). The lysimeters were placed under a rain shelter to control water regimes properly and the rest of the environmental variables were not controlled. Therefore, the plants were exposed to temperature conditions following the seasonal pattern (Figure 2).

The plants were grown under a combination of two water and nitrogen regimes. The low and high nitrogen (LN and HN, respectively) regimes were applied the day before transplanting by over saturating half of the lysimeters (60 lysimeters) with a full strength (100%) Hoagland's nutrient solution (Hoagland and Arnon 1938) and the other half with a diluted Hoagland's nutrient solution (25%) and then they were left for one day to drain the excess irrigation. The lysimeter water capacity (equivalent to field capacity) was calculated as the difference between the weights of the lysimeters with wet substrate (after drainage of excess nutrient solution) and dry substrate (prior to saturation). For the high-watered (HW) regime, the lysimeters were maintained at 100% of lysimeter capacity until the experiment was harvested (three weeks after anthesis of the modern genotypes) by adding nutrient solution every 3 days. For the low-watered (LW) regime, irrigation was withheld two weeks after transplanting until substrate reached 40% of lysimeter total water capacity (which was about two weeks before heading) and then the lysimeters were maintained at this water content up to experiment harvest. From transplanting to the end of the experiment (middle of grain filling) plants were grown for about 18 weeks. The idea underlying this was to mimic a typical growth pattern under field conditions, including a slow decrease in water content, thus avoiding the situation typical of pot plants where severe water stress may develop in a few days.

Leaf gas exchange measurements

Leaf gas exchange measurements were conducted two weeks before harvest, which corresponded to anthesis time of the old genotypes and one week after anthesis for the modern genotypes. Measurements were conducted on the flag leaf blade of the main stem of three plants inside each lysimeter. Measurements were made with an IRGA LI-COR 6400 open system (LI-COR Inc., Lincoln, NE, USA), between 10.00 and 14.00 hours (solar time), under light saturated conditions (1500 μmol m^{-2} s^{-1}), 25°C, CO_2 concentration of 400 μmol mol^{-1} and a relative humidity (RH) between 60–70%. The gas exchange parameters measured were net CO_2 assimilation rate (A_{Sat}), transpiration rate (E), stomatal conductance (g_s) and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a). Further, the instantaneous water use efficiency (WUE_{Inst}) was calculated as the ratio A_{Sat}/E.

Plant growth and root traits

Days to heading (DH) were measured as the number of days after transplanting when 50% of the ears emerged. The number of spikes per plant (NSP) was determined one day before harvest (at 3 weeks after anthesis of the modern genotypes). At harvest, flag leaf blades of all plants were cut and scanned immediately with an Epson scanner (Epson Stylus SX130, Tokyo, Japan), images obtained at 300 dpi and the area of the flag leaf blades calculated using ImageJ 1.46r, a public-domain Java image processing and analysis program (http://imagej.nih.gov/ij/) created by the National Institutes of Health (US Department of Health and Human Services). The flag leaf blades and the rest of leaf blades of the entire lysimeter were placed separately inside paper envelopes, dried at 60°C for 48 h and the total dry biomass of the flag leaf blades and rest leaf blades measured. In addition, the specific leaf area (SLA, $cm^2\ g^{-1}$) of the flag leaf was calculated according to Cornelissen et al. (2003):

$$\text{SLA} = \frac{\text{Leaf blade area (cm}^2\text{)}}{\text{Leaf dry biomass (g)}}$$

The rest of the aboveground biomass was harvested, also placed inside paper envelopes, dried at 60°C for 48 h and weighed. Afterwards the aerial biomass (AB) was calculated as the total sum of spike (SB), culm (CB) and leaf blade (LB) dry biomass.

After harvesting the aerial parts, the lysimeter was separated into two halves and the inside substrate column was cut horizontally into five equal sections (layers), each 30 cm long, from the column’s upper surface to the bottom end: 0–30 cm; 30-60 cm; 60-90 cm; 90-120 cm; and 120-150 cm. Roots in each substrate column section were cleaned of substrate with tap water, further rinsed with distilled water, dried superficially with paper towel and a sample of 15 to 20 living root segments (roots white to light brown in colour) of 20-25 cm long each (representing in total *ca.* 10% of the roots in the soil section fresh weight) were kept inside plastic bags in the -4 ºC fridge for further root length measures. The rest of the root segments in the soil section were placed inside a paper envelope and dried at 60°C for 48 h. Later, the root samples were defrosted, spread by forceps in an acrylic box (A4 size) filled with a 0.5-1 cm height of distilled water to minimize the number of overlaps and the box placed over an Epson scanner (Epson Stylus SX130, Tokyo, Japan) for root digitisation. When the root sample was too large to complete in one scan, more scans were performed. The root segments were digitalized using the negative option (roots appeared in black, while the background was white) at 300 dpi resolution and 24 bit bmp image format. The length of the root segments was measured from the scanned images using ImageJ 1.46r (Figure 3) following a semi-automated process, which can be applied to all the pictures in the folder selected and do not require user interaction for each image, therefore allowing a huge number of images to be analysed in a few minutes.

Root dry weight was measured for each of the five sections separately and the root dry biomass (RB) was calculated as the sum of the root dry weight of the five sections. Further, the ratio of aerial dry biomass to root dry biomass (AB RB^{-1}) was calculated.

Root weight density of each soil section (RWD_{sec}, g m^{-3}) was calculated following Elazab et al. (2012):

$$RWD_{sec} = \frac{RB_{sec}}{\Pi * R^2 * L}$$

Where: RB_{sec} = root dry biomass in the soil section (g); R= lysimeter radius (0.07 m); and L= length of the lysimeter section (0.30 m long).

Specific root length at each of the five soil sections (SRL_{sec}, m g^{-1}) was determined from the analysed images as follows (Elazab et al. 2012):

$$SRL_{sec} = \frac{RL_{segments}}{RL_{segments}}$$

Where: $RL_{segment}$ = total length of the root segments (m) obtained from the scanned root images; and $RB_{segment}$ = root dry biomass of the scanned segments (g).

Root length of each soil section (RL_{sec}, m) was estimated according to the following equation:

$$RL_{sec} = SRL_{sec} \quad x \; RB_{sec}$$

The summation of the root lengths of the five sections was used to calculate the whole root length (RL).

Root length density of each soil section (RLD_{sec}, cm cm^{-3}) was calculated as follows (Elazab et al. 2012):

$$RLD_{sec} = \frac{RL_{sec}}{\Pi \text{ x } R^2 \text{ x } L}$$

The cumulative plant transpiration and water use efficiency

The amount of water transpired in each lysimeter (W_t) was calculated during the growth cycle by weighing the lysimeter just prior to irrigation. Direct evaporation from the substrate surface was avoided by adding 2 cm of coarse perlite aggregates to the top surface of each lysimeter. Then the cumulative plant transpiration (T_{cum}) between consecutive irrigations was calculated as W_t divided by the number of plants in the lysimeter. Further, the time-integrated water use efficiency of the plant was calculated as the ratio of the AB to the T_{cum} (WUE_{AB}).

Stable isotope analysis

Dry mater of the flag leaf blades was finely milled and samples of ~0.7 mg were weighed into tin capsules for $^{13}C/^{12}C$ ratio (R) and flag leaf nitrogen content (N_{FL}, %)

analyses and other samples of ~1 mg were weighed into silver capsules for $^{18}O/^{16}O$ ratio analysis.

The total N concentration and the stable carbon isotope analyses were conducted at the Scientific Facilities of the University of Barcelona. Analyses were performed using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Schwerte, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan), operating in continuous flow mode. The $^{13}C/^{12}C$ ratios (R) of plant material were expressed in δ notation (Coplen 2008), as the carbon isotope composition ($\delta^{13}C$):

$$\delta^{13}C\ (‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

Where: sample refers to plant material and standard to Pee Dee Belemnite (PDB) calcium carbonate. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 3, polyethylene foil; IAEA CH6, sucrose; USGS 40, L-glutamic acid) were used with an analytical precision of 0.1‰.

Further, the specific leaf nitrogen (SLN) of the flag leaf blades was calculated as the ratio between the flag leaf blades dry N content in dry matter and the flag leaf blade surface (g m^{-2}).

The analysis of the ^{18}O stable isotope was conducted at Iso-Analytical Limited Laboratory, (Crewe, Cheshire, UK). The $^{18}O/^{16}O$ ratios of the different plant parts were determined by an isotope ratio mass spectrometer (IRMS, Europa Scientific Geo 20–20) with a triple Faraday cup collector array to monitor the masses 28, 29 and 30. Results were also expressed in δ notation as oxygen isotope composition ($\delta^{18}O$) values:

$$\delta^{18}O\ (‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

using secondary standards of known $^{18}O/^{16}O$ ratios (IAEA-CH-6, sucrose; IAEA-C-3, cellulose; IAEA-601, benzoic acid) calibrated against Vienna Standard Mean Oceanic Water (VSMOW) with an analytical precision of 0.2‰.

Statistical analyses

Combined analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure to calculate the effects of water regimes, nitrogen regime, genotypes and all the possible interactions between them.

Mean separation of water regimes, nitrogen regimes and genotypes for the different parameters was performed by the independent sample t-test ($P \leq 0.05$). Relationships between parameters were analysed using the Pearson linear correlation and the correlations were built from nitrogen and genotype means within each water treatment. Data were analysed using the SPSS 16 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using SigmaPlot 11.0 for Windows (Sysat Software Inc., Point Richmond, CA, USA).

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Conflict of interest

The authors declare that they have no competing interests.

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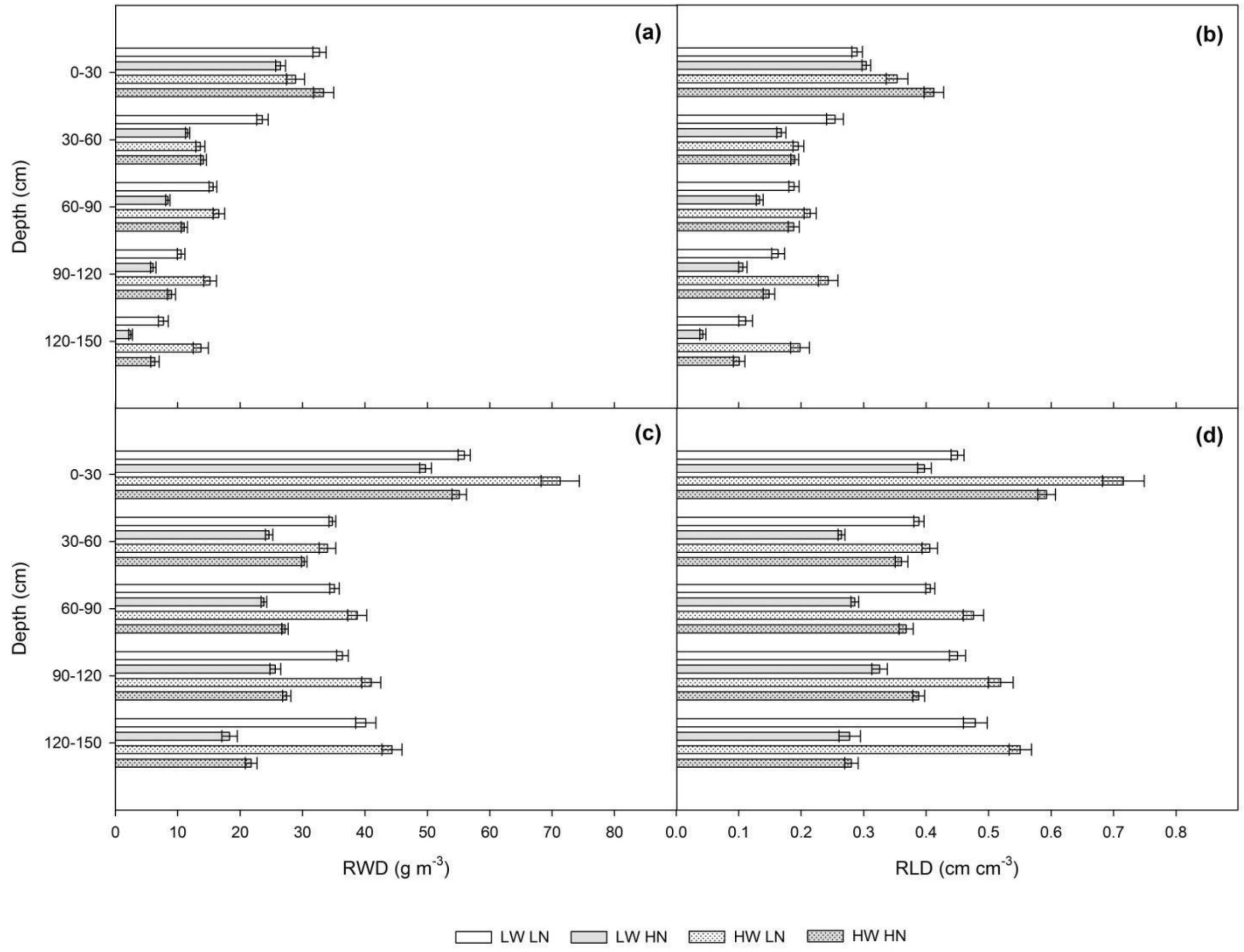
Figure 1. Effect of water and nitrogen regimes on: root weight density (RWD) of modern (a) and old (c) genotypes and root length density (RLD) of modern (b) and old (d) genotypes at the different soil column depths studied.



Data includes: LW LN, low-watered and low nitrogen regime (white bars); LW HN, low-watered and high nitrogen regime (grey bars); HW LN, high-watered and low nitrogen regime (white dotted bars); and HW HN, high-watered and high nitrogen regime (grey dotted bars). Error bars represent the standard error of the mean (SEM).

Figure 2. Monthly average minimum (T_{min}) and maximum (T_{max}) air temperatures (Cº) and RH (%) during the growing period.

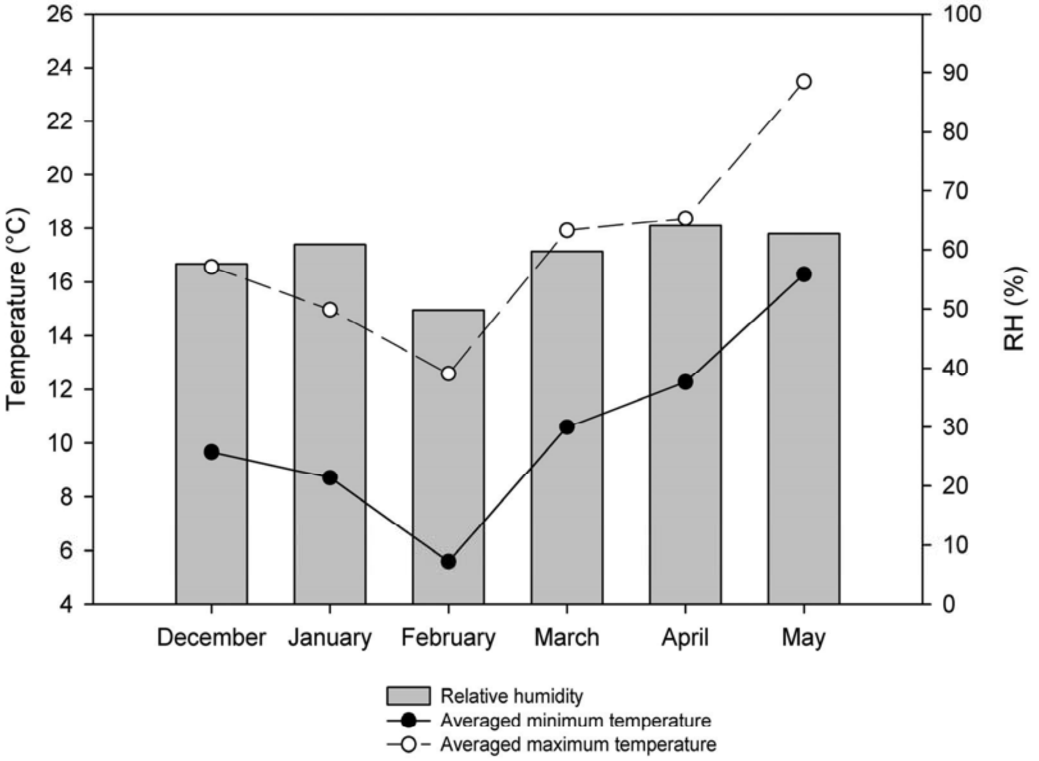


Figure 3. The original scanned root image on the left and the root image analysed with ImageJ on the right.

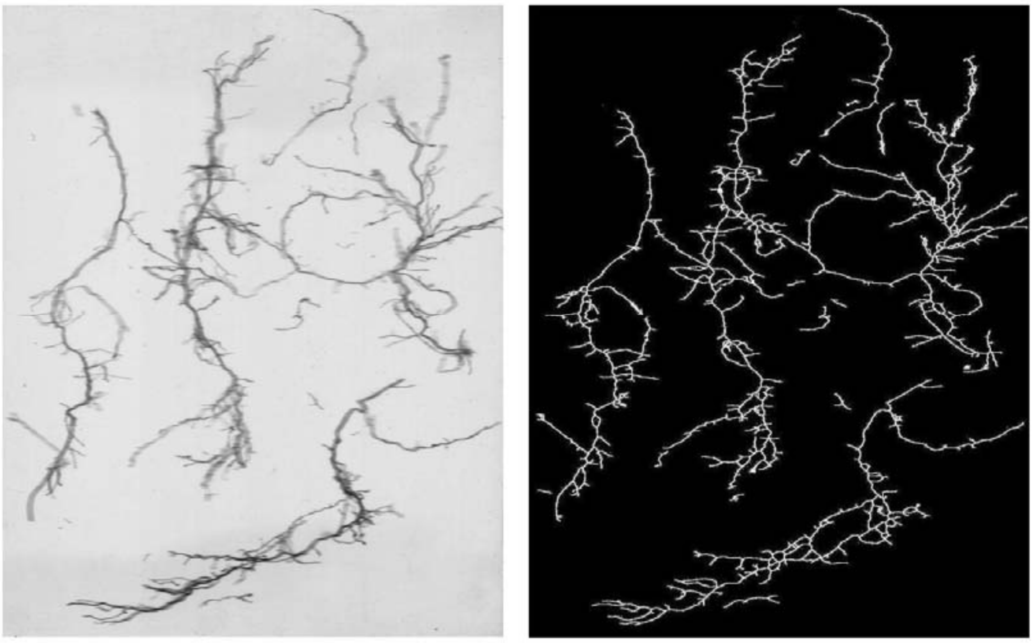


Table 1. Genotypic mean values of the different growth parameters studied under different water and nitrogen supplies.

	WR		NR		G		ANOVA		
	LW	HW	LN	HN	Modern	Old	WR	NR	G
AB	4.33 a	7.81 b	5.11 a	7.06 b	4.70 a	7.49 b	364.97***	115.21***	219.46***
SB	1.49 a	2.22 b	1.51 a	2.21 b	2.12 b	1.57 a	18.36***	11.71***	42.53***
CB	2.14 a	4.10 b	2.76 a	3.49 b	1.78 a	4.50 b	107.90***	19.10***	191.79***
LB	0.70 a	1.49 b	0.84 a	1.37 b	0.80 a	1.41 b	18.95***	8.13***	16.67***
RB	0.13 a	0.15 b	0.17 b	0.11 a	0.09 a	0.20 b	0.02*	0.08***	0.37***
RL	73.39 a	92.25 b	95.57 b	69.63 a	54.56 a	112.05 b	14919.61**	18194.65***	98346.96***
NSP	1.31 a	1.95 b	1.40 a	1.86 b	1.58 a	1.68 a	11.26***	5.59***	2.73^{NS}
PH	89.63 a	100.63 b	97.94 b	92.32 a	68.70 a	121.56 b	3635.50***	949.21**	87205.85***
SLA	196.81 a	234.37 a	210.66 a	220.52 a	239.62 b	191.56 a	9097.10^{NS}	23.74^{NS}	224414.90*
AB RB^{-1}	40.13 a	69.97 b	35.37 a	75.42 b	68.09 b	41.57 a	8659.54**	42491.48***	69261.80***
N_{FL}	3.52 a	3.89 b	3.30 a	4.09 b	3.37 a	4.03 b	1.74*	14.35***	19.00**
SLN	2.80 a	2.98 a	2.45 a	3.33 b	2.25 a	3.53 b	1.07^{NS}	22.87**	65.41*
DH	99.10 a	102.45 b	102.85 b	98.70 a	97.20 a	104.35 b	336.17*	516.68*	10790.18***

Aerial biomass (AB, g plant^{-1}), spike dry biomass (SB, g plant^{-1}), culm dry biomass (CB, g plant^{-1}), leaf blade dry biomass (LB, g plant^{-1}), root dry biomass (RB, g plant^{-1}), root length (RL, m plant^{-1}), number of spikes per plant (NSP), plant height (cm), specific leaf area (SLA, cm^2 g^{-1}), the ratio of aerial dry biomass to root dry biomass (AB RB^{-1}), flag leaf nitrogen content (N_{FL}, %), specific leaf nitrogen (SLN, g m^{-2}) and days to heading (DH). Data shown are the means of the ten genotypes studied over three replicates for each water and nitrogen combination. LW, low-watered; HW, high-watered; LN, low nitrogen; HN, high nitrogen; Modern, modern genotypes; Old, old genotypes; WR, water regime; NR, nitrogen regime; and G, genotype. Means followed by different letters were significantly different ($P \leq 0.05$) by the independent samples t-test ($P \leq 0.05$). The associated sum of squares type III and probabilities (^{NS}, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) are shown.

Table 2. Genotypic mean values of the different studied water status parameters and stable isotopes under different water and nitrogen supplies.

	WR		**NR**		**G**		**ANOVA**		
	LW	HW	LN	HN	Modern	Old	WR	NR	G
A_{sat}	18.56 a	21.30 b	21.52 b	18.25 a	18.69 a	21.04 b	192.68***	297.78***	326.39*
g_s	0.20 a	0.26 b	0.27 b	0.18 a	0.22 a	0.24 a	0.12***	0.24***	0.15^{NS}
E	4.20 a	5.37 b	5.63 b	3.89 a	4.66 a	4.88 a	44.84***	82.69***	37.01^{NS}
C_i/C_a	0.54 a	0.59 b	0.60 b	0.53 a	0.59 b	0.55 a	0.10***	0.15***	0.11*
WUE_{Inst}	4.62 b	4.18 a	4.02 a	4.81 b	4.19 a	4.62 b	8.29***	16.01***	8.16**
WUE_{AB}	3.16 a	2.94 a	2.52 a	3.59 b	2.63 a	3.49 b	0.34^{NS}	32.84***	20.55***
$\delta^{13}C_{FL}$	-28.66 b	-29.71 a	-30.20 a	-28.17 b	-30.23 a	-28.14 b	18.40***	106.21***	115.54***
$\delta^{18}O_{FL}$	30.68b	30.10a	30.37a	30.41a	30.85b	29.93a	6.81**	0.32^{NS}	26.18***
T_{cum}	1.36 a	2.60 b	1.99 a	1.95 a	1.81 a	2.15 b	40.68***	0.09^{NS}	4.23***

Net CO_2 assimilation rate at light saturation (A_{sat}, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), the ratio of intercellular to ambient CO_2 concentration (C_i/C_a), instantaneous water use efficiency (WUE_{Inst}, mmol CO_2 mmol H_2O^{-1}), the time-integrated water use efficiency (WUE_{AB}, g L^{-1}), the stable carbon ($\delta^{13}C$, ‰) and oxygen ($\delta^{18}O$, ‰) isotope compositions of the flag leaf blades (*FL*) and the plant cumulative transpiration (T_{cum}, L $plant^{-1}$). Data shown are the means of the studied ten genotypes over three replicates for each water and nitrogen combination. LW, low-watered; HW, high-watered; LN, low nitrogen; HN, high nitrogen; Modern, modern genotypes; Old, old genotypes; WR, water regime; NR, nitrogen regime; and G, genotype. Means followed by different letters were significantly different ($P \leq 0.05$) by the independent samples t-test ($P \leq 0.05$). The associated sum of squares type III and probabilities (^{NS}, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) are shown.

Table 3. Genotypic mean values of the different root traits studied under different water and nitrogen supplies.

	WR		**NR**		**G**		**ANOVA**		
	LW	HW	LN	HN	Modern	Old	WR	NR	G
RWD.1	41.05 a	46.59 a	46.78 a	40.77 a	30.33 a	57.97 b	1387.54^{NS}	534.17^{NS}	22158.22***
RWD.2	23.61 a	22.67 a	26.35 b	19.88 a	15.69 a	30.99 b	3.71^{NS}	872.14**	6722.45***
RWD.3	20.68 a	23.05 a	26.31 b	17.33 a	12.89 a	31.30 b	228.35^{NS}	1949.60***	9366.05***
RWD.4	19.55 a	22.76 a	25.51 b	16.69 a	10.16 a	32.70 b	406.52^{NS}	1967.84***	14994.36***
RWD.5	18.05 a	21.12 a	26.90 b	12.23 a	7.79 a	31.61 b	870.49^{NS}	5396.90***	17609.78***
RLD.1	0.36 a	0.51 b	0.45 a	0.42 a	0.34 a	0.54 b	0.77***	0.01^{NS}	1.37***
RLD.2	0.27 a	0.28 a	0.31 b	0.24 a	0.20 a	0.36 b	0.03^{NS}	0.09**	0.79***
RLD.3	0.25 a	0.31 b	0.32 b	0.24 a	0.18 a	0.38 b	0.09**	0.14***	1.07***
RLD.4	0.26 a	0.32 b	0.34 b	0.24 a	0.17 a	0.42 b	0.15*	0.31***	2.02***
RLD.5	0.24 a	0.28 b	0.34 b	0.18 a	0.12 a	0.40 b	0.17*	0.74***	2.61***
SRL.1	94.71 a	121.09 b	103.41 a	112.24 a	120.61 b	94.28 a	10355.63***	718.75^{NS}	29183.17***
SRL.2	119.61 a	138.01 b	127.72 a	129.76 a	139.67 b	117.21 a	6397.40*	0.15^{NS}	38150.60**
SRL.3	134.37 a	148.90 a	131.64 a	151.68 b	156.35 b	126.02 a	3314.66^{NS}	5734.64**	24772.29**
SRL.4	149.80 a	162.11 a	147.52 a	164.42 b	178.09 b	132.54 a	981.78^{NS}	2991.97*	68374.21***
SRL.5	161.93 a	169.17 a	147.11 a	185.24 b	193.06 b	138.58 a	3660.81^{NS}	24858.13**	127567.68***

Root weight density (RWD, g m^{-3}), root length density (RLD, cm cm^{-3}) and specific root length (SRL, g m^{-1}). The number following the acronym of the trait refers to the soil column depth where the trait was measured: 1 refers to 0-30 cm; 2 refers to 30-60 cm; 3 refers to 60-90 cm; 4 refers to 90-120 cm; and 5 refers to 120-150 cm. Data shown are the means of the ten genotypes studied over three replicates for each water and nitrogen combination. LW, low-watered; HW, high-watered; LN, low nitrogen; HN, high nitrogen; Modern, modern genotypes; Old, old genotypes; WR, water regime; NR, nitrogen regime; and G, genotype. Means followed by different letters were significantly different ($P \leq 0.05$) by the independent samples t-test ($P \leq 0.05$). The associated sum of squares type III and probabilities (^{NS}, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) are shown.

Table 4. Pearson correlation coefficients of the linear relationships between traits.

	AB	RB	RL	AB RB^{-1}	N$_{FL}$	A_{Sat}	g_s	E	C_i/C_a	WUE_{Inst}	WUE_{AB}	$\delta^{13}C_{FL}$	$\delta^{18}O_{FL}$	T_{cum}
AB		-0.56	-0.26	0.77**	0.36	-0.62	-0.77**	-0.84**	-0.81**	0.87**	0.92**	0.86**	0.19	-0.09
		-0.78**	-0.70*	0.92**	0.71*	-0.83**	-0.76*	-0.80**	-0.73*	0.74*	0.92**	0.95**	0.05	0.10
RB	-0.02		0.88**	-0.92**	0.01	0.59	0.68*	0.67*	0.66*	-0.47	-0.83**	-0.83**	0.03	0.84**
	-0.06		0.94**	-0.93**	-0.52	0.53	0.46	0.48	0.46	-0.42	-0.90**	-0.78**	-0.48	0.40
RL	0.01	0.98**		-0.71*	-0.06	0.32	0.34	0.33	0.36	-0.17	-0.55	-0.58	0.19	0.85**
	-0.16	0.94**		-0.85**	-0.58	0.56	0.48	0.51	0.53	-0.47	-0.83**	-0.71*	-0.33	0.43
AB RB^{-1}	0.44	-0.70*	-0.68*		0.13	-0.56	-0.74*	-0.75*	-0.84**	0.72*	0.94**	0.96**	0.22	-0.61
	0.61	-0.80**	-0.80**		0.61	-0.67*	-0.62	-0.64*	-0.59	0.56	0.96**	0.89**	0.29	-0.22
N$_{FL}$	0.42	0.00	-0.03	0.04		0.34	0.14	0.04	-0.38	0.54	0.19	0.15	-0.25	0.36
	0.45	-0.40	-0.32	0.48		-0.78**	-0.85**	-0.86**	-0.91**	0.91**	0.67*	0.81**	-0.17	0.06
A_{Sat}	-0.85**	0.34	0.29	-0.56	-0.39		0.93**	0.91**	0.45	-0.32	-0.71*	-0.57	-0.18	0.45
	-0.63	0.03	0.11	-0.31	-0.21		0.94**	0.96**	0.87**	-0.83**	-0.80**	-0.87**	0.16	-0.03
g_s	-0.79**	0.52	0.46	-0.59	-0.53	0.92**		0.99**	0.73*	-0.59	-0.85**	-0.74*	-0.29	0.43
	-0.73*	0.22	0.30	-0.51	-0.35	0.93**		0.99**	0.91**	-0.89**	-0.75*	-0.82**	0.10	0.01
E	-0.77**	0.50	0.46	-0.53	-0.58	0.91**	0.99**		0.76*	-0.67*	-0.88**	-0.78**	-0.27	0.37
	-0.75*	0.22	0.30	-0.52	-0.36	0.92**	0.99**		0.93**	-0.91**	-0.77**	-0.85**	0.12	-0.03
C_i/C_a	-0.79**	0.40	0.35	-0.45	-0.58	0.82**	0.95**	0.96**		-0.92**	-0.83**	-0.82**	-0.36	0.24
	-0.80**	0.32	0.39	-0.63	-0.47	0.85**	0.94**	0.94**		-0.98**	-0.72*	-0.84**	0.12	0.00
WUE_{Inst}	0.63*	-0.40	-0.38	0.29	0.65*	-0.67*	-0.85**	-0.90**	-0.95**		0.79**	0.80**	0.30	-0.01
	0.76*	-0.44	-0.47	0.69*	0.47	-0.73*	-0.89**	-0.91**	-0.96**		0.66*	0.80**	-0.17	0.16
WUE_{AB}	0.96**	-0.25	-0.22	0.64*	0.35	-0.87**	-0.84**	-0.82**	-0.82**	0.66*		0.96**	0.15	-0.46
	0.98**	-0.26	-0.35	0.75*	0.46	-0.67*	-0.79**	-0.81**	-0.85**	0.84**		0.95**	0.30	-0.30
$\delta^{13}C_{FL}$	0.78**	-0.20	-0.16	0.34	0.55	-0.69*	-0.80**	-0.80**	-0.87**	0.78**	0.80**		0.25	-0.49
	0.98**	-0.12	-0.23	0.65*	0.44	-0.52	-0.63	-0.66*	-0.70*	0.70*	0.96**		0.10	-0.07
$\delta^{18}O_{FL}$	-0.01	0.28	0.32	0.03	-0.49	0.32	0.35	0.42	0.27	-0.32	-0.04	-0.36		0.15
	0.24	0.08	-0.07	0.08	-0.57	-0.34	-0.32	-0.34	-0.20	0.27	0.29	0.29		-0.67*
T_{cum}	0.20	0.90**	0.92**	-0.63	0.06	0.07	0.28	0.29	0.22	-0.28	-0.06	-0.09	0.24	
	0.29	0.74*	0.73*	-0.39	-0.01	0.07	0.14	0.14	0.02	-0.10	0.08	0.23	-0.12	

The correlations were calculated within each water regime using the genotype means of both high and low nitrogen regimes (*n*=10). For each correlation matrix, the upper and lower triangle represents the low and high water regimes, respectively. For each correlation value, the upper and the lower value refers to modern and old genotypes, respectively. Probabilities (*, $P \leq 0.05$; **, $P \leq 0.01$) are shown. Traits included were: Aerial biomass (AB, g plant^{-1}), root dry biomass (RB, g plant^{-1}), root length (RL, m plant^{-1}), the ratio of aerial dry biomass to root dry biomass (AB RB^{-1}), flag leaf nitrogen content (N$_{FL}$, %), Net CO_2 assimilation rate ($A_{sat.}$ µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol CO_2 m^{-2} s^{-1}), transpiration rate (*E,* mmol H_2O m^{-2} s^{-1}), the ratio of intercellular to ambient CO_2 concentration (C_i/C_a), instantaneous water use efficiency ($WUE_{Inst.}$ mmol CO_2 mmol H_2O^{-1}), the time-integrated water use efficiency (WUE_{AB}, g L^{-1}), the stable carbon ($\delta^{13}C$, ‰) and oxygen ($\delta^{18}O$, ‰) isotope compositions of the flag leaf blades (*FL*) and the plant cumulative transpiration (T_{cum}, L plant^{-1}).

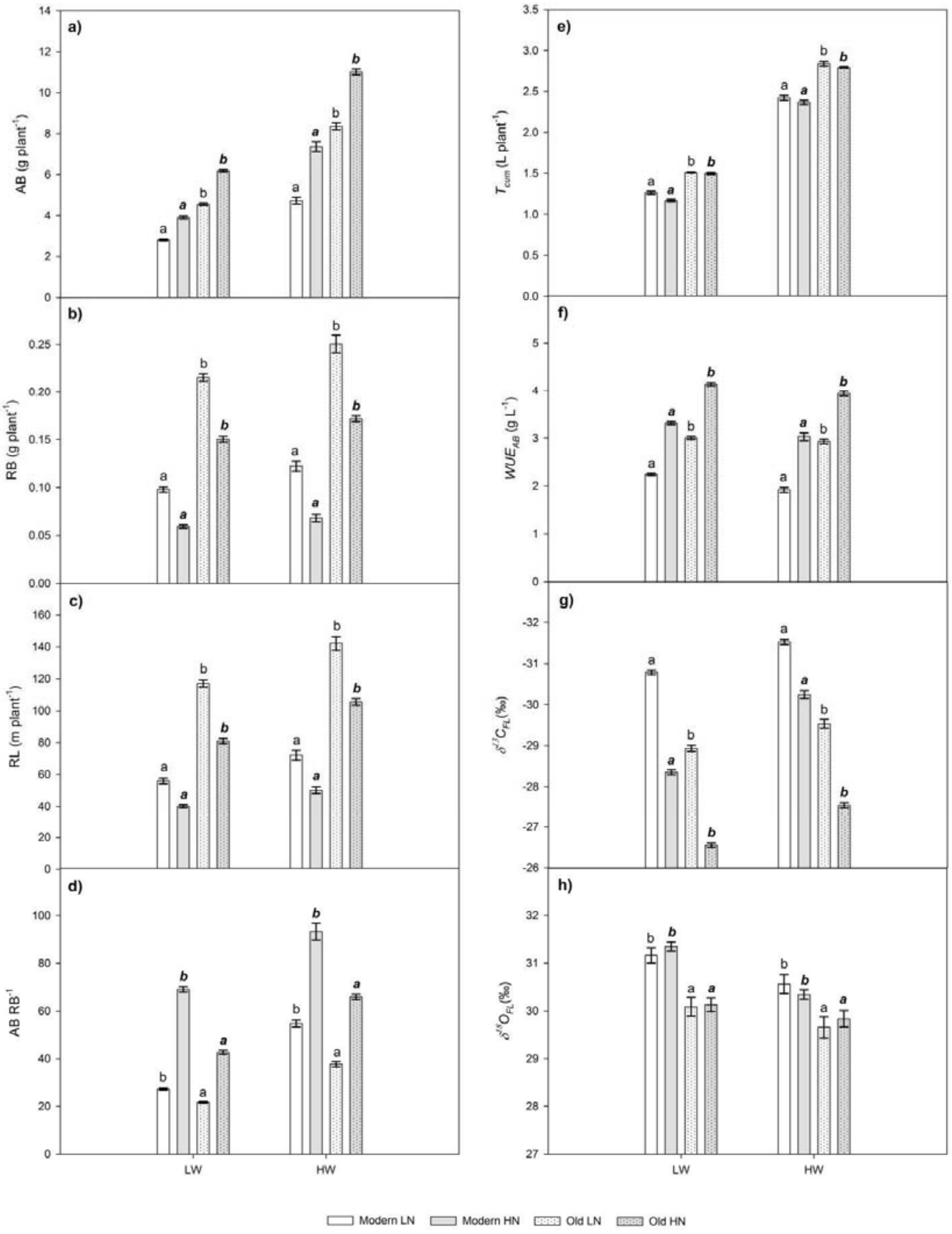
Table 5. Pearson correlation coefficients of root traits with AB, WUE_{AB}, stable isotopes and T_{cum} across the five different root layers studied.

		RWD.1	RWD.2	RWD.3	RWD.4	RWD.5	RLD.1	RLD.2	RLD.3	RLD.4	RLD.5	SRL.1	SRL.2	SRL.3	SRL.4	SRL.5
LW	AB	-0.26	-0.63*	-0.49	-0.26	-0.62	0.38	-0.2	-0.19	-0.17	-0.59	0.53	0.55	0.62	0.66*	0.75*
		-0.67*	-0.86**	-0.83**	-0.56	-0.62	-0.38	-0.63	-0.81**	-0.44	-0.42	-0.1	0.25	0.28	0.36	0.72*
	WUE_{AB}	-0.53	-0.73*	-0.75*	-0.53	-0.77*	0.19	-0.28	-0.47	-0.45	-0.75*	0.65*	0.62	0.76*	0.69*	0.72*
		-0.52	-0.83**	-0.85**	-0.77**	-0.83**	-0.13	-0.64*	-0.84**	-0.70*	-0.67*	0.12	0.22	0.41	0.34	0.86**
	$\delta^{13}C$	-0.59	-0.79**	-0.79**	-0.49	-0.76*	0.15	-0.36	-0.54	-0.43	-0.79*	0.72*	0.65*	0.73*	0.70*	0.6
		-0.51	-0.86**	-0.78**	-0.55	-0.70*	-0.11	-0.64*	-0.86**	-0.54	-0.51	0.15	0.25	0.18	0.16	0.83**
	$\delta^{18}O$	-0.18	0.03	0.08	0.35	0.05	0.15	-0.1	0.31	0.44	0.02	0.62	0.09	0.5	0.47	-0.4
		-0.02	-0.1	-0.47	-0.67*	-0.53	0.43	-0.09	-0.33	-0.48	-0.52	0.52	0.04	0.62	0.46	0.31
	T_{cum}	0.69*	0.42	0.82**	0.80**	0.76*	0.31	0.24	0.81**	0.83**	0.80*	-0.38	-0.31	-0.55	-0.2	-0.39
		-0.28	0.03	0.19	0.63	0.62	-0.53	0.13	0.15	0.70*	0.71*	-0.47	0.09	-0.44	-0.08	-0.38
HW	AB	0.48	0.45	-0.21	-0.27	-0.24	0.51	0.42	-0.04	-0.32	-0.22	-0.16	-0.47	0.22	0.07	-0.01
		0.24	0.27	-0.01	-0.09	-0.26	-0.01	0.02	-0.06	-0.03	-0.35	-0.39	-0.46	-0.15	0.22	0.15
	WUE_{AB}	0.27	0.27	-0.39	-0.48	-0.43	0.32	0.26	-0.27	-0.52	-0.42	0.14	-0.08	0.32	0.47	0.34
		0.08	0.13	-0.19	-0.29	-0.41	-0.21	-0.1	-0.19	-0.22	-0.49	-0.05	-0.13	0.42	-0.16	0.49
	$\delta^{13}C$	0.42	0.18	-0.38	-0.4	-0.38	0.47	0.12	-0.14	-0.46	-0.38	0.09	-0.39	0.31	0.03	0.06
		0.17	0.23	-0.09	-0.19	-0.35	-0.06	0	-0.07	-0.14	-0.47	-0.33	-0.41	0.02	0.23	0.12
	$\delta^{18}O$	-0.01	0.23	0.2	0.25	0.15	0	0.55	0.1	0.35	0.21	-0.14	0.38	-0.08	-0.12	0.32
		0.33	0.38	0.15	0.04	0.08	-0.04	0.15	0.32	-0.26	-0.08	-0.38	-0.28	0.22	-0.57	-0.34
	T_{cum}	0.76*	0.75*	0.75*	0.80**	0.78**	0.68*	0.76*	0.85**	0.79**	0.80**	-0.46	-0.15	-0.33	-0.37	-0.40
		0.68*	0.58	0.69*	0.75*	0.59	0.83**	0.46	0.43	0.81**	0.58	-0.42	-0.25	-0.51	-0.09	-0.31

The correlations were calculated within each water regime using the genotypes means of both high and low nitrogen regimes (n=10). For each correlation value, the upper and the lower values refer to the modern and old genotypes, respectively. LW, low-watered; and HW, high-watered. Probabilities (*, $P \leq 0.05$; **, $P \leq 0.01$) are shown. Traits included were: root weight density (RWD, g m^{-3}), root length density (RLD, cm cm^{-3}), specific root length (SRL,, g m^{-1}), aerial biomass (AB, g plant^{-1}), the time-integrated water use efficiency (WUE_{AB}, g L^{-1}), the stable carbon ($\delta^{13}C$, ‰) and oxygen ($\delta^{18}O$, ‰) isotope compositions of the flag leaf blades (*FL*) and the plant cumulative transpiration (T_{cum}, L plant^{-1}). The number following the acronym of the trait refers to the soil column depth where the trait was measured: 1 refers to 0-30 cm; 2 refers to 30-60 cm; 3 refers to 60-90 cm; 4 refers to 90-120 cm; and 5 refers to 120-150 cm.

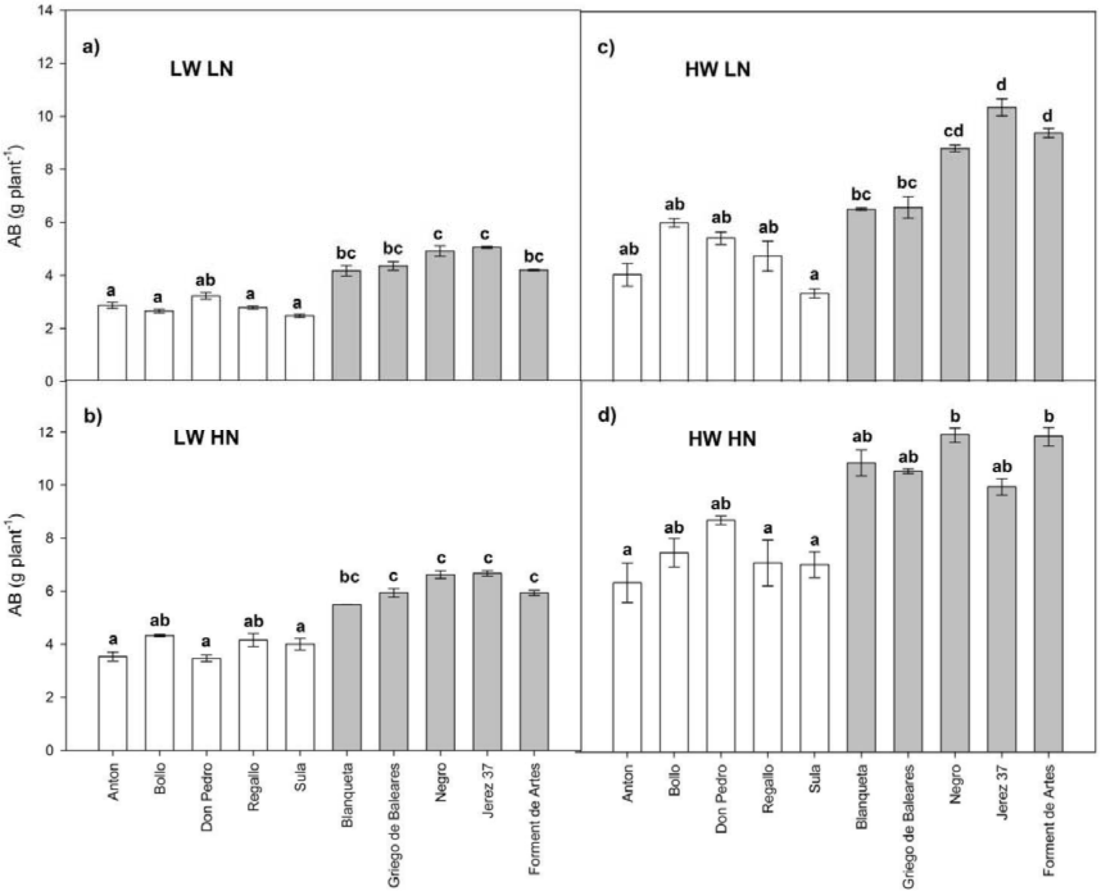
Supporting information

Figure S1. Aerial biomass (AB, a), root dry biomass (RB, b), root length (RL, c), the ratio of aerial biomass to root dry biomass (AB RB^{-1}, d), cumulative plant transpiration (T_{cum}, e), time-integrated water use efficiency (WUE_{AB}, f) and stable carbon ($\delta^{13}C_{FL}$, g) and oxygen ($\delta^{18}O_{FL}$, h) isotope compositions of the flag leaf blade means for the studied genotypes.



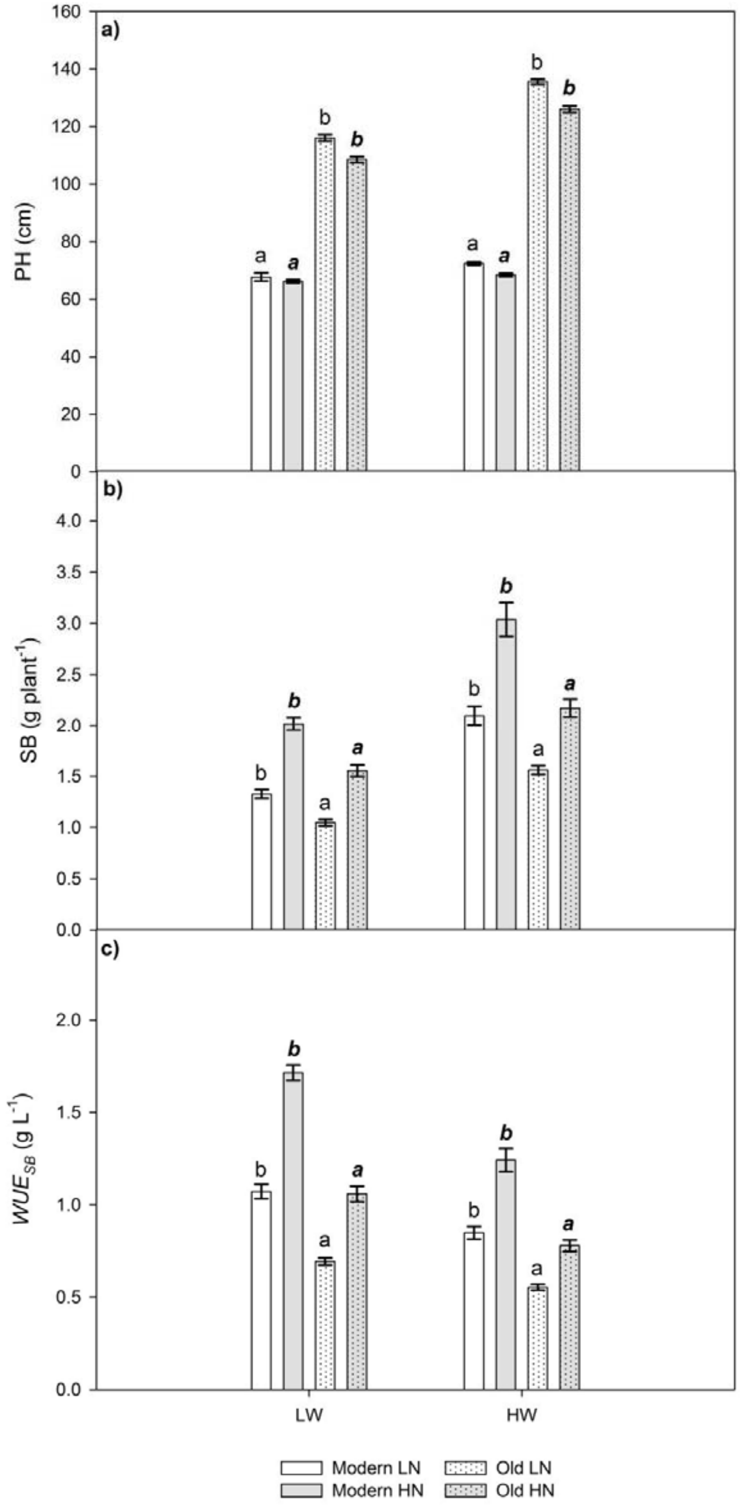
Data include: low-watered regime (LW); high-watered regime (HW); modern genotypes under low nitrogen (Modern LN, white bars); modern genotypes under high nitrogen (Modern HN, grey bars); old genotypes under low nitrogen (Old LN, white dotted bars); and old genotypes under high nitrogen (Old HN, grey dotted bars). Error bars represent the standard error of the mean (SEM). Means followed by different letters were significantly different (*$P \leq 0.05$) by an independent samples t-test. Non-italic and italic letters are for the LN and HN genotypic mean comparisons, respectively.

Figure S2. Aerial biomass (AB) means for the studied genotypes.



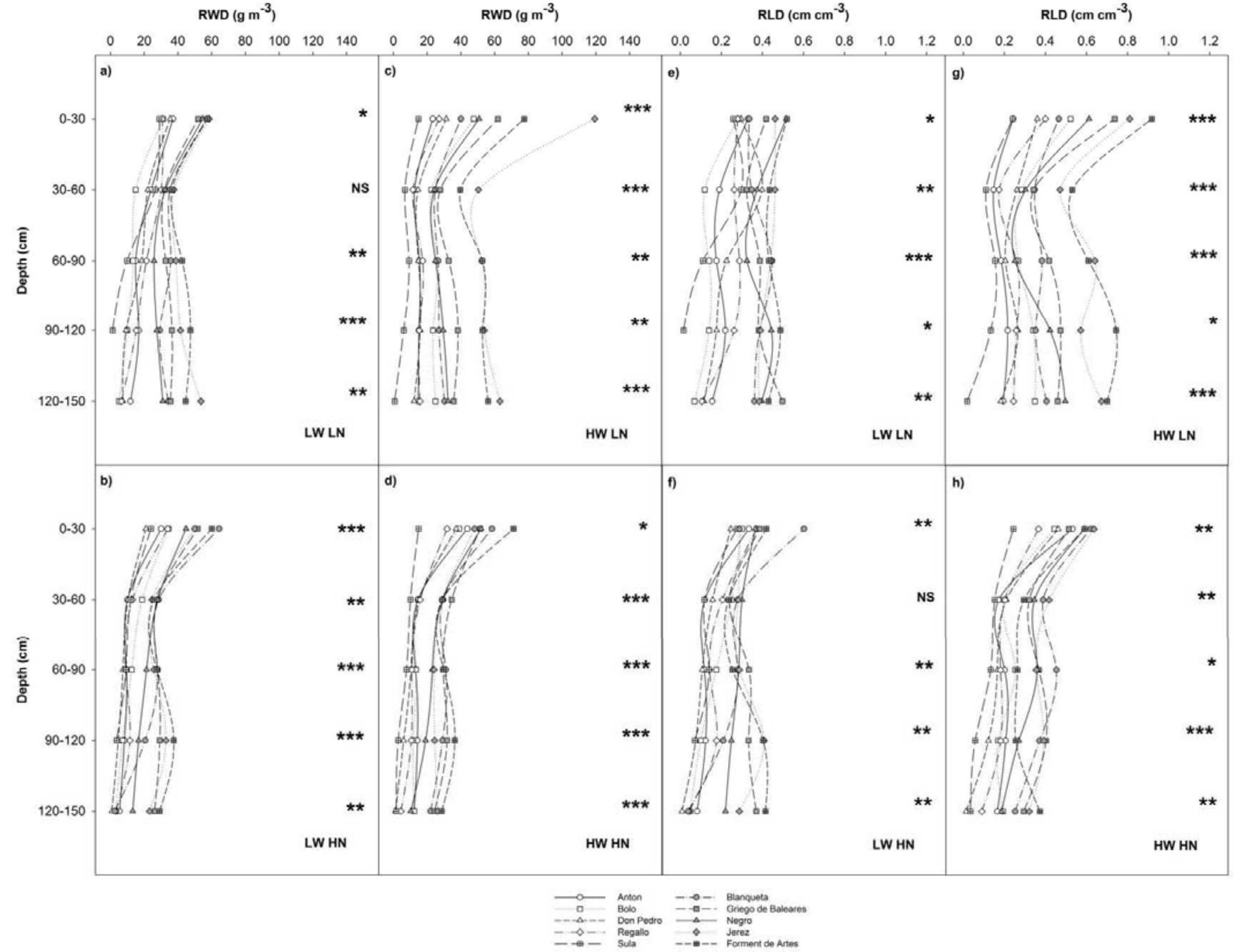
The genotypes were: Anton, Bolo, Don Pedro, Regallo, Sula, Blanqueta, Griego de Baleares, Negro, Jerez 37 and Forment de Artes**.** White bars refer to the modern genotypes and grey bars refer to the old genotypes. Data include: LW LN, low-watered and low nitrogen treatment; LW HN, low-watered and high nitrogen treatment; HW LN, high-watered and low nitrogen treatment; and HW HN, high-watered and high nitrogen treatment. Error bars represent the standard error of the mean (SEM). Means followed by different letters were significantly different (*$P \leq 0.05$) by Duncan's multiple range test.

Figure S3. Plant height (PH, a), spike dry biomass (SB, b) and water use efficiency of spike (*WUE$_{SB}$*).



Data include: low-watered regime (LW); high-watered regime (HW); modern genotypes under low nitrogen (Modern LN, white bars); modern genotypes under high nitrogen (Modern HN, grey bars); old genotypes under low nitrogen (Old LN, white dotted bars); and old genotypes under high nitrogen (Old HN, grey dotted bars). Error bars represent the standard error of the mean (SEM). Means followed by different letters were significantly different (*$P \leq 0.05$) by the independent samples t-test. Non-italic and italic letters are for the LN and HN genotypic means comparisons, respectively.

Figure S4. Harvested root weight density (RWD, a-d) and root length density (RLD, e-h) at different substrate column depths.

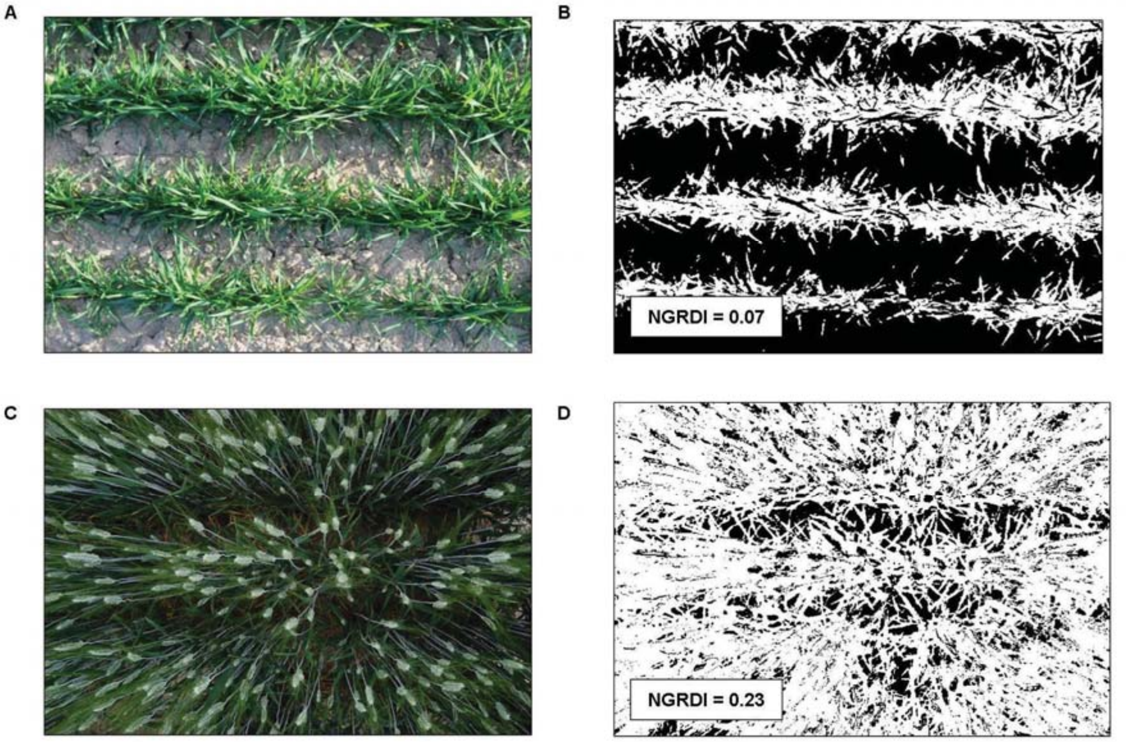


Data include the genotypes: Anton (white circles), Bolo (white squares), Regallo (white diamonds), Sula (white crossed squares), Blanqueta (grey circles), Griego de Baleares (grey squares), Negro (grey triangles), Jerez 37 (grey diamonds) and Foment de Artes (grey crossed squares). LW LN, low-watered low nitrogen; LW HN, low-watered high nitrogen; HW LN, high-watered low nitrogen; and HW HN, high-watered high nitrogen regime. NS, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$ results from ANOVA test for the different genotypes at the different soil column depths studied are shown.

Chapter 4

The combined use of vegetation indices and stable isotopes to predict durum wheat grain yield under contrasting water conditions

Elazab A, Bort J, Zhou B, Serret MD, Nieto-Taladriz MT, Araus JL (2015) The combined use of vegetation indices and stable isotopes to predict durum wheat grain yield under contrasting water conditions. *Agricultural Water Management* **158**, 196-208.



Digital image analysis of durum wheat genotypes which were grown in the Experimental Field Facilities of INIA, Madrid, Spain. 2011/2012 cycle

Resumen

Los estudios de mejora del rendimiento del trigo duro en condiciones de estrés abiótico se encuentran, a menudo, limitados por la falta de métodos de seguimiento adecuados que apoyen el manejo y la gestión de los cultivos y por la falta de herramientas de fenotipeado eficientes para la mejora de los mismos. Los objetivos de este estudio fueron: (1) comparar el rendimiento de las plantas de trigo duro bajo diferentes regímenes de riego evaluando el crecimiento y el estado hídrico de las plantas; y (2) comprender cómo dichas características pueden predecir el rendimiento de grano (GY, por sus siglas en inglés). En este sentido, se cultivaron cinco genotipos modernos de trigo duro en régimen de secano (RF, por sus siglas en inglés) y de riego suplementario (SI, por sus siglas en inglés). Se estudiaron dos tipos de características fisiológicas: (1) índices de vegetación: *Índice Normalizado de Diferencia de Vegetación (NDVI*, por sus siglas en inglés) y el Índice Normalizado de Diferencia de color Rojo-Verde (NGRDI, por sus siglas en inglés); y (2) la composición en isótopos estables de carbono y de oxígeno ($\delta^{13}C$ y $\delta^{18}O$, respectivamente) de diferentes órganos de la planta. El NGRDI en la fase de antesis y la $\delta^{13}C$ en los granos maduros fueron los caracteres mejor correlacionados (positiva y negativamente, respectivamente) con el GY. Ambos caracteres en combinación explicaron como mínimo, el 50 % de la variabilidad en el GY dentro de cada régimen hídrico. Los modelos de “sendero” producidos para las condiciones de RF y SI destacaron el papel importante del NGRDI y de la $\delta^{13}C$ en la predicción del GY. Además, el estudio mostró la importancia de la utilización de los índices de vegetación derivados de imágenes digitales rojo-verde-azul (RGB) como una técnica económica para evaluar la biomasa aérea y el GY bajo diferentes condiciones de disponibilidad hídrica.

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The combined use of vegetation indices and stable isotopes to predict durum wheat grain yield under contrasting water conditions

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ABSTRACT

Improving durum wheat performance to abiotic stresses is often limited by a lack of proper monitoring methods in support of crop management and efficient phenotyping tools for breeding. The objectives of this study were: (1) comparing the performance under contrasting water treatments of different physiological traits, which evaluate plant growth and water status; and (2) understanding how these traits can predict grain yield (GY) performance under contrasting water conditions. Thus, five modern durum wheat genotypes were subjected to rainfed (RF) and supplemental irrigation (SI) treatments. Two categories of physiological traits were tested; (1) the vegetation indices: the Normalized Difference Vegetation Index (NDVI) and the Normalized Green Red Difference Index (NGRDI); and (2) the stable carbon and oxygen isotope compositions ($\delta^{13}C$ and $\delta^{18}O$) of different plant parts. The NGRDI at anthesis and the $\delta^{13}C$ of mature grains were the traits best correlated (positively and negatively, respectively) with GY. Both traits in combination explained at least 50% of variability in GY within each water treatment. The produced path models for RF and SI conditions highlighted the particular role of NGRDI and $\delta^{13}C$ in predicting GY. In addition, the study showed the potential of using vegetation indices derived from digital Red-Green-Blue (RGB) images as a low-cost technique for assessing aerial biomass (AB) and GY under different water availabilities.

1. Introduction

Durum wheat is one of the most important crops in Mediterranean environments (FAO, 2012), with water stress being the main constraint limiting productivity (Oweis et al., 2000). This limitation is likely to increase in the future because climatic change is expected to decrease precipitation and increase evapotranspiration in the Mediterranean region (Lobell et al., 2008). Crop management and breeding may improve the performance of durum wheat under stress conditions. However, the lack of efficient tools to monitor the performance of agronomical practices or to undertake appropriate phenotyping in breeding programmes limits the efficiency of both avenues.

Information obtained from physiological assays, such as gas exchange and pressure–volume measurements, often provide accurate data on the immediate plant water status, transpiration or photosynthesis (Ferrio et al., 2003). However, it is difficult to upscale the information at the canopy level over a large temporal and/or spatial scale represented by the entire crop cycle (Ferrio et al., 2003; Lambers et al., 1998). Actually, the most successful traits for evaluation integrate crop performance in time (throughout the crop cycle) and space (at the canopy level) in terms of the ability to capture resources (e.g. radiation, water, and nutrients) and how efficiently these resources are used (Araus et al., 2002, 2008). Among the categories of integrative traits it is worth mentioning the analysis of stable isotopes in plant samples, together with the use of proximal (remote) sensing techniques at the field level (Aparicio et al., 2000, 2002, 2004; Araus et al., 2009, 2013; Casadesús et al., 2007; Casadesús and Villegas, 2014; French et al., 2015; Gumma et al., 2015; Hunt et al., 2013; Rahimi et al., 2014; Rorie et al., 2011; Sakamoto et al., 2012a,b; Torbick and Salas, 2015; Usman et al., 2014). A combination of these approaches has been proposed, for example, in breeding (Araus and Cairns, 2014; White et al., 2012), but to our knowledge reports of such studies are scarce. Whereas stable carbon and oxygen isotopes deal with the availability and use of water by the crop, or in other words deal with the water status (Araus et al., 2013), remote sensing-derived indices inform on the radiation uptake and the photosynthetic

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(assimilatory) capacity of the plants (associated with aerial plant growth and biomass) (Araus and Cairns, 2014; Gamon et al., 1995). Therefore, these traits may help to elucidate how crop responses to growing conditions are defined in terms of final yield.

In C_3 plants the carbon isotope composition ($\delta^{13}C$; frequently expressed as a discrimination against surrounding air, $\Delta^{13}C$) measured in plant tissues was determined as negatively correlated with the ratio of intercellular CO_2 leaf concentration to ambient CO_2 (C_i/C_a) and positively correlated with the ratio of net assimilation to water transpired (A/E). Therefore, $\delta^{13}C$ is positively related to water use efficiency (*WUE*) (Araus et al., 2013; Farquhar and Richards, 1984; Monneveux et al., 2006). Genetic variability for $\delta^{13}C$ has been reported in wheat (e.g. Araus et al., 2003a,b, 2013; Condon et al., 1987, 1993, 2002, 2004; Rebetzke et al., 2002). Further, $\delta^{13}C$ is a highly heritable trait that is relatively easy to manipulate in breeding populations (Condon and Richards, 1992; Rebetzke et al., 2002). Correlations between $\delta^{13}C$ and grain yield (GY) are normally high, and either negative or positive according to the plant tissue sampled and environmental conditions tested (Araus et al., 2003a,b; Condon and Richards, 1992; Voltas et al., 1999). High $\delta^{13}C$ has been used as a selection trait for high *WUE* at the seedling stage in commercial wheat varieties for the summer dominant rainfall environments of Australia where crop yield relies on the water accumulated before planting (Condon et al., 2004; Rebetzke et al., 2002; Richards et al., 2011). However, in the Mediterranean basin environments characterized by precipitation after planting and moderate to medium drought during the reproductive part of the crop, the efficient use of water (*EUW*) by the crop rather than the *WUE* is the factor affecting productivity (Blum, 2009). Since genotypes exhibiting a higher crop water status, and thus a higher *EUW*, are those with a lower *WUE* (therefore lower $\delta^{13}C$), these genotypes with a lower $\delta^{13}C$ of mature grains (or of any other plant part developed at the end of the crop) may reflect better growing conditions and therefore exhibit higher GY (Araus et al., 2013). In fact, negative phenotypic and genotypic correlations between $\delta^{13}C$ and both GY and aerial biomass (AB) have been reported (Araus et al., 1998, 2003a, 2013; Merah et al., 2001; Monneveux et al., 2006), meaning that genotypes that are able to maintain higher water use (even if it is at the expense of a lower *WUE*) are the most productive (Araus et al., 2008, 2013). In the case of Australia, the high $\delta^{13}C$ genotypes, which were selected for the summer dominant rainfall environments of Australia, have almost no increased GY in either the winter dominant rainfall environments (Mediterranean environments) or in the environments with highly variable rainfall of Australia (Condon et al., 2004). Overall, high $\delta^{13}C$ (and therefore *WUE*) is a 'conservative' trait in terms of water use and crop growth rate, and thus in the absence of soil water deficit, high $\delta^{13}C$ genotypes tend to grow slower than low $\delta^{13}C$ genotypes, resulting in lower AB and GY (Condon and Richards, 1992; Condon et al., 2004). However, the contradictory results of the relationship between $\delta^{13}C$ and GY may also be due to the fact that frequently these studies have used sets of genotypes that have variations not only in $\delta^{13}C$, but in heading and/or anthesis dates as well. Phenology could strongly influence GY and $\delta^{13}C$, being responsible for the negative relationships between $\delta^{13}C$ and GY (Araus et al., 2002, 2003a; Condon et al., 2004). Therefore, when studying the relationship between $\delta^{13}C$ and GY it is necessary to test genotypes with a similar phenology (Araus et al., 1998, 2003a; Condon et al., 2004; Rebetzke et al., 2002; Richards et al., 2011).

The oxygen isotope composition ($\delta^{18}O$) of plant tissues is known to reflect the evaporative conditions throughout the crop cycle (Barbour et al., 2000) and thus it has been proposed as a proxy method for measuring transpiration as well as an indicator of genotypic differences in stomatal conductance (g_s) (Araus et al., 2013; Barbour et al., 2000; Cabrera-Bosquet et al., 2011; Elazab et al., 2012; Ferrio et al., 2007). Leaf $\delta^{18}O$ has been negatively correlated with the transpiration rate (T) (Barbour et al., 2000; Barbour and Farquhar, 2000; Cabrera-Bosquet et al., 2009a,b; Ferrio et al., 2007) and under well-watered conditions in wheat the $\delta^{18}O$ of flag leaves correlates negatively with GY and g_s (Barbour et al., 2000). Cabrera-Bosquet et al. (2009b) found negative correlations in maize between the $\delta^{18}O$ of grains and GY under well-watered and moderate water stress conditions, whereas under severe water stressed conditions the correlations were positive. However, the correlation of $\delta^{18}O$ analyzed in mature grains of wheat with GY is frequently weak or absent (Araus et al., 2013; Ferrio et al., 2007). Nevertheless, the combined measurements of $\delta^{13}C$ and $\delta^{18}O$ in the same plant tissue may help in separating the independent effects of photosynthetic capacity (A) and g_s on $\delta^{13}C$ because $\delta^{18}O$ is not affected by photosynthesis (Araus et al., 2013; Barbour and Farquhar, 2000; Elazab et al., 2012).

The assessment of AB is important for monitoring crop growth because it could reflect the effect of stresses on crop growth and senescence (Araus et al., 2008; Royo and Villegas, 2011). Larger green AB represents higher potential canopy photosynthesis and thus more yield. Therefore, the effect of water stress in limiting plant growth has a subsequent impact on reducing the photosynthetic potential at the crop level and thus GY. In that sense a number of studies have revealed that spectral reflectance or/and digital imaging by ground-based remote sensing has the potential to provide precise, non-destructive instantaneous quantitative estimates of AB and GY (Aparicio et al., 2004; Raun et al., 2001). The Normalized Difference Vegetation Index (NDVI) is among the most usual of spectral reflectance indices, and it is related to the photosynthetically active AB (Ferrio et al., 2005). The NDVI has been used as an indicator of AB and GY in durum wheat (Aparicio et al., 2000, 2002, 2004; Casadesús et al., 2007; Ferrio et al., 2005) and bread wheat (Gutiérrez-Rodríguez et al., 2004; Lobos et al., 2014). The introduction of low-cost portable and easy to handle active spectroradiometers (such as GreenSeeker and Crop Circle) has become a very useful alternative as they can measure the NDVI directly. Moreover, these spectroradiometers are active in the sense that they have their own light source, and thus can be used under diverse atmospheric conditions (Araus et al., 2009; Marti et al., 2007). As a limitation, NDVI values saturate at high green biomass densities (i.e. high leaf area index), which means that this technique may have a low precision at key moments of the crop cycle (e.g. anthesis), especially at high plant densities under favourable agronomic conditions.

In recent years the use of digital Red-Green-Blue (RGB) images has been proposed as an alternative to develop vegetation indices that may replace spectroradiometrical based NDVI (Casadesús et al., 2007; Casadesús and Villegas, 2014; Hunt et al., 2013; Sakamoto et al., 2012a). The price, size, and the easy use of conventional digital cameras make them viable alternatives to assess AB and GY in cereals (Casadesús et al., 2007; Casadesús and Villegas, 2014; Mullan and Reynolds, 2010). A number of studies have used digital RGB imaging to measure different colour parameters such as: greenness; intensity of green, red and blue; and derived normalized indices from the green, red and blue bands (Casadesús et al., 2007; Gitelson et al., 2002; Kipp et al., 2014; Mullan and Reynolds, 2010). Such information has allowed estimation of a wide range of crop traits in durum and bread wheat such as early vigour, leaf area index, leaf senescence, AB and GY (Casadesús et al., 2007; Casadesús and Villegas, 2014; Gitelson et al., 2002; Mullan and Reynolds, 2010). Moreover, a large number of digital RGB images (i.e. photos) can be obtained with a minimum effort (Casadesús and Villegas, 2014) and processed with suitable (either open-access or commercial photo editing) software to extract parameters related to green AB (Casadesús et al., 2007). Among the different indices derived from RGB images, the Normalized Green Red Differences Index (NGRDI), which uses the Green and Red regions of the spectrum, is considered one of the most promising indices. Gitelson et al. (2002) reported that NGRDI was more efficient than NDVI at estimating AB in wheat. Hunt et al. (2005) used NGRDI to estimate the nutrient status and AB in maize, soybean and alfalfa, whereas Motohka et al. (2010) found the NGRDI to be superior to the NDVI in explaining the seasonal growth patterns of canopies of different species including deciduous and coniferous trees, and grassland and rice fields. However, to the best of our knowledge no studies reporting correlations between NGRDI and GY have been published until now.

Besides the choice of the most appropriate trait (or combination of traits) for predicting GY performance under different water regimes, it is also crucial to determine the key time(s) for their evaluation. Measuring these traits at more or less frequent intervals during the crop cycle together with measurements in the harvestable components would be unfeasible (or at best impractical) (Araus and Cairns, 2014). Moreover, the relative importance of each trait in explaining plant performance and the appropriate time to measure them may change depending on the severity, time and duration of the stress and the phenological stage when it occurs. Beyond that, a mechanistic explanation of how the different physiological traits can predict GY performance is not always clear, but the existence of a statistical relationship between the trait measured and the GY is used as proof of concept for the adequacy of the trait. This study tests an approach based on correlations and stepwise and path analyses to dissect in a mechanistic manner how physiological traits can predict GY performance under different water conditions.

The first objective of this study was to compare the performance under contrasting water conditions of different physiological traits, which evaluate plant growth and water status, in terms of their relationships with GY and AB at harvest. Traits tested were the spectral reflectance index, NDVI, the digital imaging-derived index, NGRDI, and δ^{13}C and δ^{18}O. Measurements were performed at different phenological stages (for vegetation indices) and in different plant parts (for the isotopic compositions). The second objective was to dissect how these physiological traits can predict GY performance through the different agronomical yield components.

2. Material and methods

2.1. Plant materials and experimental conditions

Five durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.) genotypes (Anton, Bolo, Don Pedro, Regallo and Sula) were tested. The genotypes represented modern Spanish commercial varieties released after 1990 with a similar phenology. The experiment was carried out in the Experimental Station of Aranjuez (40°03′ N, 3°31′ E, and 500 m above sea level) of the Spanish Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. The soil was Entisol Fluvent Xerofluvent (with 4.9 g kg^{-1} organic matter in the upper 0.4 m, total nitrogen content of 0.37 g kg^{-1}, carbonate content of 233 g kg^{-1}, electric conductivity of 0.16 d Sm^{-1} and alkaline pH of 8.1). The experimental design was a split-plot with six replicates and with water treatments (two treatments) as the main plot factor and the five genotypes as the split-plot factors. The trial was planted in autumn (November 18, 2011) in 7 m^2 plots (7 rows of 5 m long, and 0.20 m apart) and a plant density of 350 seeds/m^2. Climatic conditions during the 2011–2012 growing season are detailed in Table 1. Water treatments were rainfed (RF) and supplemental irrigation (SI), the SI was supplied from sprinklers providing an extra ≈360 mm distributed in three equal amounts at around mid-tillering, heading, and grain filling stages, respectively (Table 1). The plots were fertilized with phosphorus and potassium before sowing and protected from weeds, insects, pests and diseases by recommended agronomic practices. Moreover, plots were fertilized with ammonium sulfate and urea applied prior to sowing (64.3 kg N ha^{-1}) and at the beginning of tillering during late February (128.0 kg N ha^{-1}), respectively, totalling 192.3 kg N ha^{-1}.

2.2. Agronomic measurements

Days to heading (DH) were measured as the number of days from sowing until ears had emerged on 50% of the stems. Plant height (PH) was measured at grain filling as the distance from the ground level to the ear tip. Other growth traits were determined by destructive sampling at harvest. For each plot, plants from a 1 m length of the central row were harvested and the number of ears/m^2 (NE m^{-2}) calculated. A subsample of ten representative ears was collected to assess the number of grains/ear (NGE^{-1}) and thousand grain weight (TGW). Grain yield (GY) and aerial biomass (AB) was determined for each plot by harvesting mechanically.

2.3. Canopy reflectance measures

Spectral measurements and digital RGB images were taken at mid-tillering (stage 23), anthesis (stage 65), and grain filling (stage 87) of Zadoks' decimal code (Zadoks et al., 1974).

Canopy reflectance measurements were taken using a GreenSeeker hand-held Optical Sensor (Ntech Industries, Inc., Ukiah, CA, USA). The sensor measures the Normalized Difference Vegetation Index (NDVI) using its own light source. The sensor calculates the NDVI using measurements of the spectral reflectance in red (660 nm) and the near infrared (770 nm) wavelengths (Tucker and Sellers, 1986):

$$\mathrm{NDVI} = [(\mathrm{NIR} - \mathrm{VIS})/(\mathrm{NIR} + \mathrm{VIS})], \quad (1)$$

where NIR and VIS refer to the near infrared and the visible red wavelengths, respectively.

The GreenSeeker sensor was mounted on a hand-held tripod to take measurements at the same angle and distance from crop surface and the measurements were taken with the nadir view to avoid the non-Lambertian characteristics of vegetation. Distance between the sensor and the canopy top was kept constant at around 0.80 m. The operator carrying the GreenSeeker walked parallel to the plot, directing the sensor to the central row of the plot to avoid pointing at the soil, maintaining constant speed and holding the GreenSeeker trigger down without pausing in the plot. The GreenSeeker recorded 10–15 NDVI counts/plot. These counts were averaged for each plot to give a single value per plot. The measurements were taken close to solar noon (between 11 a.m. and 1 p.m.).

2.4. Image acquisition

The digital images were taken at the same time as the GreenSeeker measures. For each plot and time of measurement three digital images from different points were obtained per plot in a zenithal position, at about 0.8 m above the top of the central row canopy. The three images were averaged and a single value per plot calculated. The digital images were taken using a NIKON D40 digital RGB camera. The zoom was set at a focal length of 18 mm; using FOV of 66°43′ and 46°51′ for the horizontal and vertical angles, respectively, representing an area of ≈0.73 m^2; the shutter speed was set at 1/125 and the aperture was left automatic. Images were taken without flash. The image size was 3008 × 2000 pixels, and the digital pictures were saved in JPEG format.

Image analysis was performed with ImageJ 1.46r, a public-domain Java image processing and analysis programme (http://imagej.nih.gov/ij/) created by the National Institutes of Health (US

Table 1
Environmental growing conditions at the INIA's experimental station.

Month	T_{Max} (°C)	T_{Min} (°C)	RH_{Max} (%)	RH_{Min} (%)	Rad_T (W/m^2)	ETP_T (mm)	ASI (mm)	P_T (mm)	RF P_T/ETP_T	SI $(ASI+P_T)/ETP_T$	Growth stage
Nov	22.73	−1.25	100.00	40.95	7.94	30.06	0.00	58.67	1.95	1.95	Germination
Dec	16.56	−5.82	100.00	30.72	7.40	19.18	0.00	10.35	0.54	0.54	Seedling growth
Jan	17.11	−6.23	100.00	27.95	9.41	23.09	0.00	11.37	0.49	0.49	Seedling growth
Feb	22.66	−11.35	100.00	7.96	14.08	43.87	0.00	2.23	0.05	0.05	Seedling growth and start of tillering
Mar	26.50	−5.62	100.00	8.50	17.78	81.58	120.00	25.58	0.31	1.78	Tillering
Apr	23.61	−1.25	100.00	23.49	18.69	96.50	120.00	49.94	0.52	1.76	Booting, Heading, and Anthesis
May	36.42	2.79	100.00	12.81	25.27	145.15	120.00	25.79	0.18	1.00	Grain filling
Jun	40.77	8.59	95.80	11.05	27.99	186.52	0.00	0.61	0.00	0.00	Ripening
Total						625.95	360.00	184.54			

Monthly average maximum (Max) and minimum (Min) air temperatures (T), relative humidity (RH), total monthly accumulated irradiance (Rad_T), potential evapotranspiration (ETP_T), the amount of supplemental irrigation (ASI) and precipitation (P_T), and the ratio of the total water input (P_T plus ASI where appropriate) to ETP_T in the rainfed (RF) and supplemental irrigation (SI). The ETP calculation was based on the Penman–Monteith equation.

Department of Health & Human Services). It runs on any computer with a Java 1.5 or later virtual machine. The programme applies the chosen image operations (Fig. 1) to all the pictures in the folder selected and does not require user interaction for each image, therefore allowing a huge number of images to be analyzed in a few minutes. Colour channel information (digital numbers: DNs) was separated into the three standard colour channels: Red, Green and Blue and the Normalized Green Red Difference Index (NGRDI) was calculated according to Hunt et al. (2005):

$$\text{NGRDI} = [(\text{Green} - \text{Red})/(\text{Green} + \text{Red})], \qquad (2)$$

No direct radiometric calibration (transformation of the DNs to physical units of reflectance) for the Green and Red channels was performed because the wavelengths of the digital RGB camera channels were not available from the manufacturer. Thus, an indirect calibration was done using 1.5 m × 1.5 m black and white panels placed within the field and images of these panels together were captured by the digital RGB camera just prior capturing the plots images in the three studied growth stages. Then the NGRDI values of the black and white panels measured at the mid-tillering stage were used to calibrate the NGRDI values of the same panels measured at anthesis and the grain filling stage using the straight-line calibration option of the ImageJ programme.

2.5. Stable isotope analysis

Samples of flag leaf blades (10 flag leaf blades/plot) and mature grains (1 kg/plot) were collected at anthesis and harvest, respectively. Dry matter from flag leaf blades and mature grains was finely milled and samples of ~1 mg were weighed into tin capsules for $^{13}C/^{12}C$ ratio analysis and other samples of ~1 mg were weighed into silver capsules for $^{18}O/^{16}O$ ratio analysis.

The stable carbon isotope analysis of the two plant organs was conducted at the Scientific Facilities of the University of Barcelona. Analyses were performed using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Schwerte, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan), operating in continuous flow mode. The $^{13}C/^{12}C$ ratios (R) of plant material were expressed in δ notation (Coplen, 2008), as carbon isotope composition ($\delta^{13}C$):

$$\delta^{13}\text{C}\ (‰) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000, \qquad (3)$$

where sample refers to plant material and standard to Pee Dee Belemnite (PDB) calcium carbonate. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 3, polyethylene foil; IAEA CH6, sucrose; USGS 40, L-glutamic acid) were used with an analytical precision of 0.1‰.

The analysis of the ^{18}O stable isotope was conducted at Iso-Analytical Limited Laboratory (Crewe, Cheshire, UK). The $^{18}O/^{16}O$ ratios of the different plant parts were determined by an isotope ratio mass spectrometer (IRMS, Europe Scientific Geo 20-20). Results were also expressed in δ notation as oxygen isotope composition ($\delta^{18}O$) values:

$$\delta^{18}\text{O}\ (‰) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000, \qquad (4)$$

using secondary standards of known $^{18}O/^{16}O$ ratios (IAEA-CH-6, sucrose; IAEA-C-3, cellulose; IAEA-601, benzoic acid) calibrated against Vienna Standard Mean Oceanic Water (VSMOW) with an analytical precision of 0.2‰.

2.6. Statistical analysis

The hypothesis of zero difference between means was tested with analysis of variance (ANOVA) performed using the general linear model (GLM) procedure to calculate the effects of water treatment, genotype and water treatment by genotype interactions on the studied parameters. Mean separation of genotypes for the different parameters was performed by Duncan's multiple range test ($P < 0.05$) and the independent sample *t*-test ($P < 0.05$) was used when comparing the water treatment means.

Relationships between parameters were analyzed within each water treatment (RF, SI) alone and with both combined using Pearson linear correlation. Stepwise multiple linear regression was used to determine the independent variables (stable isotopes, digital and spectral indices) accounting for the majority of total GY (dependent variable) in both RF and SI treatments.

For better understanding of the mechanisms that could predict GY performance under contrasting conditions, a path-analysis was performed for portioning correlation coefficients into direct and indirect effects. A detailed path conceptual model was suggested for each of the two water treatments, indicating the direct and indirect responses on the dependent factor (GY) through: (1) the independent factors represented by the AB at mid-tillering and anthesis assessed via the NGRDI, and the photosynthetic and water status ($\delta^{13}C$); and (2) the effect of the above independent factors on the agronomical yield components (NE m^{-2}, NGE^{-1}, and TGW). The χ^2 goodness of fit test was used to test the null hypothesis that the reduced model (which has some of the paths deleted between variables) fits the data as efficiently as the saturated model (i.e. in which there is a direct path from each variable to every other variable). The comparative fit index (CFI) was used to compare the reduced models with the independence model where all possible paths were deleted (Bentler, 1990). A non-significant χ^2 is preferred and the CFI with values >0.9 taken as indicative of a good fit.

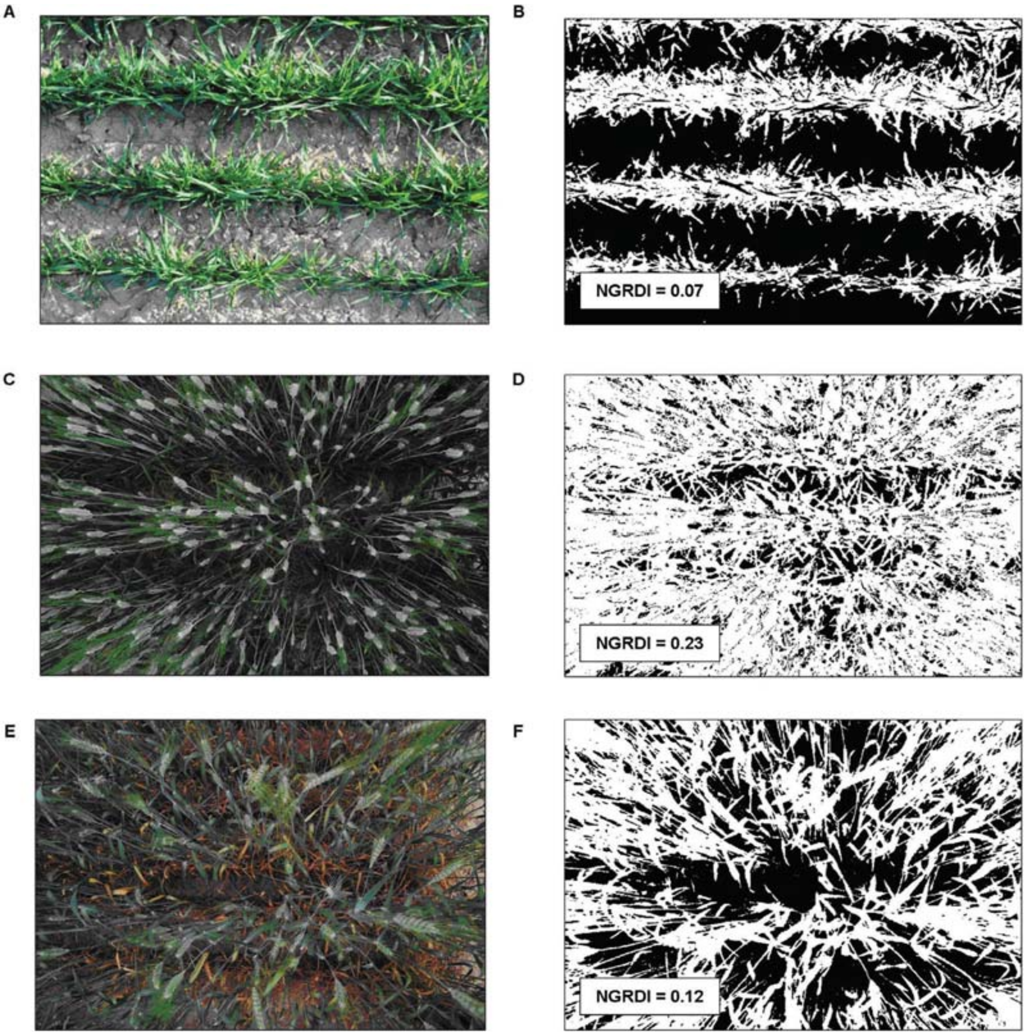

Fig. 1. Analysis of the Normalized Green Red Difference Index (NGRDI) from digital RGB images. Digital images are on the left and the corresponding NGRDI images are on the right. Pictures are of the genotype Sula at mid-tillering (A and B) and anthesis (C and D) under supplemental irrigation and the same genotype at anthesis (E and F) under rainfed conditions.

Data were analyzed using the SPSS 16 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using SigmaPlot 11.0 for Windows (Sysat Software Inc., Point Richmond, CA, USA).

3. Results

Durum wheat genotypes were exposed to two different water treatments, rainfed (RF), which was exposed to natural precipitation, and supplemental irrigation (SI), which was applied from mid-tillering onwards (Table 1). Under RF, water stress started to develop during March, coinciding with mid-tillering, when average temperatures rose by around 10 °C. While the precipitation to evapotranspirtation (P_T/ETP_T) ratio was 0.31 and continued at this level until maturity, under SI the ratio of the amount of supplemental irrigation plus precipitation to the potential evapotranspiration [$(ASI + P_T)/ETP_T$] was clearly higher than one (ca. 1.80) during March and April. However, the ratio decreased to one during May, coinciding with grain filling, and physiological ripening took place in early June when the ratio was 0. This pattern suggests that even under SI some degree of water stress occurred during grain filling.

3.1. The effect of water treatment and genotype on GY and agronomical parameters

The effect of water treatment was significant for all studied traits ($P < 0.001$) except for the thousand grain weight (TGW) (Table 2). Compared with SI, the aerial biomass (AB), grain yield (GY), number of grains/ear (NGE^{-1}), number of ears/m^{-2} (NE m^{-2}), plant height (PH) and days to heading (DH) were significantly lower by 50%, 60%, 33%, 41%, 16% and 5% under RF, respectively. Significant genotypic effects existed only for GY and NGE^{-1} ($P < 0.05$ and $P < 0.001$ for GY and NGE^{-1}, respectively). The water treatment by genotype interaction was not significant for all studied traits. The low GY genotype Don Pedro was characterized by low NGE^{-1} and high PH.

3.2. The effect of water treatment and genotype on vegetation indices and stable isotopes

The effect of water treatment was significant for almost all traits ($P < 0.001$) except for the Normalized Difference Vegetation Index and the Normalized Green Red Difference Index measured at mid-tillering (NDVI.1 and NGRDI.1, respectively), before the application of the first irrigation in the SI treatment (Tables 3 and 1,

Table 2
The effect of water treatment and genotype on grain yield, aerial biomass and agronomical yield components.

GT	Traits						
	AB ($g\,m^{-2}$)	GY ($g\,m^{-2}$)	NGE^{-1}	TGW (g)	NE m^{-2}	PH (cm)	DH
Anton	1242.36 a	454.79 b	22.70 b	46.22 a	399.46 a	91.25 ab	149.46 a
Bolo	1298.25 a	447.12 ab	19.93 a	47.15 a	425.50 a	93.00 ab	149.96 a
Don Pedro	1177.12 a	398.16 a	19.85 a	45.30 a	389.12 a	93.58 b	148.33 a
Regallo	1228.92 a	456.94 b	23.98 b	44.67 a	387.79 a	89.58 a	149.92 a
Sula	1272.87 a	478.13 b	25.36 b	45.92 a	379.98 a	90.33 ab	149.42 a
RF_{mean}	834.62 a	254.54 a	17.97 a	45.97 a	294.46 a	83.40 a	145.72 a
SI_{mean}	1653.19 b	639.51 b	26.76 b	45.73 a	498.28 b	99.70 b	153.12 b
ANOVA							
WT	***	***	***	NS	***	***	***
GT	NS	*	***	NS	NS	NS	NS
WT × GT	NS	NS	NS	NS	NS	NS	NS

Water treatments (WT), genotype (GT), aerial biomass (AB), grain yield (GY), number of grains/ear (NGE^{-1}), thousand grain weight (TGW), number of ears/m^2 (NE m^{-2}), plant height (PH) and days to heading (DH). Data shown are the genotype means in the two water treatments. Means followed by different letters were significantly different ($P \leq 0.05$) by Duncan's test. An independent samples *t*-test ($P \leq 0.05$) was used to compare between the water treatments means. RF, rainfed; SI, supplemental irrigation; WT × GT, water treatment by genotype interaction.
NS, not significant.
[*] $P \leq 0.05$.
[***] $P \leq 0.001$.

respectively). Thus, the values of both vegetation indices at anthesis and grain filling were higher in the SI than in the RF treatment. Regardless of the water treatments, the values of both NGRDI and NDVI increased from mid-tillering to anthesis and then decreased during grain filling (Table 3). No significant genotypic differences existed for either NGRDI or NDVI. Strong positive correlations were found between NGRDI and NDVI at anthesis and the grain filling stage, but no significant correlations were found at mid-tillering (Table 4).

Compared with the RF treatment, SI decreased (in absolute values) the $\delta^{13}C$ of the flag leaf ($\delta^{13}C_{FL}$) and the mature grains ($\delta^{13}C_G$) by 2.3% and 1.9‰, respectively, and as well as the $\delta^{18}O$ of the same organs ($\delta^{18}O_{FL}$ and $\delta^{18}O_G$) by 5.5‰ and 2.1‰, respectively (Table 3). The genotypic effect was significant for the carbon and oxygen stable isotope signatures in both flag leaf blades and mature grains ($P < 0.001$ for $\delta^{13}C_{FL}$ and $\delta^{18}O_{FL}$; $P < 0.01$ for $\delta^{13}C_G$; and $P < 0.05$ for $\delta^{18}O_G$). Overall, the low GY genotype Don Pedro showed almost the highest $\delta^{13}C$ and $\delta^{18}O$ in the different plant organs.

The water treatment by genotype interaction was not significant for all the stable isotopes signatures in different plant organs except for $\delta^{18}O_G$, while no significant interactions existed for either NGRDI or NDVI, regardless of the developmental stage. Although $\delta^{18}O_{FL}$ correlated positively with $\delta^{13}C_{FL}$ (0.55, $P < 0.01$) and $\delta^{13}C_G$ (0.57, $P < 0.01$) under RF treatment, $\delta^{18}O_G$ did not correlate with $\delta^{13}C_{FL}$ or $\delta^{13}C_G$ (Table 5). Under SI treatment, there were no significant associations between the carbon and oxygen isotope compositions.

3.3. *Relationships of NGRDI and NDVI with GY, AB and agronomical components*

Both NGRDI and NDVI showed highly significant and positive correlations with GY and AB across water regimes at both anthesis and the grain filling stage, while no significant associations were detected at mid-tillering (Table 4). However, the NGRDI was the only vegetation index to show significant association with GY and AB within each water regime. Thus, NGRDI at anthesis (NGRDI.2)

Table 3
The effect of water treatment and genotype on the studied stable isotopes and vegetation indices.

GT	Traits									
	$\delta^{13}C_{FL}$ (‰)	$\delta^{13}C_G$ (‰)	$\delta^{18}O_{FL}$ (‰)	$\delta^{18}O_G$ (‰)	NGRDI.1	NGRDI.2	NGRDI.3	NDVI.1	NDVI.2	NDVI.3
Anton	−27.73 ab	−25.69 a	29.70 a	30.88 a	0.05 a	0.19 a	0.11 a	0.20 a	0.74 a	0.44 a
Bolo	−27.33 b	−25.16 b	30.90 c	31.11 a	0.06 a	0.20 a	0.12 a	0.22 a	0.76 a	0.47 a
Don Pedro	−27.61 b	−25.02 b	30.98 c	31.73 b	0.05 a	0.18 a	0.11 a	0.18 a	0.72 a	0.45 a
Regallo	−28.11 a	−25.58 a	30.21 ab	31.28 ab	0.06 a	0.20 a	0.13 a	0.17 a	0.77 a	0.50 a
Sula	−27.32 b	−25.31 ab	30.66 bc	31.36 ab	0.05 a	0.20 a	0.12 a	0.17 a	0.74 a	0.49 a
RF_{mean}	−26.48 b	−24.42 b	33.25 b	32.32 b	0.05 a	0.14 a	0.05 a	0.19 a	0.63 a	0.28 a
SI_{mean}	−28.76 a	−26.28 a	27.73 a	30.22 a	0.05 a	0.25 b	0.18 b	0.17 a	0.86 b	0.67 b
ANOVA										
WT	***	***	***	***	NS	***	***	NS	***	***
GT	***	**	***	*	NS	NS	NS	NS	NS	NS
WT × GT	NS	NS	NS	*	NS	NS	NS	NS	NS	NS

Water treatments (WT), genotype (GT), the stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope compositions of the flag leaf blade (FL) and mature grains (G), and the Normalized Green Red Difference Index (NGRDI) and Normalized Difference Vegetation Index (NDVI). For NGRDI and NDVI, the number following the acronym refers to the stage when the measure was conducted: 1 refers to mid-tillering, 2 to anthesis and 3 to the grain filling stage. Data shown are the genotype means in the two water treatments. Means followed by different letters were significantly different ($P \leq 0.05$) by Duncan's test. An independent samples *t*-test ($P \leq 0.05$) was used to compare between the water treatments means. RF, rainfed; SI, supplemental irrigation; WT × GT, water treatment by genotype interaction.
NS, not significant.
[*] $P \leq 0.05$.
[**] $P \leq 0.01$.
[***] $P \leq 0.001$.

Table 4
Pearson correlation coefficients of the relationships between traits measured across both water treatments.

	AB (g m^{-2})	GY (g m^{-2})	NGE^{-1}	TGW (g)	NE m^{-2}	PH (cm)	DH	$\delta^{13}C_{FL}$ (‰)	$\delta^{13}C_{G}$ (‰)	$\delta^{18}O_{FL}$ (‰)	$\delta^{18}O_{G}$ (‰)	NGRDI.1	NGRDI.2	NGRDI.3	NDVI.1	NDVI.2	NDVI.3
AB (g m^{-2})																	
GY (g m^{-2})	**0.94****																
NGE^{-1}	**0.62****	**0.78****															
TGW (g)	−0.05	0.07	−0.02														
NE m^{-2}	**0.91****	**0.82****	**0.36****	−0.23													
PH (cm)	**0.81****	**0.84****	**0.65****	0.03	**0.70****												
DH	**0.73****	**0.76****	**0.65****	−0.04	**0.63****	**0.70****											
$\delta^{13}C_{FL}$ (‰)	−**0.83****	−**0.87****	−**0.73****	0.08	−**0.71****	−**0.78****	−**0.74****										
$\delta^{13}C_{G}$ (‰)	−**0.89****	−**0.94****	−**0.71****	−0.16	−**0.76****	−**0.79****	−**0.68****	**0.86****									
$\delta^{18}O_{FL}$ (‰)	−**0.89****	−**0.93****	−**0.79****	0.03	−**0.76****	−**0.84****	−**0.76****	**0.92****	**0.90****								
$\delta^{18}O_{G}$ (‰)	−**0.82****	−**0.83****	−**0.69****	0.10	−**0.71****	−**0.73****	−**0.69****	**0.76****	**0.78****	**0.85****							
NGRDI.1	−0.06	−0.01	−0.09	0.19	0.00	−0.02	−0.10	0.15	−0.03	0.08	0.08						
NGRDI.2	**0.96****	**0.97****	**0.72****	0.00	**0.85****	**0.86****	**0.77****	−**0.89****	−**0.92****	−**0.94****	−**0.84****	−0.04					
NGRDI.3	**0.86****	**0.90****	**0.74****	−0.07	**0.76****	**0.85****	**0.77****	−**0.86****	−**0.83****	−**0.90****	−**0.77****	−0.06	**0.91****				
NDVI.1	−0.10	−0.14	−0.24	−0.01	−0.01	−0.19	−0.18	0.21	0.16	0.15	0.04	0.16	−0.13	−0.21			
NDVI.2	**0.84****	**0.89****	**0.78****	−0.04	**0.69****	**0.82****	**0.71****	−**0.86****	−**0.83****	−**0.92****	−**0.84****	−0.04	**0.90****	**0.92****	−0.14		
NDVI.3	**0.85****	**0.89****	**0.75****	−0.06	**0.74****	**0.85****	**0.76****	−**0.85****	−**0.82****	−**0.89****	−**0.77****	−0.05	**0.90****	**0.98****	−0.23	**0.92****	

Traits included were: aerial biomass (AB), grain yield (GY), number of grains/ear (NGE^{-1}), thousand grain weight (TGW), number of ears/m^2 (NE m^{-2}), plant height (PH), days to heading (DH), stable carbon isotope composition of the flag leaf blade ($\delta^{13}C_{FL}$) and mature grains ($\delta^{13}C_{G}$), oxygen isotope composition of the flag leaf blade ($\delta^{18}O_{FL}$) and mature grains ($\delta^{18}O_{G}$), and the Normalized Green Red Difference Index (NGRDI) and Normalized Difference Vegetation Index (NDVI). For NGRDI and NDVI, the number following the acronym refers to the stage when the measure was conducted: 1, mid-tillering; 2, anthesis and 3, grain filling.

** $P \leq 0.01$.

Bold figures indicate significant correlations.

Table 5
Pearson correlation coefficients of the linear relationships between traits.

	AB (g m^{-2})	GY (g m^{-2})	NGE^{-1}	TGW (g)	NE m^{-2}	PH (cm)	DH	$\delta^{13}C_{FL}$ (‰)	$\delta^{13}C_{G}$ (‰)	$\delta^{18}O_{FL}$ (‰)	$\delta^{18}O_{G}$ (‰)	NGRDI.1	NGRDI.2	NGRDI.3	NDVI.1	NDVI.2	NDVI.3
AB (g m^{-2})		**0.60****	−0.20	−0.19	**0.75****	−0.33	−0.04	0.08	−0.30	−0.05	−0.05	**0.41***	**0.69****	0.28	0.29	0.04	0.07
GY (g m^{-2})	**0.56****		**0.50****	0.01	0.23	−0.11	0.27	−0.02	−**0.58****	−**0.38***	−0.31	0.27	**0.67****	0.21	0.09	0.22	0.11
NGE^{-1}	−0.36	0.23		−0.03	−**0.65****	0.04	0.36	−0.26	−0.29	−0.32	−0.24	−0.33	0.09	−0.04	−0.29	0.18	0.08
TGW (g)	−0.02	**0.37***	0.01		−0.32	0.20	0.30	0.22	−0.01	−0.07	−0.35	−0.16	−0.06	−0.09	0.02	−0.18	−0.09
NE m^{-2}	**0.74****	0.35	−**0.61****	−0.36		−0.24	−0.20	0.19	−0.08	0.07	0.15	**0.60****	**0.44***	0.24	**0.40***	0.03	0.04
PH (cm)	0.10	0.01	−0.18	0.08	0.06		0.26	0.18	−0.06	0.23	−0.06	0.07	−0.28	−0.24	−0.21	0.03	−0.05
DH	−0.01	−0.10	−0.01	−0.13	0.04	−0.22		0.02	−0.15	−0.27	−0.31	0.01	0.14	0.15	−0.11	0.30	0.12
$\delta^{13}C_{FL}$ (‰)	−0.17	−0.25	−0.09	0.07	−0.18	−0.01	−0.20		**0.43***	**0.55****	−0.22	0.22	−0.10	−0.16	0.23	−0.20	−0.17
$\delta^{13}C_{G}$ (‰)	−**0.51****	−**0.74****	−0.04	−**0.52****	−0.23	−0.02	0.17	0.31		**0.57****	−0.13	0.04	−**0.47****	−0.02	0.12	−0.13	0.02
$\delta^{18}O_{FL}$ (‰)	−0.05	−0.20	−**0.41***	0.11	0.06	0.05	0.30	0.33	0.34		0.10	0.17	−0.22	0.06	0.06	−0.35	0.22
$\delta^{18}O_{G}$ (‰)	−0.27	−0.10	−0.06	0.31	−0.21	0.18	0.12	0.21	0.25	**0.42***		−0.09	−0.18	0.11	−0.29	−0.04	0.30
NGRDI.1	−0.21	0.16	0.21	**0.38***	−0.26	0.11	−0.13	0.19	−**0.38***	−0.15	0.13		**0.43***	0.19	0.22	0.26	0.17
NGRDI.2	**0.75****	**0.64****	−0.18	0.17	**0.52****	0.25	−0.04	−**0.46***	−**0.58****	−0.22	−0.19	−0.12		0.20	0.11	0.01	0.00
NGRDI.3	−0.09	−0.01	0.22	−0.15	−0.04	0.21	0.05	−0.19	0.01	−0.09	0.18	−0.04	−0.01		0.04	0.23	**0.73****
NDVI.1	0.16	0.16	−0.03	−0.03	0.15	0.09	−0.02	−0.11	−0.11	−**0.37***	−0.22	0.08	0.26	−0.16		−0.05	0.03
NDVI.2	−0.06	0.11	**0.44***	0.00	−0.24	0.06	−0.23	−0.34	−0.14	−**0.51****	−0.36	−0.01	0.17	**0.47****	0.14		0.29
NDVI.3	−0.17	−0.11	0.22	−0.11	−0.15	0.09	−0.01	0.03	0.12	0.09	0.12	0.00	−0.14	**0.82****	−0.24	**0.44***	

The upper-right and lower-left triangle contains correlation coefficients of traits under rainfed and supplemental irrigation, respectively. Traits included were: aerial biomass (AB), grain yield (GY), number of grains/ear (NGE^{-1}), thousand grain weight (TGW), number of ears/m^2 (NE m^{-2}), plant height (PH), days to heading (DH), stable carbon isotope composition of the flag leaf blade ($\delta^{13}C_{FL}$) and mature grains ($\delta^{13}C_{G}$), stable oxygen isotope composition of the flag leaf blade ($\delta^{18}O_{FL}$) and mature grains ($\delta^{18}O_{G}$), and the Normalized Green Red Difference Index (NGRDI) and Normalized Difference Vegetation Index (NDVI). For NGRDI and NDVI, the number following the acronym refers to the stage when the measure was conducted: 1 refers to mid-tillering, 2 to anthesis and 3 to the grain filling stage.

* $P \leq 0.05$.

** $P \leq 0.01$.

Bold figures indicate significant correlations.

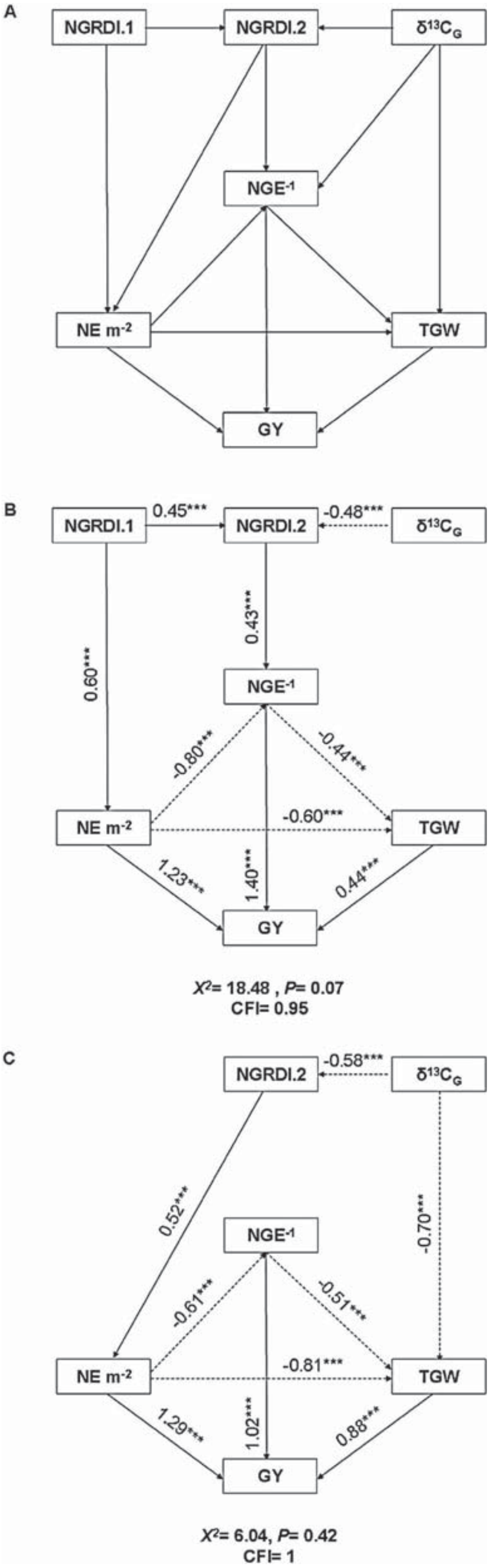

Fig. 2. Path-analysis showing the direct and indirect relations of the most promising traits and grain yield. The path model quantifying the relative contribution of direct and indirect effects of water regime during the reproductive period (represented by grain δ^{13}C), and growth and aerial biomass (represented by the Normalized Green Red Difference Index, NGRDI at mid-tillering and anthesis) and yield components on the grain yield (GY) is shown in (A). The water treatments are as follows: (B) RF, rainfed; (C) SI, supplemental irrigation. The single headed arrow between two variables denotes a hypothesis of direct causation. Dashed lines indicate negative relationships. Overall chi-squared (χ^2) goodness of fit statistics and the comparative fit index (CFI) for each path model are shown at the bottom centre of each panel (a non-significant χ^2 is preferred and the CFI with values > 0.9 taken as indicative of a good fit). $\delta^{13}C_G$, stable carbon isotope composition of mature grains; NGRDI.1 and NGRDI.2 refer to the vegetation index measured at mid-tillering and anthesis, respectively; NE m^{-2}, number of ears m^{-2}; NGE^{-1}, number of grains/ear; TGW, thousand grain weight; GY, grain yield. The regression weights and probabilities are shown: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

was positively correlated with GY and AB under both RF and SI (Table 5), whereas the NGRDI.1 correlated positively with AB only under RF (Table 5). However, regardless of the stage of measurement, NDVI did not correlate with GY and AB within either of the water regimes (Table 5). Correlations across water regimes of the vegetation indices with the agronomical components were highly significant even when they were notably lower than those of GY and AB (Table 4). The only agronomical component that did not correlate was TGW. However, within each water regime the vegetation indices did not correlate with other agronomical component except for NE m^{-2}, TGW and NGE^{-1}, and again NGRDI performed better than NDVI (Table 5).

3.4. *Relationships of δ^{13}C and δ^{18}O with GY, AB, agronomical components and vegetation indices*

Across water regimes δ^{13}C and δ^{18}O in both the flag leaf and mature grains showed strongly significant and negative correlations with GY, AB and the agronomical components except for TGW (Table 4). Moreover, all the isotope signatures were negatively correlated with the vegetation indices NGRDI and NDVI at all crop stages except at mid-tillering (Table 4). The $\delta^{13}C_G$ had the best correlation (negative) with GY and NGRDI.2 under both the RF and SI treatments and also with AB and TGW under SI, while the $\delta^{13}C_{FL}$ did not correlate with GY or any other growth parameters or vegetation index under RF and correlated only with NGRDI.2 under SI (Table 5). The $\delta^{18}O_{FL}$ correlated (negatively) with GY only under RF and correlated (also negatively) with NGE^{-1} and both NDVI.1 and the NDVI at anthesis (NDVI.2) under SI treatment (Table 5).

3.5. *Comparative relationships of the stable isotopes and vegetation indices with GY*

A stepwise analysis was performed within each water treatment having GY as a dependent variable and the different stable isotope compositions and vegetation indices as independent variables. Irrespective of the water treatment (SI or RF), NGRDI.2 and $\delta^{13}C_G$ were the first two variables chosen by the model, explaining 50% and 58% of the total variability in GY under RF and SI treatments, respectively (Table 6).

A path-coefficient analysis was conducted for each water treatment aimed at developing a conceptual model (Fig. 2A). This described GY as being a combination of the two categories of phenotypical traits and the agronomical yield components. The final objective of the model was to dissect how these physiological traits directly or indirectly assess GY performance within each water regime. Only $\delta^{13}C_G$, NGRDI.1 and NGRDI.2 were included in the path model because they were the best individual performers in terms of their relationship with GY and the agronomical components. For both water regimes the path model provided a perfect fit to the data (Fig. 2B and C), as CFI ≈ 1 and χ^2 was not significant. The $\delta^{13}C_G$ showed a significant direct (negative) effect on the biomass at anthesis (NGRDI.2) under both RF and SI treatments, and also a significant direct (negative) effect on the TGW under SI. The NGE^{-1} and NE m^{-2} showed the strongest direct (positive)

Table 6
Stepwise analysis explaining grain yield variation across genotypes within each water treatment.

WT	Trait	Variable chosen	Final r^2	Final stepwise model
RF	GY	NGRDI.2	0.42^{***}	$GY = 8.39 \times 10^{-16} + 0.67$ NGRDI.2
		NGRDI.2, $\delta^{13}C_G$	0.50^{***}	$GY = 1.48 \times 10^{-15} + 0.51$ NGRDI.2 $- 0.34\ \delta^{13}C_G$
SI	GY	$\delta^{13}C_G$	0.52^{***}	$GY = 5.62 \times 10^{-15} - 0.74\ \delta^{13}C_G$
		$\delta^{13}C_G$, NGRDI.2	0.58^{***}	$GY = 5.70 \times 10^{-15} - 0.55\ \delta^{13}C_G + 0.32$ NGRDI.2

Grain yield (GY) as a dependent variable and the stable carbon isotope composition of mature grains ($\delta^{13}C_G$) and the Normalized Green Red Difference Index measured at anthesis (NGRDI.2). WT, water treatment; RF, rainfed; SI, supplemental irrigation. The GY variability attributed to the variables included in the model is shown by r^2.

*** $P \leq 0.001$.

effect on GY under RF and SI treatments, respectively. The NGRDI.1 showed significant direct (positive) effects on NE m^{-2} and NGRDI.2 under RF. The NGRDI.2 showed a significant direct (positive) effect on the NE m^{-2} under SI treatment, but not under RF as it showed a significant direct (positive) effect on the NGE^{-1}. The NGE^{-1} and TGW showed significant (positive) effects on the final GY, with the effect of NGE^{-1} being stronger than TGW under both water treatments (especially under RF). In summary, the GY under RF treatment depends indirectly on the NGRDI.1 and NGRDI.2 (indicators of green AB at mid-tillering and anthesis, respectively) with these two vegetation indices having a direct effect on the NE m^{-2} and NGE^{-1}, respectively. GY also depends on the $\delta^{13}C_G$ (indicator of water status during the reproductive stage) with this isotope composition having a direct effect on NGRDI.2. Under SI treatment the GY depends on NGRDI.2 through an effect on the NE m^{-1}, whereas $\delta^{13}C_G$ had a direct effect on NGRDI.2 as well as on grain filling through its direct effect on TGW.

4. Discussion

The effect of water stress was clear, with grain yield (GY) and total aerial biomass (AB) at harvest decreasing strongly under rainfed (RF) compared with supplemental irrigation (SI) conditions. Moreover, the number of ears/ground area (NE m^{-2}) and number of grains/ear (NGE^{-1}) together with plant height (PH) and days to heading (DH) significantly decreased, whereas thousand grain weight (TGW) was not affected (Table 2). Similar patterns of response to water stress have been reported previously in durum wheat (Araus et al., 2013; Casadesús et al., 2007). The NGE^{-1} and NE m^{-2} were the most drought stress affected GY components, while the TGW remained stable (Giunta et al., 1993).

4.1. *Effect of water treatment on carbon and oxygen stable isotope compositions*

RF treatment significantly increased $\delta^{13}C$ and $\delta^{18}O$ in both flag leaves and mature grains compared with SI (Table 3). These findings are in general agreement with previous studies on durum wheat (Araus et al., 2013; Cabrera-Bosquet et al., 2011; Elazab et al., 2012; Ferrio et al., 2007). An increase in the $\delta^{13}C$ of the plant dry matter may be due to either greater photosynthetic capacity (A) or lower stomatal conductance (g_s) or to both factors together, which consequently decreases the ratio of intercellular CO_2 leaf concentration to ambient CO_2 (C_i/C_a) (Farquhar and Richards, 1984; Cabrera-Bosquet et al., 2009a; Condon et al., 2004). Usually the lower g_s associated with water stress (or the presence of other factors such as salinity that cause a subsequent water stress) is considered the main factor affecting $\delta^{13}C$ (Araus et al., 1998, 2003b; Richards et al., 2011; Yousfi et al., 2009).

Whatever the water treatment, $\delta^{13}C$ was higher in the grains than in the flag leaf, which also agrees with previous studies in durum wheat (Araus et al., 1993; Merah et al., 2001; Monneveux et al., 2006). The higher $\delta^{13}C$ of grains could reflect the decrease in soil moisture availability and the increasing evaporative demand during the final stages of the crop cycle, which are typical of Mediterranean conditions (Cabrera-Bosquet et al., 2009a,b; Elazab et al., 2012). Moreover, the ears are known to have lower permeability to CO_2 diffusion than leaves, which may contribute to a constitutively higher $\delta^{13}C$ in mature grains compared to the flag leaf (Araus et al., 1993; Tambussi et al., 2005).

Under SI, the $\delta^{18}O$ of grains ($\delta^{18}O_G$) was higher than in the flag leaf ($\delta^{18}O_{FL}$). According to previous studies, the increase in evaporative demand later in the crop cycle and the low g_s and high position of the ear in the plant may cause the ear to be at a higher temperature than the flag leaf, and therefore there could be a greater ^{18}O enrichment of photoassimilates in the ear than in the flag leaf (Cabrera-Bosquet et al., 2011; Elazab et al., 2012). However, under RF, the $\delta^{18}O$ was higher in the flag leaf compared with the grains. According to several authors (Araus et al., 2013; Barbour et al., 2000; Barbour and Farquhar, 2000; Cabrera-Bosquet et al., 2011; Ferrio et al., 2007) $\delta^{18}O_G$ is affected by several factors. In the case of the RF trial, the decrease in the $\delta^{18}O$ of grain was probably the result of rainfall that occurred during grain filling (Table 1). A second factor may have been biochemical fractionation during the translocation to the growing grains of assimilates accumulated before anthesis. Therefore, the environmental signal associated with the $\delta^{18}O$ of photoassimilates may be lost during transport of these assimilates to the growing grains. In fact, while the $\delta^{18}O_{FL}$ correlated positively with the $\delta^{13}C_{FL}$ and $\delta^{13}C_G$ in the RF treatment, which was in accordance with a common factor (differences in g_s) affecting the three isotope signatures (Barbour et al., 2000; Barbour and Farquhar, 2000), the $\delta^{18}O_G$ did not correlate with $\delta^{13}C_G$ (Table 5). In the case of the SI treatment, neither the $\delta^{18}O_G$ nor the $\delta^{18}O_{FL}$ correlated with the $\delta^{13}C_{FL}$ or $\delta^{13}C_G$. In this case the mixture of water sources (irrigation and rainfall) with different $\delta^{18}O$ may also have affected the environmental signal of photoassimilates. Therefore, while the $\delta^{18}O_G$ and even the $\delta^{18}O_{FL}$ do not appear appropriate to indicate differences in water status within a given growing condition, the $\delta^{13}C_{FL}$ and even $\delta^{13}C_G$ seem less affected by the set of miscellaneous factors (other than the water regime during growth) that affect $\delta^{18}O$.

4.2. *Relationships of carbon and oxygen stable isotope compositions with GY*

The $\delta^{13}C_G$ was negatively correlated with GY, whatever the water treatment, and was negatively correlated with AB to a lesser degree (Table 5). Comparable negative relationships have been frequently reported in durum wheat (Araus et al., 2013; Merah et al., 2001; Monneveux et al., 2006). Such a negative relationship has also been reported frequently for cereals under Mediterranean conditions, particularly in growing conditions yielding beyond 2 tonnes ha^{-1} (Araus et al., 2003a,b, 2013; Rebetzke et al., 2008). The results in Table 2 showed that GY of the SI and RF treatments were beyond the 2 tonnes ha^{-1} (around 6.4 and 2.6 tonnes ha^{-1}, for SI and RF treatments, respectively). Positive relationships of the $\delta^{13}C_G$ with GY and AB have usually only been reported in cereals under very severe drought conditions (Reynolds et al., 2007; Voltas et al.,

1999) and in some cases relationships of the $\delta^{13}C_G$ with GY may even disappear for environments yielding below 2.5 tonnes ha^{-1} (Araus et al., 2003a,b). The positive relationship between $\delta^{13}C_G$ and GY may be understood in the sense that higher water use efficiency (*WUE*) increases yield under water stress (Farquhar and Richards, 1984; Condon et al., 2004; Araus et al., 2003a,b, 2008, 2013).

The negative correlation of the $\delta^{13}C_G$ with GY that is frequently reported under Mediterranean environments characterized by terminal (i.e. during grain filling) drought stress may be the result of a miscellaneous set of factors (Araus et al., 2013; Condon et al., 2004; Monneveux et al., 2006; Tambussi et al., 2005). Genotypic differences in phenology may be involved because genotypes with a short growth cycle (i.e. early heading, anthesis and/or maturity) may escape the terminal drought stress typical of Mediterranean conditions, therefore exhibiting lower $\delta^{13}C_G$ and higher GY than genotypes with longer crop cycles. However, this is not the case in the present study because DH and $\delta^{13}C_G$ were not correlated within either of the two water treatments. Low grain $\delta^{13}C$ may reflect a greater reliance of grain filling on pre-anthesis stem reserves stored earlier in the season when plants are less stressed. Therefore, genotypes that are exposed to severe stress during grain filling will be more dependent on pre-anthesis assimilate reserves, which have $\delta^{13}C$ values that are relatively lower than assimilates produced during grain filling (Condon et al., 2004; Monneveux et al., 2006). This implies a close correlation between leaf and grain $\delta^{13}C$ (Monneveux et al., 2006), which is the case in our study, where a significant positive correlation existed between $\delta^{13}C_{FL}$ and $\delta^{13}C_G$ under RF treatment (Table 5), while such a correlation did not exist under SI conditions. Alternatively, the genotypes capable of extracting more water from the soil (e.g. due to more efficient root systems, osmotic adjustment, faster soil cover, etc.) may have greater yields while exhibiting lower $\delta^{13}C_G$ (Blum, 2009). Therefore, the negative relationship between $\delta^{13}C_G$ and GY may indicate that even under water stress conditions the high GY genotypes are those capable of maintaining a higher transpiration and thus higher water use. In other words, this means a more efficient use of water (*EUW*) even if it is at the expense of a lower *WUE*, especially during the grain filling period (Araus et al., 2002, 2013; Blum, 2009).

The negative relationship found under RF between $\delta^{18}O_{FL}$ and GY (Table 5) supports a higher transpiration rate being present in genotypes with the greatest yield. Nevertheless, apart from the results under RF conditions discussed above, the $\delta^{18}O_G$ and $\delta^{18}O_{FL}$ did not correlate with GY and AB under either of the water treatments (Table 5). Indeed, concerns about weak or absent $\delta^{18}O$ signatures, particularly in mature grains, has raised doubts about the use of $\delta^{18}O$ to evaluate, for example, the genotypic performance under different water treatments (Araus et al., 2013; Cabrera-Bosquet et al., 2009a,b; Elazab et al., 2012; Ferrio et al., 2007). In the pioneering study of Barbour et al. (2000) on bread wheat, negative correlations of $\delta^{18}O_{FL}$ and $\delta^{18}O_G$ with GY under irrigated conditions were reported, with the $\delta^{18}O_{FL}$ having the best correlation with GY. Araus et al. (2013) reported for three consecutive crop cycles in durum wheat that the $\delta^{18}O_G$ was correlated much more weakly than $\delta^{13}C_G$ with GY, irrespective of the water conditions.

4.3. The effect of water treatment on vegetation indices

The NGRDI and NDVI values increased from mid-tillering to anthesis and then dropped at grain filling (Table 3). It is widely reported for wheat that crop green AB reaches its maximum around anthesis and then decreases during grain filling due to senescence (Aparicio et al., 2002, 2004; Kanemasu, 1974). NDVI and NGRDI values at anthesis and grain filling were lower under RF than SI, in agreement with the effect of water stress on green AB (Aparicio et al., 2000, 2004). Water stress is known to inhibit plant growth and accelerate senescence with a subsequent decrease in vegetation indices measured either with a spectroradiometer or with a digital RGB camera (Casadesús and Villegas, 2014; Gitelson et al., 2002; Mullan and Reynolds, 2010). The results presented in Table 2 confirmed the same, where wheat plants under RF treatment were one week earlier in heading than the SI treatment plants, and thus the RF treatment plants were more senescent than the SI treatment plants during grain filling. Thus, at grain filling plants under RF exhibited NGRDI.3 and NDVI.3 values that were 72% and 58% lower, respectively, than the SI plants (Table 3).

4.4. Relationship of vegetation indices with GY

The NGRDI measured at anthesis was the only vegetation index positively correlated with GY for both the RF and SI treatments (Table 5). Our study contradicts previous studies reporting grain filling as the best stage for vegetation indices to correlate with GY under well-watered conditions when the leaf area index (LAI) had already decreased to values around 2 (Aparicio et al., 2000; Gutiérrez-Rodríguez et al., 2004). Casadesús et al. (2007) reported in durum wheat that NDVI and digital-image-derived indices measured at anthesis correlated positively with GY under severe water stress conditions (with LAI around 2), but failed to correlate under well-watered conditions. Our study agrees with Aparicio et al. (2000) and Ferrio et al. (2005) who reported anthesis as the best crop stage for GY prediction under RF conditions.

Also, the NGRDI measured at mid-tillering showed a positive correlation with AB under the RF treatment (Table 5), which suggests a role of early plant vigour in the final AB. Indeed, early vigour is a very important trait in rainfed Mediterranean conditions (Botwright et al., 2002; Casadesús and Villegas, 2014) because it prevents loss of water due to evaporation from the soil surface (Richards et al., 2011).

The superior GY prediction of NGRDI compared to NDVI may be due to differences in their formulation. Whereas the NDVI uses light reflected in the visible (VIS) (usually red) and near infrared (NIR) regions of the spectrum, the NGRDI is only formulated using the VIS region (Green and Red). Our results at early growth stages are in accordance with Hunt et al. (2005) who reported the efficiency of vegetation indices derived from VIS regions of the spectrum (using NGRDI) to detect variation between vegetation and soil at early growth stages. This contradicts the studies by Aparicio et al. (2002, 2004) who stressed the importance of NDVI and other vegetation indices derived from spectral reflectance in the VIS and NIR regions measured at early growth stages. The difference in reflectance between the NIR and VIS on bare soil is very small compared with the differences between the NIR and VIS spectra associated with green AB (Kipp et al., 2014). This may create problems when measuring NDVI at early stages of the crop cycle or under very severe stress conditions (which decrease the LAI) because there is a large amount of bare soil present (Aparicio et al., 2004; Kipp et al., 2014). However, the VIS region (Green and Red) used in the NGRDI calculation is characterized by high light absorption of the red and reflection of the green wavelengths due to the presence of chlorophyll in leaves. By contrast, NDVI uses spectral regions (red and NIR) that are absorbed to some extent by bare soil (Kipp et al., 2014). As a result, the NGRDI is able to assess AB better than the NDVI under conditions where bare soil is exposed at early growth stages.

The reasons why NDVI failed to assess GY and AB at anthesis under both water treatments (Table 5) may be different from the problems with measurements at early stages of the crop cycle. This is more closely related to saturation of the reflectance spectra in the red and the NIR regions due to a high LAI at anthesis, and this means that increases in leaf area do not result in a parallel increase in NDVI values (Aparicio et al., 2000; Casadesús et al., 2007; Ferrio et al., 2005). However, NGRDI does not saturate in the same way because it depends mainly on changes in pigment colour and therefore it is better at detecting differences in green AB and GY at anthesis. Different authors have reported that vegetation indices derived from the VIS region are more sensitive for detecting differences in green AB at high canopy densities (i.e. when no bare soil is exposed) than the NDVI, in spite of the small changes in leaf colour associated with senescence (Gitelson et al., 2002; Kanemasu, 1974; Motohka et al., 2010). Moreover, vegetation indices that only use the VIS region depend on the pigment's (mostly chlorophyll) reflectance characteristics, which are basically the same in all plant species (Gitelson et al., 2002). By contrast, NIR reflectance is more species-specific because it depends on many factors such as canopy architecture, leaf anatomy, cell structure, etc. (Gitelson et al., 2002; Kanemasu, 1974).

4.5. *The combined use of vegetation indices and stable isotopes to predict variations in GY*

The stepwise analysis (Table 6) confirmed that the $\delta^{13}C_G$ and the NGRDI do not account for the same portion of effect on GY. In fact, drought affects yield through a combined effect on plant growth and rate of photosynthesis (Du et al., 2013). Thus, the combination of a vegetation index as an indicator of plant growth together with the analysis of the stable carbon isotope signature in dry matter as a time-integrated indicator of photosynthetic and transpirative gas exchange may cover adequately the factors affecting GY performance under a given water regime. In our study both NGRDI at anthesis and $\delta^{13}C_G$ were chosen as the two significant variables of a stepwise model explaining 50–58% of the GY variability within each water treatment (Table 6). However, the order of both variables changed, with NGRDI at anthesis and $\delta^{13}C_G$ being the first variables chosen under RF and SI, respectively. This may reflect differences in environmental conditions, with plant growth being the most affected trait under the water stress conditions of RF. By contrast under SI, terminal (during grain filling) water stress is the main factor determining GY performance, whereas constitutive (i.e. in the absence of water stress) differences in plant growth that exist at anthesis have a minor role. Although the concept has been proposed (Araus and Cairns, 2014; White et al., 2012), to our knowledge the application of a combination of vegetation indices with stable isotopes signatures to capture differences in GY has not been reported in the literature.

For better understanding the mechanisms that could predict GY performance under contrasting water conditions, a path-analysis was run to model the relations between the best physiological traits informing on growth and photosynthesis with the agronomical yield components and ultimately GY (Fig. 2). Under RF, both the $\delta^{13}C_G$ (an indicator of photosynthetic and water status during the reproductive stage) and the NGRDI at mid-tillering (early vigour) had an indirect effect on the GY via the NGRDI at anthesis (photosynthetic area at anthesis), which subsequently affected NGE^{-1}. In fact, NGE^{-1} depends on the availability of assimilates going to the ear, which will determine the number and fertility of flowers and further grain set (Serrago et al., 2013). In addition, the NGRDI at mid-tillering informs on tiller density and thus on NE m^{-2} and then on GY, even if that yield component affects NGE^{-1} and TGW negatively. Although the positive effect of better growth increases the photosynthetic area from crop establishment to anthesis (higher NGRDI at mid-tillering and anthesis), and despite a better water status during the crop reproductive stage (lower $\delta^{13}C_G$) that increases the number of grains/ground area basis via higher NE m^{-2} and NGE^{-1}, both the NE m^{-2} and NGE^{-1} may negatively affect TGW. However, the overall positive effects of a higher NE m^{-2} and NGE^{-1} on GY are by far much stronger than TGW. The results of the simple correlations and the path-analysis showed that NGE^{-1} is the most important yield component contributing to GY under RF treatment, which is in accordance with previous studies in wheat under drought conditions (García del Moral et al., 2003; Simane et al., 1993).

The indirect effect of the NGRDI at mid-tillering on the GY via NE m^{-2} revealed the role of early plant vigour on the tillering ability of the plant and the consequent formation of fertile tillers (i.e. NE m^{-2}). We may conclude that under Mediterranean rainfed conditions early vigour (assessed through NGRDI at mid-tillering) had a positive effect on GY through a larger number of fertile ears and contributing to a higher photosynthetic area during the reproductive stage (further assessed through NGRDI at anthesis) of the crop (López-Castañeda et al., 1995; Richards et al., 2011). An improved water status during the reproductive stage had a positive effect on GY primarily through a larger photosynthetic area and a subsequent improvement in ear fertility (NGE^{-1}) (García del Moral et al., 2003; Simane et al., 1993). The potential effect of good water status increasing TGW was probably offset by the negative effect of NGE^{-1} on TGW (García del Moral et al., 1991, 2003; Simane et al., 1993).

Under SI treatment, early vigour (through NGRDI at mid-tillering) had no clear effect on GY, probably due to the good water conditions during March and April, which coincided with the second part of tillering and further stem elongation, allowing strong crop establishment. The water conditions during May, coinciding with the reproductive period, were less optimal than in previous months (the evapotranspiration was almost equal to the total water supply), which caused a mild water stress. Under such circumstances, plants exhibiting better water conditions during the reproductive stage (lower $\delta^{13}C_G$) experienced a direct positive effect on grain filling (i.e. increasing TGW). An indirect effect through a larger photosynthetic area during the reproductive stage (higher NGRDI at anthesis) is also involved. A larger photosynthetic area was positively related to a larger number of fertile tillers (higher NE m^{-2}), which affected GY positively despite the negative effect on the size of the ear (lower NGE^{-1}) and the grains (lower TGW).

4.6. *Conclusions and the way ahead*

The results of the present study showed that the $\delta^{13}C$ of mature grains is the best stable isotope to detect GY variation under both RF and SI treatments. Moreover, in agreement with previous studies, the use of $\delta^{18}O$ as a predictor of plant transpiration is not encouraging (Araus et al., 2013; Ferrio et al., 2007), probably due to miscellaneous factors other than the crop water status that affect the final $\delta^{18}O$ signature of assimilates, especially in the mature grains. The study also showed that a digital imaging-derived index (NGRDI) was better than the NDVI in detecting variability in GY and AB. Indeed, the combination of a digitally derived vegetation index with $\delta^{13}C_G$ may predict a substantial percentage of variability in GY. Moreover, path-analysis of these factors (including agronomical components) determining grain yield, modelled how physiological traits may predict GY performance under different water availabilities in a Mediterranean environment. Recent studies on different cereals under field conditions have reported the applicability of digital imaging-derived indices estimating very diverse crop traits such as the chlorophyll and nitrogen content, early vigour and the LAI, AB, GY. Digital imaging is considered a high throughput, easy-to-use monitoring technique, and thus a large number of experimental plots could be phenotyped in a short time (e.g. in our study 180 images of the trial plots were taken in less than 20 min and further processed in less than 10 min). Moreover, the low-cost of RGB imaging is related to the affordability of conventional digital cameras together with the availability of open-access software for image processing, making this approach most appropriate for precision agriculture and crop phenotyping, instead of using the more expensive multispectral cameras.

However, the practical integration of digital imaging as a monitoring tool needs some considerations. Among them, that the image quality from different commercial cameras can vary and may result in different information for the same canopy (Li et al., 2010). Also, the existence of exposure problems due to changes in light intensity over time under field conditions, which require a radiometric calibration of digital numbers into reflectance, a task that is frequently not easy (Gates, 1980; Hunt et al., 2005; Kipp et al., 2014; Zhou et al., 2013). In our study we did not undertake direct radiometric calibration for the Green and Red channels of the digital RGB camera because the wavelengths of these bands were not supported by the manufacturer of the digital camera. However, we suggest using the NGRDI values of the calibration panels at the mid-tillering stage to standardize the NGRDI values of the same panels during later growth stages. In addition, measuring the AB at critical crop stages (mid-tillering-anthesis and grain filling) that are characterized by clear differences in the AB quantity and leaf characteristics (e.g. colour and orientation), could compensate for error effects that result from the use of the non-radiometerically calibrated images.

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Chapter 5

Detecting terminal heat stress effects on maize biomass and grain yield by remote sensing techniques

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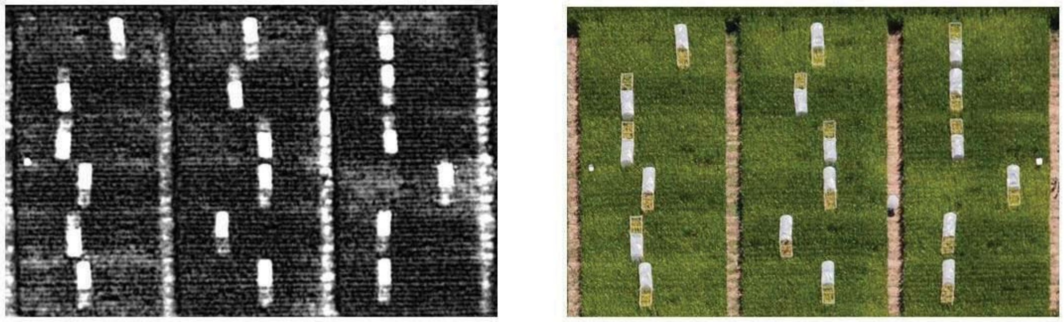
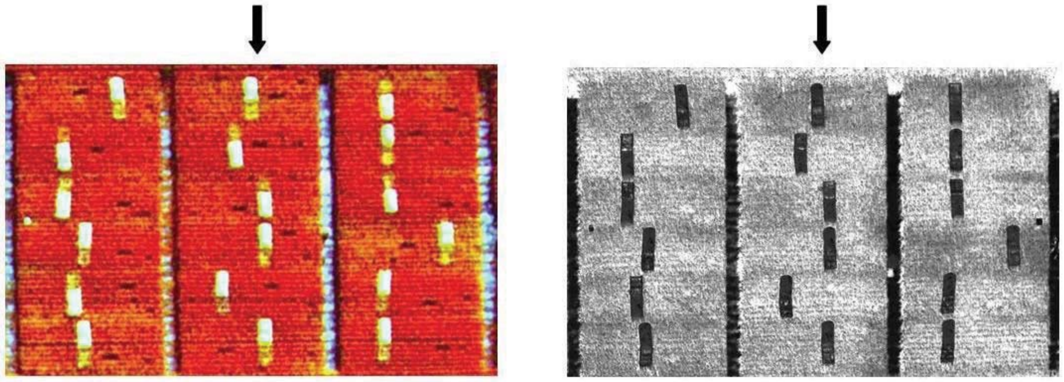


Red band image

Digital RGB image



False colour NDVI image

Gray scale NGRDI image

Spectral and Digital images analysis of maize field in Algerri, LLeida, Spain. 2011 and 2012 cycles

Resumen

El objetivo de este estudio ha sido comparar la potencialidad de dos técnicas diferentes de teledetección para estudiar los efectos del estrés térmico en condiciones de campo en sobre la biomasa aérea (AB) y el rendimiento de grano (GY) de un cultivo de maíz. Dicho trabajo se realizó en condiciones de campo durante dos temporadas consecutivas de cultivo. Se aplicaron dos tratamientos térmicos antes y después de la floración (pre y post-floración, respectivamente), además del tratamiento de control a temperatura ambiente. Se midieron dos índices de vegetación. Por un lado el NDVI, mediante una cámara multiespectral, y por el otro el NGRDI empleando una cámara digital RGB. Las imágenes fueron tomadas desde una avioneta tipulada. Ninguno de los dos índices pudo predecir el GY de las plantas con tratamiento térmico anterior a la floración debido al efecto del estrés por calor en el desarrollo de los granos, que no se pudieron detectar por los índices de vegetación diseñados, ya que éstos están diseñados para captar diferencias en dosel verde del cultivo. Por el contrario, tanto el NGRDI como el NDVI se correlacionaron positivamente con el GY y la AB en el tratamiento térmico posterior a la floración y en el control. Sin embargo, el NGRDI mostró, en ambos tratamientos, más correlación con la AB y el GY que el NDVI. Puesto que el NGRDI se basa únicamente en la reflectancia en la región visible (VIS, por sus siglas en inglés) del espectro (verde y rojo) sin depender de las regiones de infrarrojo cercanas (NIR, por sus siglas en inglés), ofrece mejores resultados que el NDVI. Esto se debe a que a diferencia del NDVI, el NGRDI no se satura a índices de área foliar elevados y fue más eficiente capturando pequeñas diferencias en el color de las hojas debidas a la senescencia de las mismas a causa del estrés térmico. Además, el NGRDI parece comportarse de manera más independiente que el NDVI respecto a variaciones interanuales en el estado del cultivo, por lo que el NGRDI fue capaz de predecir la AB y el GY mejor que el NDVI cuando se combinaron los datos de los dos años consecutivos de crecimiento. La determinación del GY y la AB mediante el análisis de imágenes digitales facilita una nueva herramienta potencial de bajo coste para evaluar el efecto de las condiciones de estrés en el maíz.

Detecting terminal heat stress effects on maize biomass and grain yield by remote sensing techniques

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Abstract

The objective of this study was to compare the performance of two different remotely sensed techniques in detecting the effects of heat stress under field conditions on final maize aerial biomass (AB) and grain yield (GY). The study was conducted under field conditions for two consecutive growing seasons. Two heat treatments were applied before and after flowering (pre- and post-flowering, respectively) in addition to the control treatment at ambient air temperature. Remote sensing measurements were taken with a multispectral band camera to measure the normalized difference vegetation index (NDVI) and a digital Red/Green/Blue (RGB) camera to measure the normalized green red difference index (NGRDI). Both indices failed to predict the GY of pre-flowering heat-treated plants due to grain set establishment problems that could not be detected by vegetation indices which are designed to capture differences in green canopy area. In contrast, both the NGRDI and the NDVI correlated positively with GY and AB in the control and post-flowering heat treatment. However, for both treatments the NGRDI exhibited higher correlations with AB and GY than the NDVI. Since the NGRDI is formulated based only on the reflectance in the visible regions (VIS) of the spectrum

(Green and Red) without dependence on the near infrared regions (NIR), it performs better than the NDVI. This is because it overcame the reported saturation patterns at high leaf area index and was more efficient at capturing even small differences in leaf colour due to the leaf senescence imposed by the heat stress. Also, the NGRDI seemed to be a more seasonally independent parameter than the NDVI, which is more affected by temporal variability within the field, and thus the NGRDI predicted AB and GY better than the NDVI when combining the data of the two growing seasons. Determination of GY and AB by digital image analysis offers a potential new low-cost tool to assess the effect of stress conditions in maize.

Key words: Carbon isotope composition, digital RGB images, multispectral images, vegetation indices, water indices

1. Introduction

Maize (*Zea mays* L.) is a major cereal crop for both livestock feed and human nutrition and it is grown in a wide range of agro-ecological environments throughout the world (http://www.iita.org/maize). The world production in 2012 was 872 million Mg with the largest producer, the United States, producing 31.40 %, and Ukraine, the primary European producer producing 2.40 %, while Spain produced 0.48 % (4.2 million Mg) of the world total production (FAO, 2012). Field crops are usually exposed to a series of abiotic stresses (e.g. drought, nitrogen deficiency, above optimal temperatures), which affects their production according to the timing, duration and intensity of the stress (Rattalino Edreira and Otegui, 2012). Heat stress due to high air temperatures is a serious threat to maize production and the problem will increase according to current climate change predictions (Cairns et al., 2013; Lobell et al., 2008, 2011). Raising the temperature above the optimal level for a period of time can cause irreversible damage to plant growth and development (Wahid et al., 2007). Air temperature will increase significantly over the next decades due to continuing emissions of greenhouse gases, whereas precipitation changes are far less certain (Hawkins et al., 2013). According to several reports, global warming is expected to increase the potential annual losses of up to 10 million Mg of maize, which would affect 140 million people in developing countries (Tassawar et al., 2007).

Heat stress, when occurring during reproductive stages, sharply decreases grain yield (GY) in maize. While about 50% of the final dry matter accumulation at maturity is gained during the post-silking period, with the majority of such accumulation taking place in the grain (Ning et al., 2013), heat stress also strongly affects final grain number (Cairns et al., 2012; Rattalino Edreira and Otegui, 2012, 2013). Indeed, the optimum temperature for grain development has been reported to lie between 27 and 32 ºC (Keeling and Greaves, 1990). A rise in temperature over the optimum encourages pollen shedding to start earlier, whereas silking is delayed, therefore increasing the anthesis-to-silking interval (ASI) to the point that the silking period no longer coincides with anthesis. Rises in temperature also reduce pollen viability and silk receptivity, therefore resulting in low grain number per ear and poor GY (Samuel et al., 1986; Schoper et al., 1987). The reduction in crop photosynthetic capacity during grain filling is the other dominant response of heat stress (with temperatures around or slightly above 40ºC), which affects grain yield negatively by accelerating leaf senescence due to loss of leaf chlorophyll (Chl) (Farooq et al., 2011; Hatfield et al., 2008; Hodgins and van Huystee, 1986; Peng and Gitelson, 2011; Prasad and Staggenborg, 2008; Rattalino Edreira and Otegui, 2012, 2013) and increasing N remobilization from leaves to feed the demand of N by the actively growing grains (Prasad and Staggenborg, 2008; Rajcan and Tollenaar, 1999). Moreover, temperatures above 37.5ºC inhibit photosynthesis and as temperatures increase to 45ºC the rate of photosynthesis may be inhibited by 95% (Berry and Bjorkman, 1980; Crafts-Brandner et al., 2002). As a consequence the availability of photosynthates to the grains decreases and this in turn affects GY (Prasad and Staggenborg, 2008). At the canopy level, the previously mentioned limitations cause a reduction in radiation use efficiency in terms of biomass production per unit of light intercepted by the canopy (Cicchino et al., 2010; Rattalino Edreira and Otegui, 2012).

Estimating leaf area index (LAI, the ratio of total leaf area per ground area) and the green leaf area index (GLAI, the ratio of green photosynthetic leaf area per ground area) is one way to assess the effect of heat (or any other stress that accelerates senescence) on potential canopy photosynthesis and thus GY (Lopes et al., 2011; Nguy-Robertson et al., 2012). However, direct estimation of GLAI has many limitations, being both time- and labour-intensive work that requires destructive sampling during the growth cycle (Babar et al., 2006). Leaf senescence can be assessed by destructive sampling of some leaves and further laboratory analysis of the chlorophyll (Chl) content or determined directly in the field using Chl meters (e.g. SPAD Chl meter) based on the evaluation of the vegetation index using the radiation transmitted through the leaf in the Red and near infrared (NIR) regions of the spectrum (Ling et al., 2011).

The problem with these approaches is that results are very dependent on the specific leaves sampled, so they require many measurements to obtain a representative average (Teal et al., 2006) and are thus considered time-consuming processes, which frequently prevents upscaling of the conclusions at the whole canopy level. An indirect, fast and low-cost alternative to assess GLAI, such as visual ranking is very dependent on staff training and subjectivity, sometimes making reproducibility low, particularly when plants are in reproductive stages (Gitelson et al., 2014). Other approaches such as, measuring light extinction through the canopy with a ceptometer is time-consuming and lacking in precision when GLAI is high (Facchi et al., 2010; Keane et al., 2005; Seidel et al., 2011).

During recent decades the use of remote sensing techniques has attracted much attention in monitoring the effect of growing conditions, and particularly of stresses, on photosynthesis capacity and yield of crops (Dobrowski et al., 2005; Hoyos-Villegas et al., 2014). Remote sensing techniques have many advantages, such as speed of measurement, application at the whole canopy level and the possibility to estimate several parameters simultaneously, such as photosynthetic capacity, LAI, GLAI and the total Chl content of the canopy (Araus et al., 2001; Babar et al., 2006; Gitelson et al., 2014). Spectroradiometrical techniques based on evaluating the radiation reflected by the canopy in the visible (VIS) and NIR regions of the spectrum is the most common approach (Araus and Cairns, 2014; Babar et al., 2006). It is based on the fact that absorption of specific wavelengths is known to be associated with distinct morphological and physiological characteristics reflecting crop growth (Svensgaard et al., 2014). Vegetation indices were the first spectral indices developed and are still the most utilized in agriculture (Araus and Cairns, 2014). Vegetation indices have been mostly used in precision agriculture as fast, instantaneous and non-destructive methods to determine crop N status (Filella et al., 1995; Roberts et al., 2011; Zhang et al., 2006), and thus are predictors of the potential yield increases from in-season N fertilizer application (Raun et al., 2001, 2002; Roberts et al., 2011; Teal et al., 2006).

The normalized difference vegetation index (NDVI), which uses the NIR and VIS (mostly Red) reflectance in its calculation is one of the most widely used vegetation indices for Chl and N canopy content, GLAI, aerial biomass (AB) and GY estimation (Casadesús et al., 2007; Nguy-Robertson et al., 2012; Raun et al., 2001, 2002; Teal et al., 2006). Moreover, in compared to spectroradiometers, the use of multispectral cameras allows assessment of within-field spatial variability in the NDVI, or other spectral indices, which may be more convenient in areas such as precision agricultural or crop phenotyping (Lan et al., 2010; Warren and Metternicht, 2005). However, the NDVI (as well as other vegetation indices) tends to show a saturation pattern at moderate to high GLAI values, which decreases the NDVI sensitivity in detecting variations in the above traits (Gitelson et al., 2002; Kanemasu, 1974; Nguy-Robertson et al., 2012).

The use of vegetation indices derived from the VIS region, such as the normalized green red differences index (NGRDI), which uses the Green and Red regions of the spectrum, has been proposed as an alternative to the NDVI in GLAI estimation in maize and other cereals. The main reason for this is the fact that the difference between the Green and Red reflectance is maintained at high GLAI, and thus the NGRDI is not prone to saturation, unlike the NDVI (Gitelson et al., 2002; Hunt et al., 2005, 2013; Kanemasu, 1974; Sakamoto et al., 2012a, b). The NGRDI has been used to estimate different traits such as canopy Chl content (leaf senescence), GLAI, LAI and AB (Gitelson et al., 2002; Hunt et al., 2005, 2013; Kanemasu, 1974; Sakamoto et al., 2012a, b). Another advantage of the NGRDI is that it can be obtained with conventional RGB cameras. The price and the ease of use of digital cameras compared with multispectral cameras means that the NGRDI is a viable alternative to the NDVI (Hunt et al., 2005, 2013; Sakamoto et al., 2012a, b). However, to the best of our knowledge, no studies reporting correlations between the NGRDI and GY have been published until now.

Heat stress may cause water stress, even under well-irrigated conditions, due to an increase in evapotranspiration (Farooq et al., 2011; Prasad and Staggenborg, 2008; Wahid et al., 2007). In that sense, different water indices derived from the reflectance in the NIR region between 850–970 nm have been proposed to inform on plant water status at the canopy because they can assess changes in relative water content, leaf water potential, and stomatal conductance due to the growing conditions (Babar et al., 2006; Peñuelas et al., 1993). Normalized water indices, such as the NWI-1 and the NWI-2, are among the most frequently used water indices. The lower the value of the NWI-1 or the NWI-2 the greater the amount of water content in the canopy and the better the water status of the crop (Babar et al., 2006).

For a long time the carbon isotope composition ($\delta^{13}C$) has been used successfully in C_3 cereals to detect GY variations in response to changing water conditions (Farquhar and

Richards, 1984; Farquhar et al., 1989) due to water stress. In the case of a C_4 plant like maize, variations in the $\delta^{13}C$ in response to water conditions are small (Henderson et al., 1999), but they are still adequate for use in maize as an indicator of water conditions during growth (Araus et al., 2010; Cabrera-Bosquet et al., 2009)

The objective of this study was to compare the performance of the spectral (NDVI) and digital (NGRDI) imaging approaches in assessing GY and AB at maturity in maize plants submitted to heat stress during the reproductive stage (before and after flowering). Also, the remote sensing parameters, the NWI-1 and the NWI-2, were measured and the $\delta^{13}C$ of mature grains analysed to detect whether the applied heat treatments were accompanied by water stress or not.

2. Materials and methods

2. 1. Plant materials and experimental conditions

Field experiments were conducted during the 2011 and 2012 growing seasons at Algerri, Lleida, NE Spain (41° 47′41′′ N; 0°38′52′′ E, 342 m above sea level). The soil was a mixture of Gypsic Xerochrept and Calcixerollic Xerochrept (SSS, 1990) with 1.5 % s.ms.s organic matter, electric conductivity of 0.21 dSm^{-1}, an alkaline pH of 8.2 in the upper 0.3 m, and total nitrogen content at 1 m of soil depth of 225 and 175 kg ha^{-1} in 2011 and 2012, respectively. The treatments consisted of a factorial combination of six treatments of nitrogen (N) fertilization and three temperature conditions. N treatments combined three doses [0, 100, 200 Kg N ha^{-1}] and two timings [at V4 and at 15 days before silking]. The temperature treatments consisted of a control (plots grown under natural temperature throughout the growing-season), high temperature during the critical period (Pre-flowering, from 7 d before silking to 9 d after silking) and high temperature during the effective grain filling period (Post-flowering, from 14 to 32 d after silking). The field experiments were arranged in a split-plot design with three replicates where the main plots were assigned to the six N treatments and the sub-plots to the temperature treatments (Fig. 1).

Two similar maize long cycle hybrids (FAO 700) were grown, Pioneer PR31N28 in 2011 and Pioneer PR33Y72 in 2012. Maize plants were sown in spring (31st and 20th of March 2011 and 2012, respectively) in 112 m^2 plots (8 rows of 20 m long, and 0.70 m apart) at a plant density of 84000 plants ha^{-1}. Heat treatments were applied by placing a transparent plastic cover mounted in a wooden frame of 3-3.5 m height and 1.5 m width x 1.5 m length over the crop and the plastic was removed at the end of each heating period (details in Ordoñez et al., submitted).

The maize crops were irrigated by a drip irrigation system and the soil water content was maintained near field capacity throughout the growing season. The plots were fertilized with phosphorus and potassium before sowing and protected from weeds, insects, pests and diseases by recommended agronomic practices. Climatic conditions during the 2011 and 2012 growing season are detailed in Table 1. Harvest of the different combinations of N and heat treatments was carried out between 22 August and 6 September for the 2011 growing season and between 30 August and 6 September for the 2012 growing season.

2. 2. Agronomic measurements

Aerial biomass (AB), grain yield (GY) and GY components were determined by destructive sampling at maturity (detected by black layer formation). Two rows of 1 m in length per single row; *i.e.* 1.4 m^2 including *c.* 11-12 plants per experimental unit per sample were harvested to assess the number of grains per plant (NGP) and hundred grain weight (HGW). AB and GY were determined for each plot by mechanical harvesting and the harvest index (HI) was calculated as the ratio of GY to AB x 100.

2. 3. Spectral and digital imaging

Spectral and digital RGB images were captured twice in each of the two growing seasons directly after the termination of the pre-flowering (9 days after silking) and post-flowering (32 days after silking) heat treatments. The spectral and digital RGB cameras were mounted in a hole in the bottom of a manned aircraft, and the images were taken at ≈ 80 m above the ground surface. The spectral and digital images were taken simultaneously at solar noon (between 11 a.m. and 1 p.m.).

Spectral images were taken using a 6-band multispectral camera (Mini MCA-6, Tetracam, Inc., California, USA). The camera consisted of six independent image sensors and optics with user-configurable spectral filters. The image had a resolution of 1280 × 1024 pixels with 10-bit radiometric resolution and an optical focal length of 9.6 mm, yielding an angular FOV of 55.50 m × 44.33 m and 4.33 cm pixel spatial resolution at 80 m flight altitude. Six 25 mm diameter band pass filters of 10 nm FWHM (Andover Corporation, NH, USA), with centre wavelengths at 450 nm, 670 nm, 780 nm, 860 nm, 900 nm, and 950 nm bands were mounted in the multispectral image camera. The multispectral image camera was calibrated using the empirical line method (Smith and Milton, 1999) by placing 1.5 m x 1.5 m black and white targets in a location within the field and captured in the same image used for the trial analysis. Field spectral measurements were taken on the calibration targets with a narrow-bandwidth visible/near infrared portable field spectroradiometer (model FieldSpec UV/VNIR, Analytical Spectral Devices, Inc., Boulder, CO, USA) at the same time as the spectral and digital images were captured.

Digital images were taken using a Canon EOS 5D Mark III digital RGB camera with a Canon EF 24–70mm lens set at the maximum focal length. The shutter speed and the aperture were left automatic. Images were taken without flash. The image size was 3840 x 2560 pixels, and was saved in a JPG format. The wavelengths of the digital RGB camera bands were not available from the manufacturer. The NGRDI measured values of the black and white targets of the first growing season (2011) were used in the calibration of the NGRDI values of the same targets in the second growing season (2012).

Analyses of the spectral and digital images were performed using ImageJ 1.46r, a public-domain Java image processing and analysis program (http://imagej.nih.gov/ij/) created by the National Institutes of Health (US Department of Health & Human Services). It runs on any computer with a Java 1.5 or later virtual machine. An example of the processed images is shown in Fig. 1.

Three parameters were extracted from the multiband spectral images: the normalized difference vegetation index (NDVI) informing the vegetation status, and the normalized water index 1 (NWI-1) and the normalized water index 2 (NWI-2), both informing on the water status of the plant.

The NDVI was measured using the Red (670 nm) and the NIR (780 nm) bands according to Raun et al. (2001):

$$\mathrm{NDVI} = [(R_{780}-R_{670})/(R_{780}+R_{670})] \quad (1)$$

The 860 nm, 900 nm and 950 nm NIR bands were used for the calculation of the NWI-1 and the NWI-2 according to Babar et al. (2006):

$$\mathrm{NWI\text{-}1} = [(R_{950}-R_{900})/(R_{950}+R_{900})] \quad (2)$$

$$\mathrm{NWI\text{-}2} = [(R_{950}-R_{860})/(R_{950}+R_{860})] \quad (3)$$

The colour channel information (digital numbers) of the three main colour channels (Red, Green and Blue) was extracted from the image taken by the digital RGB camera. Then a digital index equivalent to the NDVI (informing on the vegetation status) called the normalized green red difference index (NGRDI) was calculated according to Hunt et al. (2005):

$$\mathrm{NGRDI} = [(\mathrm{Green}-\mathrm{Red})/(\mathrm{Green}+\mathrm{Red})] \quad (4)$$

2. 4. Chlorophyll content

Leaf chlorophyll (Chl) content was measured simultaneously with aerial digital and spectral measurements after the termination of the pre-flowering and the post-flowering heat treatments to assess whether the applied heat treatments caused plant senescence. The measurements were performed on the flag leaves of the 11-12 plants per experimental unit (the same plants were used later to assess the agronomic components). Measurements were performed indirectly using a portable chlorophyll meter (Minolta SPAD 502 Meter, Plainfield, IL, USA). Four measurements were performed per leaf in the middle portion of the leaf, avoiding leaf veins because their thickness and paleness can affect SPAD readings.

2. 5. Stable isotope analysis

Samples of mature grains (1 kg per plot) were collected at harvest. Dry matter was finely milled and samples of ~1 mg were weighed into tin capsules for $^{13}C/^{12}C$ ratio analysis.

The stable carbon isotope analysis of mature grains was conducted at the Scientific Facilities of the University of Barcelona. Analyses were performed using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Schwerte, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan), operating in continuous flow mode. The $^{13}C/^{12}C$ ratios (R) of plant material were expressed in δ notation (Coplen, 2008), as carbon isotope composition ($\delta^{13}C$):

$$\delta^{13}C(‰) = [(R_{sample}/R_{standard})-1] \times 1000 \quad (5)$$

where sample refers to plant material and standard to Pee Dee Belemnite (PDB) calcium carbonate. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 3, polyethylene foil; IAEA CH6, sucrose; USGS 40, L-glutamic acid) were used with an analytical precision of 0.1‰.

2. 6. Statistical analysis

Combined analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure to calculate the effects of growing seasons (GS), heat treatments (T) and the growing season by heat treatment interaction (GS x T) on the studied traits. The N treatments were not used as a factor in the combined ANOVA, and thus each heat treatment was represented by 18 experimental units (six N treatments by three replicates) to ensure a wide range of growth and yield variability in each heat treatment. Mean separation of the heat treatments for the different parameters was performed by Duncan's multiple range test ($P < 0.05$).

Relationships between parameters were analysed in each heat treatment (control, pre-, and post-flowering), in the two growing seasons separately and with the combined data of both seasons using Pearson linear correlation.

Stepwise multiple linear regression was used to determine the independent variables (vegetation indices, water indices and stable carbon isotope composition of mature grains) accounting for the majority of variability in the dependent variables (AB and GY) for each of the three treatments (control, pre-flowering and post-flowering heat treatments) using the combined data of the two studied growing seasons.

Both NWI-1 and NWI-2 were measured in 2011 only once at 32 days after silking, while in 2012 they were measured simultaneously with the vegetation indices twice, at 9 days and 32 days after silking, respectively. For that reason the combined analysis of the two growing seasons was only undertaken with the measurements at 32 days after silking. The NWI-1 and NWI-2 data of 2012 are presented as supplementary material (Supplementary Tables 1 and 2).

Data were analysed using the SPSS 16 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using SigmaPlot 11.0 for Windows (Sysat Software Inc., Point Richmond, CA, USA).

3. Results

3. 1. The effect of growing season and heat treatment on the growth parameters

The growing season effects were significant for grain yield (GY), harvest index (HI) and number of grains per plant (NGP), while it was not significant for the aerial biomass (AB), hundred grains weight (HGW) and the leaf chlorophyll (Chl) content (Table 2). The heat treatments were significant for all the studied growth parameters, where the pre-flowering heat treatment had the lowest AB, GY, HI, NGP and HGW and the control treatment had the highest AB, GY and HGW, and both the control and post-flowering heat treatment did not differ significantly in HI and NGP. Also, the applied heat treatments (pre-flowering and post-flowering) significantly decreased the Chl content compared with the control plants (Table 2). The post-flowering heat treatment 32 days after silking had the lowest Chl content, the pre-flowering heat treatment maintained the same Chl content after the stress application (similar Chl content at both 9 and 32 days after silking), while in the control treatment Chl content decreased from 9 days after silking to 32 days after silking. The growing season by heat treatment interaction was not significant for any of the growth parameters except HGW.

3. 2. The effect of growing season and heat treatment on the water status and vegetation indices

Significant effects existed during the two growing seasons for all the studied traits except for the NGRDI measured at 9 and 32 days after silking. The applied heat treatment effects were significant for almost all of the studied traits except for the normalized water index 1 (NWI-1) and normalized water index 2 (NWI-2). The pre-flowering heat treatment had the lowest NDVI and NGRDI values at 9 and 32 days after silking, while the control treatment at 32 days after silking had the highest NDVI and NGRDI values and both the control and the post-flowering heat treatments did not differ significantly at 9 days after silking. The growing season by heat treatment interaction was not significant for almost all studied traits except for the NDVI at the different crop developmental stages studied and the carbon isotope composition ($\delta^{13}C$) of the grains.

3. 3. Relationship of the vegetation indices and water status with AB and GY

For the 2011 growing season, AB was positively correlated with the NDVI (1) (r^2 = 0.66, $P < 0.01$; and r^2 = 0.27, $P < 0.05$ for control and post-flowering heat treatments, respectively, Fig. 2a), and with the NGRDI (1) (r^2 = 0.68, P < 0.01; and r^2 = 0.29, P < 0.05 for control and post-flowering heat treatments, respectively, Fig. 2b). Also, GY was positively correlated with the NDVI (1) (r^2 = 0.79, P < 0.01; and r^2 = 0.30, P < 0.05 for control and post-flowering heat treatments, respectively, Fig. 4a), and with the NGRDI (2) (r^2 = 0.82, P < 0.01; and r^2 = 0.30 for control and post-flowering heat treatments, respectively, Fig. 4b).

For the 2012 growing season, AB was again positively correlated with the NDVI (1) (r^2 = 0.64, P < 0.01: and r^2 = 0.45, P < 0.01 for control and post-flowering heat treatments, respectively, Fig. 2c), and mores strongly with the NGRDI (1) (r^2 = 0.77, P < 0.01; and r^2 = 0.55, P < 0.01 for control and post-flowering heat treatments, respectively, Fig. 2d). Also, GY was positively correlated with the NDVI (1) (r^2 = 0.69, P < 0.01; and r^2 = 0.35, P < 0.01 for control and post-flowering heat treatments, respectively, Fig. 4c), and more strongly with the NGRDI (1) (r^2 = 0.76, P < 0.01; and r^2 = 0.38 for control and post-flowering heat treatments, respectively, Fig. 4d).

Combining the data of both growing seasons, AB was also positively correlated with the NDVI (1) (r^2 = 0.25, P < 0.01; and r^2 = 0.13, P < 0.05 for control and post-flowering heat treatments, respectively, Fig. 2e), and again more strongly with the NGRDI (1) (r^2 = 0.71, P < 0.01; and r^2 = 0.33, P < 0.01 for control and post-flowering heat treatments, respectively, Fig. 2f). Also, GY correlated negatively with the NDVI (1) (r^2 = 0.20, P < 0.05 for pre-flowering heat treatment, Fig. 4e), and far stronger positive correlations were found with the NGRDI (1) (r^2 = 0.76, P < 0.01; and r^2 = 0.31, P < 0.01 for control and post-flowering heat treatments, respectively, Fig. 4f).

For the 2011 growing season, AB was positively correlated with the NDVI (2) (r^2 = 0.45, P < 0.01 for control treatment, Fig. 3a), and even more strongly with the NGRDI (2) (r^2 = 0.47, P < 0.01; r^2 = 0.29, P < 0.05; and r^2 = 0.33, P < 0.01 for control, pre-flowering, and post-flowering heat treatments, respectively, Fig. 3b). Also, GY was positively correlated with the NDVI (2) (r^2 = 0.41, P < 0.01 for control treatment, Fig. 5a), and again in a stronger manner with the NGRDI (2) (r^2 = 0.49, P < 0.01; and r^2 = 0.47, P < 0.01 for control, and post-flowering heat treatments, respectively, Fig. 5b).

For the 2012 growing season, AB was again positively correlated with the NDVI (2) (r^2 = 0.76, P < 0.01: and r^2 = 0.42, P < 0.01 for control and post-flowering heat treatments, respectively, Fig. 3c), and in a similar way with the NGRDI (2) (r^2 = 0.76, P < 0.01; r^2 = 0.20, P < 0.05; and r^2 = 0.46, P < 0.01 for control, pre-flowering and post-flowering heat treatments, respectively, Fig. 3d). Also, GY was positively correlated with the

NDVI (2) ($r^2 = 0.68$, $P < 0.01$; and $r^2 = 0.47$, $P < 0.01$ for control and post-flowering heat treatments, respectively, Fig. 5c), and to a slightly stronger degree with the NGRDI (2) ($r^2 = 0.73$, $P < 0.01$; $r^2 = 0.22$, $P < 0.05$; and $r^2 = 0.47$, $P < 0.01$ for control, pre-flowering and post-flowering heat treatments, respectively, Fig. 5d).

Combining the data of both growing seasons, AB was also positively correlated with the NDVI (2) ($r^2 = 0.20$, $P < 0.01$ for control heat treatment, Fig. 3e), and again more strongly with the NGRDI (2) ($r^2 = 0.53$, $P < 0.01$; $r^2 = 0.18$, $P < 0.01$; and $r^2 = 0.35$, $P < 0.01$ for control, pre-flowering and post-flowering heat treatments, respectively, Fig. 3f). Also, GY showed positive correlation with the NGRDI (2) ($r^2 = 0.34$, $P < 0.01$; and $r^2 = 0.37$, $P < 0.01$ for control and post-flowering heat treatments, respectively, Fig. 5f), while no significant correlations were detected between GY and the NDVI (2).

The NWI-1 (2) and NWI-2 (2) did not show any significant correlations with AB or GY under any heat treatment in the both studied growing seasons and also the NWI-1 (1) and NWI-2 (1) in the 2012 growing season (Supplementary Table 2). For $\delta^{13}C$, only the post-flowering heat treatment showed weak but significant negative correlations with GY in the 2011 growing season ($r^2 = 0.24$, $P<0.05$) (Supplementary Table 2).

A stepwise analysis was performed combining the data of both growing seasons, having either GY or AB as a dependent variable and all the water indices, vegetation indices and $\delta^{13}C$ as independent variables. Irrespective of the heat treatment the NGRDI was always the first variable to be chosen by the model to explain the majority of variations in AB and GY (Table 4). Overall, the stepwise analysis revealed that the NGRDI (1) was the most important variable explaining variations in AB and GY in the control treatment, whereas the NGRDI (2) was the most important variable explaining variations in AB in the pre-flowering heat treatment and AB and GY in the post-flowering heat treatment. The stepwise analysis was unable to produce a significant model describing variations in GY in the pre-flowering heat treatments using the previously mentioned independent variables.

3. 4. Relationship of the vegetation indices to chlorophyll content

Data presented in Table 5 showed that neither the NDVI nor the NGRDI associated significantly with the leaf Chl content in the pre-flowering heat treatment, except in the case of the NGRDI (1) during 2012 growing season ($r^2 = 0.67$, $P < 0.01$). Overall, the NGRDI was significantly associated (positively) with the leaf Chl content in both the control and the post-flowering heat treatment in the two growing seasons, especially at

32 days after silking, while the NDVI showed significant positive associations with Chl content only in the control treatment but did not show any significant associations with the post-flowering heat treatment.

4. Discussion

4. 1. The effect of heat stress on AB, GY and GY components

Heat stress applied before flowering (pre-flowering heat treatment) had a strong effect of diminishing aerial biomass (AB) as well as grain yield (GY) and harvest index (HI). The two latter traits were affected mainly by a decrease in the number of grains per plant (NGP) and to a lesser extent in the hundred grain weight (HGW) (Table 2). This sharp decrease in AB and GY appeared to be associated not only with an accelerated leaf senescence (check Chl content Table 2) and the resultant decrease in biomass accumulation due to the decrease in assimilatory capacity (Farooq et al., 2011; Prasad and Staggenborg, 2008: Rattalino Edreira and Otegui, 2012, 2013; Wahid et al., 2007) but also with other limiting factors that are not directly related to assimilate availability per plant. Heat stress can also reduce GY due to a decline in HI (Rattalino Edreira and Otegui, 2012) and this response usually occurs around flowering and is linked to a reduction in the number of grains set (Prasad et al., 1999; Wahid et al., 2007). In the present study the silks of the pre-flowering heat-treated plants were hand pollinated with pollen from the control (non heat-treated) plants for granting adequate pollination (Cicchino et al., 2010; Rattalino Edreira et al., 2011), and thus avoiding the expected reduced pollen viability reported after heat stress (Schoper et al., 1987). However, the NGP, HI and the resultant GY did not improve. Actually, the pre-flowering heat treatment was applied during the critical period of the plant cycle (7 days after silking to 9 days after silking), which is characterized by the establishment of the number of grains and the potential grain size (Maddonni et al., 1998). Thus, the negative effects of the heat stress reducing silk receptivity and/or increasing grain abortion may be the main reasons involved in the low grain number per ear, and thus poor GY (Samuel et al., 1986; Wahid et al., 2007: Rattalino Edreira and Otegui, 2012, 2013; Prasad et al., 1999). In fact, the weaker correlations of the NGRDI and the NDVI with AB and GY (Figs. 2-5) in the pre-flowering heat treatments compared with the post-flowering heat treatment are mainly due to the increased effect that the applied stress has on diminishing the final grain number (Table 2) under the former treatment. Thus, this reduction in grain set is associated with a decrease in reproductive sink strength for assimilates (Rattalino Edreira and Otegui, 2012). Such an effect is not well captured by the vegetation indices, which inform more on the effect on potential canopy photosynthesis rather than grain set.

Heat applied after flowering (post-flowering heat treatment) caused accelerated canopy senescence during grain filling (check Chl content Table 2), and thus led to a decrease in the HI, GY and AB (Farooq et al., 2011; Prasad and Staggenborg, 2008: Rattalino Edreira and Otegui, 2012, 2013; Wahid et al., 2007). In this treatment the diminishment in GY and HI was related to a decrease in HGW and to a lesser extent in NGP (Table 2). Overall, post-flowering heat treatment had a much less negative affect on HI, GY and AB than pre-flowering heat treatment. The post-flowering heat treatment leaf senescence was delayed several weeks compared with the pre-flowering heat treatment. Thus, the reduced negative effect of the post-flowering heat treatment may have been due to the fact that flowering had already taken place, so reproductive processes were much less affected and the canopy photosynthesis was maintained for longer during the critical period of grain filling, where the number of grains and the potential grain size were established (Maddonni et al., 1998; Rattalino Edreira and Otegui, 2012, 2013; Wahid et al., 2007). This delay in stress application enabled the plants to store enough assimilates during the critical period of grain filling. These stored assimilates support the demand of newly active grains when the source strength decreases due to increased leaf senescence (Gambín et al., 2008; Maddonni et al., 1998; Rattalino Edreira and Otegui, 2012).

4. 2. The effect of heat stress on plant water status

Increased canopy temperature can affect leaf water status, including the relative water content (Farooq et al., 2011; Prasad and Staggenborg, 2008; Wahid et al., 2007). The negative effect of high temperature on plant water status may even occur under fully irrigated conditions because the increased evapotranspirative demand may exceed the water uptake capacity of the plant (Prasad and Staggenborg, 2008). There was no significant difference for the normalized water index 1 (NWI-1) or the normalized water index 2 (NWI-2) in the different applied heat treatments in the two studied growing seasons at 32 days after silking (Table 3) or at 9 days after silking in the 2012 growing season (Supplementary Table 1). Likewise, no significant correlations were detected between either indices and AB and GY in both growing seasons for the different crop developmental stages studied (Supplementary Table 2). These factors suggested that no differences in water status existed at the canopy level. The carbon isotope composition of mature grains ($\delta^{13}C$) was more efficient than the NWI-1 and NWI-2 in detecting differences in growing conditions between treatments (Table 3). However, a significantly negative, but weak, correlation of grain $\delta^{13}C$ with GY was only observed in the 2011 season (Supplementary Table 2). Nevertheless, the $\delta^{13}C$ from the control was the most negative (Table 3), which does not support a poorer water status in the heat treatments, where greater levels of stress are expected to result in a more negative $\delta^{13}C$ signal in mature grains than the non-stress conditions (Araus et al., 2010; Cabrera-Bosquet et al., 2009; Henderson et al., 1999). In fact, the less negative $\delta^{13}C$ in the heat treatments may be the consequence of grains being filled from assimilates produced before grain filling, when evapotranspirative demand is smaller and less prone to some degree of atmospheric water stress. In summary, in our study both the remotely sensed water indices and the $\delta^{13}C$ of mature grains do not support the occurrence of water stress associated with the heat treatments.

4. 3. The effect of heat stress on the vegetation indices

Regardless of the growing season, in general the applied heat treatments in the different crop developmental stages decreased both the NDVI and the NGRDI values compared with the control plants (Table 3 and Supplementary Table 1). This could be due to the fact that applied heat stress affected the assimilatory capacity of the plant through enhanced leaf senescence (Chl loss). Thus both the pre-flowering and post-flowering heat treatments caused loss of the active reflecting surface and showed lower vegetation index values than the control plants (Table 3 and Supplementary Table 1). The Chl content data suggests accelerated leaf senescence after the pre-flowering and post-flowering heat treatments application (Table 2). The reduction in the NDVI and the NGRDI of the pre-flowering heat treatment was higher than in the post-flowering heat treatment (Table 3 and Supplementary Table 1). However, the reduction in AB in the pre-flowering heat-treated plants was mainly due to reduction in GY and not in stover biomass (SB, the difference between AB and GY). Thus SB was always significantly higher than the post-flowering heat treatment and similar to the control treatment (Supplementary Table 1), and also the pre-flowering heat treatment kept the same the NDVI and the NGRDI values (same spectral signals) from the termination of the pre-flowering heat stress (9 days after silking) until the termination of the post-flowering heat stress (32 days after silking). Although LAI or GLAI were not measured in this study, an explanation for this could be that plants exposed to pre-flowering heat treatment did not use a great deal of the stem assimilates stored before stress application to feed newly formed grains because the heat stress greatly decreased NGP and HGW, and thus kept a high SB weight (Maddonni et al., 1998). On the other hand, plants exposed to post-flowering heat treatments may depend more on the assimilate reserves stored before the stress application because the heat stress is known to shorten the grain filling period due to accelerated leaf senescence (Rattalino Edreira and Otegui, 2012). As a consequence stem reserves are used (at least in part) to feed the newly developed grains (Rajcan and Tollenaar, 1999). Also, the post-flowering heat treatment resulted in the significantly lowest Chl content after the stress application (32 days after silking), whereas both the control and the pre-flowering heat-treated plants did not differ significantly in Chl content (Table 2).

4. 4. Annual variability and relationship of vegetation indices to AB and GY

Irrespective of the treatment both the NDVI and the NGRDI were lower in the 2012 than in the 2011 growing season (Supplementary Table 1). A set of miscellaneous factors are known to cause variations in the maize productivity and thus in the spectral signal from year to year (Henik et al., 2012; Liu and Wiatrak, 2012; Raun et al., 2001, 2002).

This variation could result from the inter-annual variability in environmental and agronomic conditions within the field together with the fact that different hybrids were grown each year. Indeed, the initial nitrogen (N) content at 1 m of soil depth was 225 and 175 kg ha^{-1} in 2011 and 2012, respectively. As a consequence, SB and the vegetation indices were higher in 2011 than in the 2012 growing season (Supplementary Table 1).

Whereas heat after flowering affects GY mostly through an accelerated senescence of the crop canopy, heat before flowering also has a direct effect on grain set (Samuel et al., 1986; Rattalino Edreira and Otegui, 2012, 2013). Because the vegetation indices are designed to capture differences in the photosynthetic apparatus (canopy green area) they are better at assessing the effect of post-flowering heat treatment on GY and even on AB than that of the pre-flowering heat treatment (Figs. 2-5). However, the NGRDI (2) showed weak but significant correlations with AB and to a lesser extent with GY (Fig 3 b, d, f and Fig. 5d, respectively). Actually, these correlations were mainly due to the effect of a few plants (2 or 3 outliers) that were able to produce a reasonable GY under the pre-flowering heat treatment.

NGRDI correlated better with AB and GY than the NDVI, regardless of the heat treatment and growing season (Figs. 2-5). The reason behind the superiority in performance of the NGRDI over the NDVI is mainly due to the difference in the spectral regions used in the formulation of both of the indices. The NDVI is known to be prone to saturation at moderate to high GLAI values (Gitelson et al., 2002; Kanemasu, 1974; Nguy-Robertson et al., 2012). The reported saturation patterns of the NDVI are mainly due to the fact that for a GLAI of 3, the total absorption of the Red radiation by a canopy is already around 95% of the total incident Red radiation. Further increases in GLAI will not affect the absorption and reflectance pattern of the Red radiation (Nguy-Robertson et al., 2012), while the NIR reflectance is levelled or even decreased at high GLAI (Gitelson et al., 2002). As a consequence the rate of change in the reflectance of both the Red and the NIR radiation in response to further increases in GLAI decreases and they become close to being invariant to any further increases in canopy density (Gitelson et al., 2002; Kanemasu, 1974). On the contrary, the NGRDI which uses the Green and Red reflectance in its formulation is not prone to this saturation pattern because at high plant density the Green reflectance is reported to be greater than the Red reflectance (around three times) (Gitelson et al., 2002; Kanemasu, 1974) and thus the contrast between both reflectance regions is maintained at high GLAI.

A set of additional factors may also affect the vegetation indices that use the NIR reflectance in their formulation, due to an artefactual decrease in the measured NIR reflectance signal. Among these factors it is worth mentioning the canopy architecture, where the vertical leaf orientation encourages more radiation to be trapped by the canopy, increasing the plant light absorption and hence decreasing the NIR reflectance (Gitelson et al., 2002; Suits, 1972). The decrease in actively reflecting leaf layers due to Chl loss from the lower leaves at certain stages of plant growth (i.e. before tassel extrusion in maize), results in a decrease in NIR reflectance (Borrás et al., 2003; Gitelson et al., 2002; Leamer et al., 1978). Finally, the increase in soil moisture is known to decrease NIR reflectance, which may result in a decrease of the NIR reflectance from the canopy (Daughtry et al., 1980; Gitelson et al., 2002; Huete et al., 1985; Kanemasu, 1974).

In contrast, the NGRDI was able to efficiently detect the rapid leaf colour changes from green to yellow or red due to the Chl loss induced by the applied heat stress. The significant (moderate to strong) positive associations between the NGRDI and the leaf chlorophyll (Chl) content (Table 5), especially when measured at 32 days after silking, confirmed the same idea. In fact, the NGRDI is only sensitive to changes in the visible range (VIS) of the spectrum because this index uses the Red and Green regions. Therefore, the NGRDI is only affected by the high light absorption of the Red and the high reflectance of the Green radiation due to the presence of chlorophyll, whereas this index does not take into account the NIR reflectance, which (as indicated above) is affected by other factors such as canopy structure, cell structure and soil moisture (Gitelson et al., 2002; Gitelson and Merzlyak, 1997). Also, the NIR reflectance is reported to not be sensitive to the presence or absence of the chlorophyll pigments (Gitelson el al., 2002; Knipling, 1970).

The NDVI calculations for all targets (oceans, plants, bare soils etc.) always result in a number that ranges from 0 to +1 with the active vegetation close to +1 (0.8 or 0.9), while non-green leaves and bare soils can give values close to 0 (usually around 0.1) (Araus et al., 2001; Weier and Herring, 2000). Thus, the actual variation in the NDVI is high within plant targets. The NGRDI calculations result in a number that ranges from -1 to +1 for all targets, but the actual variation in the NGRDI is small for most plant targets (Hunt et al., 2005). Data presented in Supplementary Table 1 shows that the NDVI (1) values of the 2011 growing season were higher than for the 2012 growing season by 0.13, 0.21 and 0.13 for the control, pre-flowering and post-flowering heat treatments, respectively and for the NDVI (2) were higher by 0.13, 0.21 and 0.05 for the control, pre-flowering and post-flowering heat treatments, respectively. However the NGRDI (1) values of the 2011 growing season were higher than the 2012 growing season only by 0.01, 0.01 and 0.02 for the control, pre-flowering and post-flowering heat treatments, respectively and for the NGRDI (2) were higher by 0.02, 0.02 and 0.01 for the control, pre- and post-flowering heat treatments, respectively. Thus, when combining the data from the trials of both growing seasons the correlations of the NDVI with AB and GY were lower than the correlations of each growing season when studied separately, while the NGRDI maintained these correlations when the data of both growing seasons were combined (Figs. 2-5). Also, the results of the ANOVA (Table 3) confirmed that the effect of the growing season was highly significant for both the NDVI (1) and (2) ($P<0.001$) while it was not significant for either the NGRDI (1) or

(2), and also the interaction between growing season and heat treatment was highly significant for the NDVI (1) and (2) ($P<0.001$) but it was not significant for the NGRDI (1) and (2). Moreover, the stepwise analysis combining the data of the two studied growing seasons showed that the NGRDI was the first variable in all cases to be chosen by the model to explain much of the variability in AB and GY irrespective of the applied heat treatment (Table 4). Therefore, besides being a more efficient index than the NDVI in predicting variations in AB and GY, the NGRDI also appeared to be more growing-season independent than the NDVI, which exhibited large variations from one season to another. As a consequence, the NGRDI is a better predictor of AB and GY than the NDVI across the multi-season (multi-environment) trials.

Defining the crop stage when GY components are more susceptible to stress conditions is important for guiding breeding programs and management practices aimed at increasing GY (Gambín et al., 2008). The present study showed that regardless the growing season, both the NDVIs (1 and 2) showed in most cases correlations with AB and GY that were a little bit smaller but close to those of the NGRDI in the control treatment and the post-flowering heat treatment (Figs 2-5) except the NDVI (2) in 2011 in the post-flowering heat treatment (Figs 3a and 5a) where the relationships with GY and AB were not significant. Also, the NGRDI (2) was the only vegetation index to show correlations with AB and to a lesser extent with GY in the pre-flowering heat treatment (Fig. 3 b, d, f and Fig.5d). It could be concluded that the NGRDI is a better indicator of AB and GY than the NDVI when the leaf senescence patterns develop at the end of the growing seasons, either under normal senescence or as a response to heat during grain filling. The data presented in Table 5 confirms this, where the NGRDI (2) is the only vegetation index to correlate significantly with Chl content in the post-flowering heat treatment irrespective of the growing season. In addition, the NGRDI (1) was the only vegetation index to show correlations with Chl content in the pre-flowering heat treatment in 2011. Previous studies in different crop species (Gitelson et al., 2002; Kanemasu, 1974) have suggested that AB is best assessed using vegetation indices formulated using only light reflected in the visible range of the spectrum: (1) the Blue and the Red; (2) the Green and the Red. Also, the reflectance contrast among the visible bands is much smaller than between the VIS and the NIR band, but it is enough to detect differences related to senescence patterns as previously described.

4.5. Conclusions and the way ahead

Recent studies have already stressed the usefulness of digital RGB images to assess the chlorophyll and nitrogen content of individual leaves of maize plants in greenhouse trials (Rorie et al., 2011). Moreover, a recent study done by Hunt et al. (2013) revealed that the digital imaging-derived indices were better than other spectroradimetrical and satellite imaging for detecting leaf chlorophyll content in maize fields treated with different N applications (Hunt et al., 2013). Also, Sakamoto et al. (2012 a, b) have reported on the applicability of digital imaging-derived indices in estimating the LAI, GLAI and green leaf biomass (GLB) in maize in field trials. However, our study is the first that shows the possibilities of an index based in the use of digital RGB images to assess the effect of stresses like heat on GY. The study showed that the NGRDI was better than the NDVI in detecting variability in GY and AB under the control and post-flowering heat treatment in the different crop developmental stages. Also, the NGRDI performs in a steadier manner compared with the NDVI when the data of two growing seasons are combined. However, both the NDVI and the NGRDI were unable to predict GY variations under pre-flowering heat treatment due to grain set establishment problems that could not be detected by vegetation indices which are designed to detect differences in the photosynthetic apparatus (canopy green area). The superior performance of the NGRDI is mainly due to the use of the VIS wavelength (Green and Red) in its formulation, which enhances the ability of the NGRDI to detect variations resulting from the rapid induced leaf senescence (i.e. chlorophyll loss) caused by heat stress. Moreover, the low-cost of RGB imaging is related to the affordability of conventional digital cameras together with the availability of open access software for image processing making this most appropriate approach for precision agriculture and crop phenotyping instead of using the more expensive multispectral cameras. However, the practical integration of digital imaging as a monitoring tool needs some considerations. Among them, that the image quality from different commercial cameras can vary and may result in different information for the same canopy, and also the existence of exposure problems due to the changing in the light intensity over time, which requires a radiometric calibration of digital numbers into reflectance, which is frequently not easy (Gates, 1980; Hunt et al., 2005; Kipp et al., 2013; Zhou et al., 2013). In our study we did not do direct calibrations for the Green and Red bands of the digital RGB camera because the wavelengths of these bands were not supported by the manufacturer of the digital camera. We suggested using the NGRDI values of the calibration targets from the first year to calibrate the NGRDI values of further years. However, the most important problem for the practical implementation of RGB images is shared with all the other remote sensing techniques and deals with a fast (preferably automated) image processing and data delivery (Araus and Cairns, 2014).

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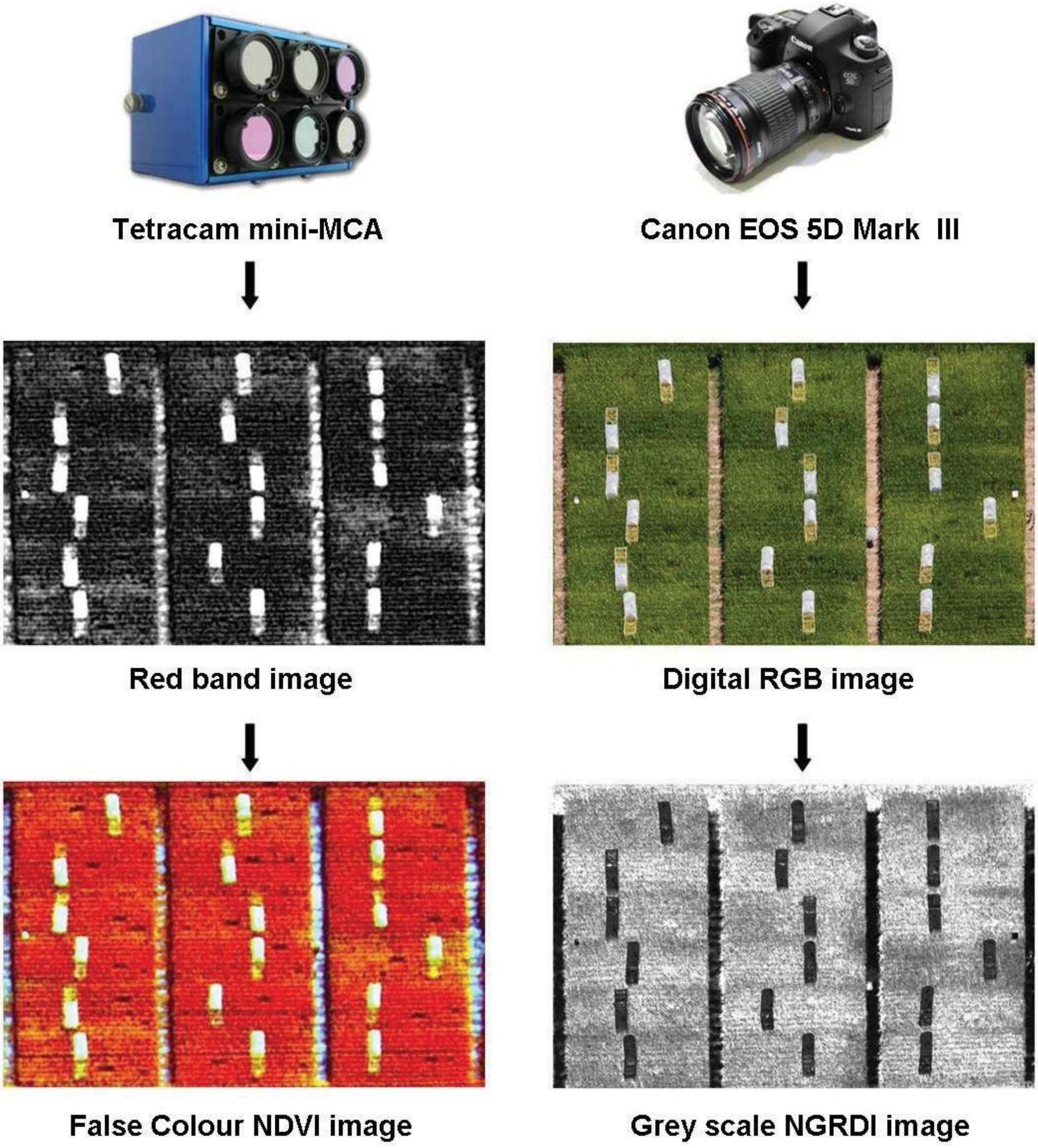
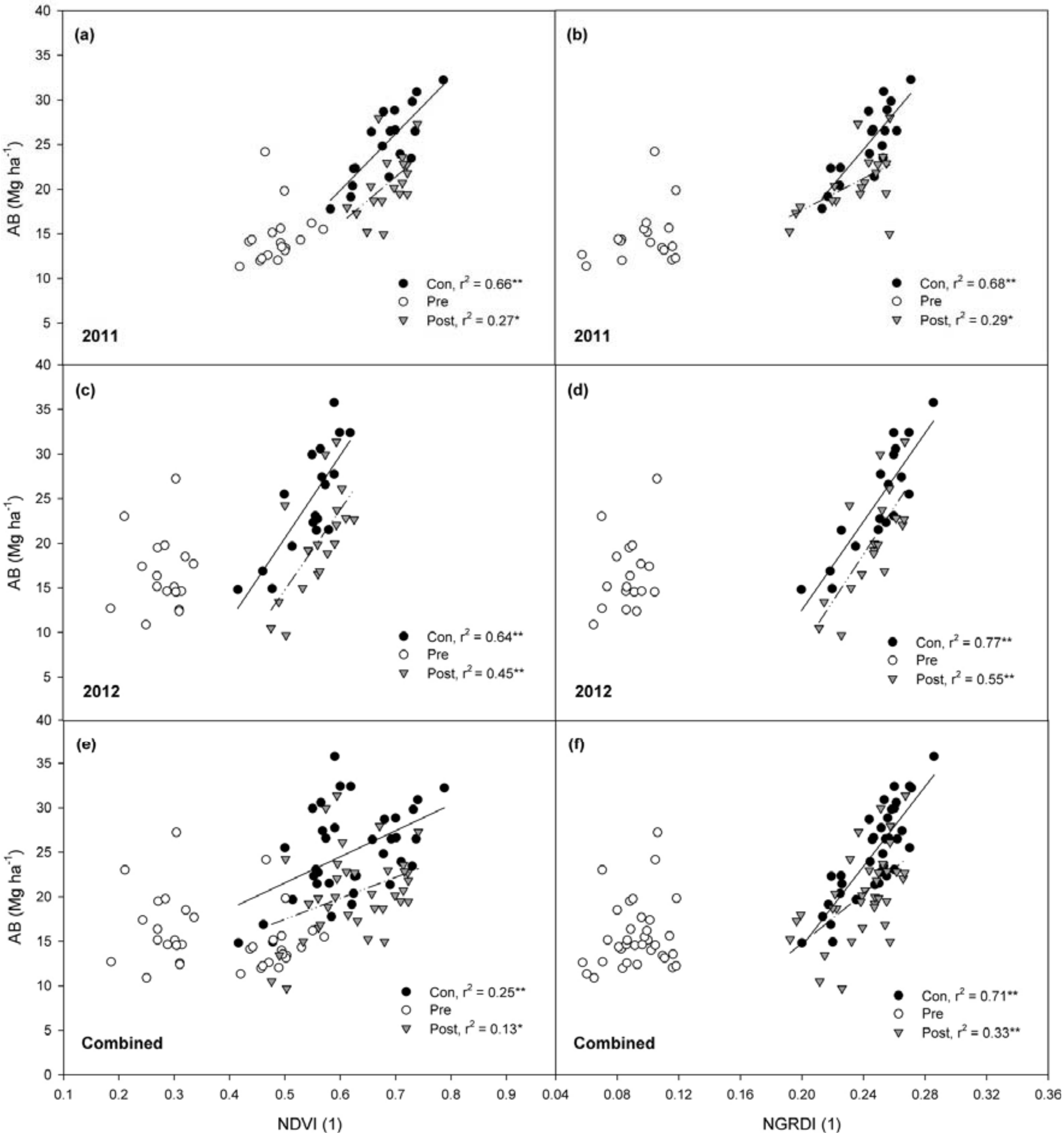
Fig. 1. The analysed NDVI and NGRDI images during the post-flowering heat treatment.



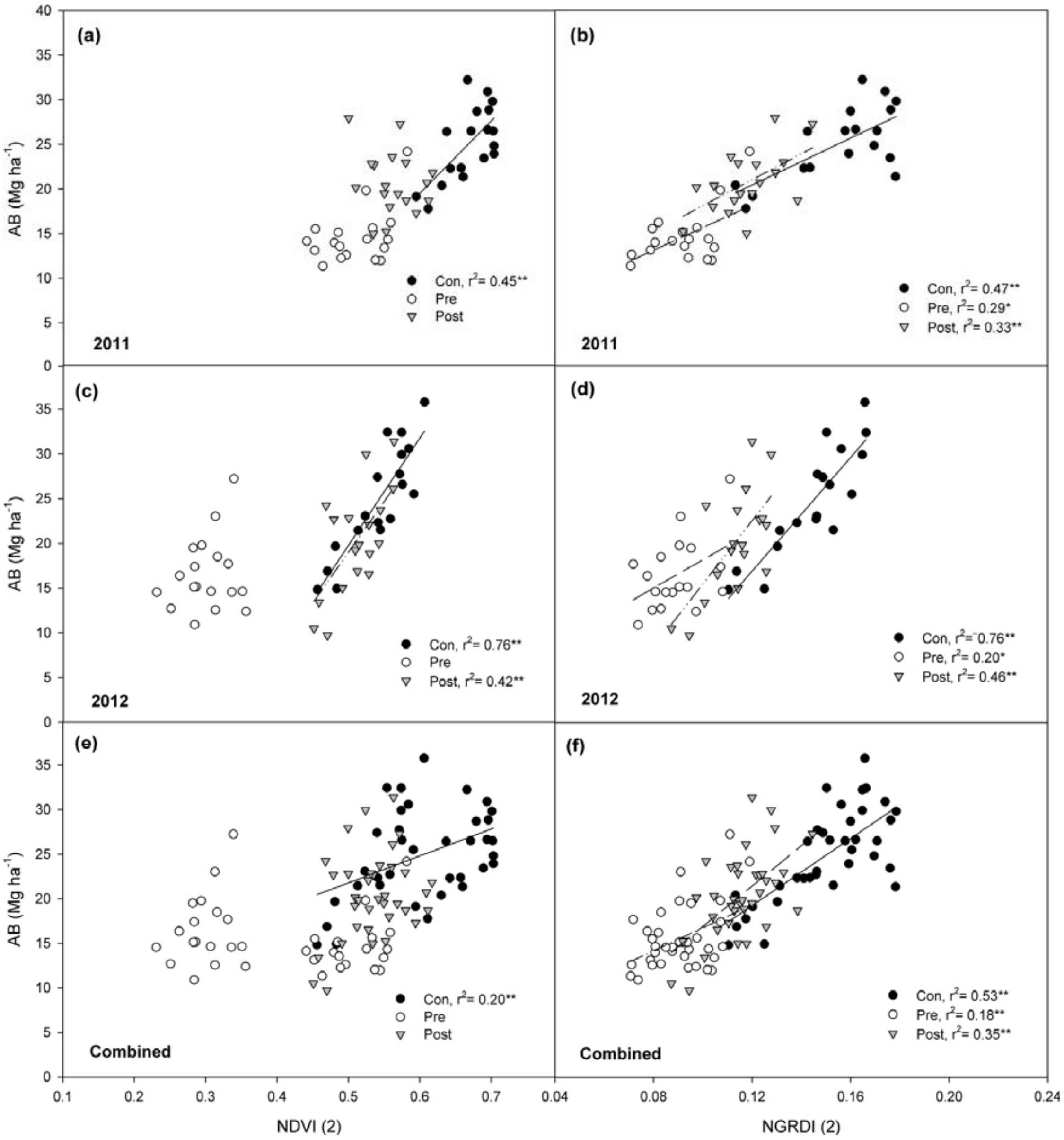
In the upper and right images, sub-plots with plastic covers and other sub-plots with yellowish/ less green plants represent the post-flowering and the pre-flowering heat treatments, respectively. The different green colour degrees in the main big plots related to the different applied nitrogen treatments.

Fig. 2. Relationship of the NDVI (1) and NGRDI (1) to AB.



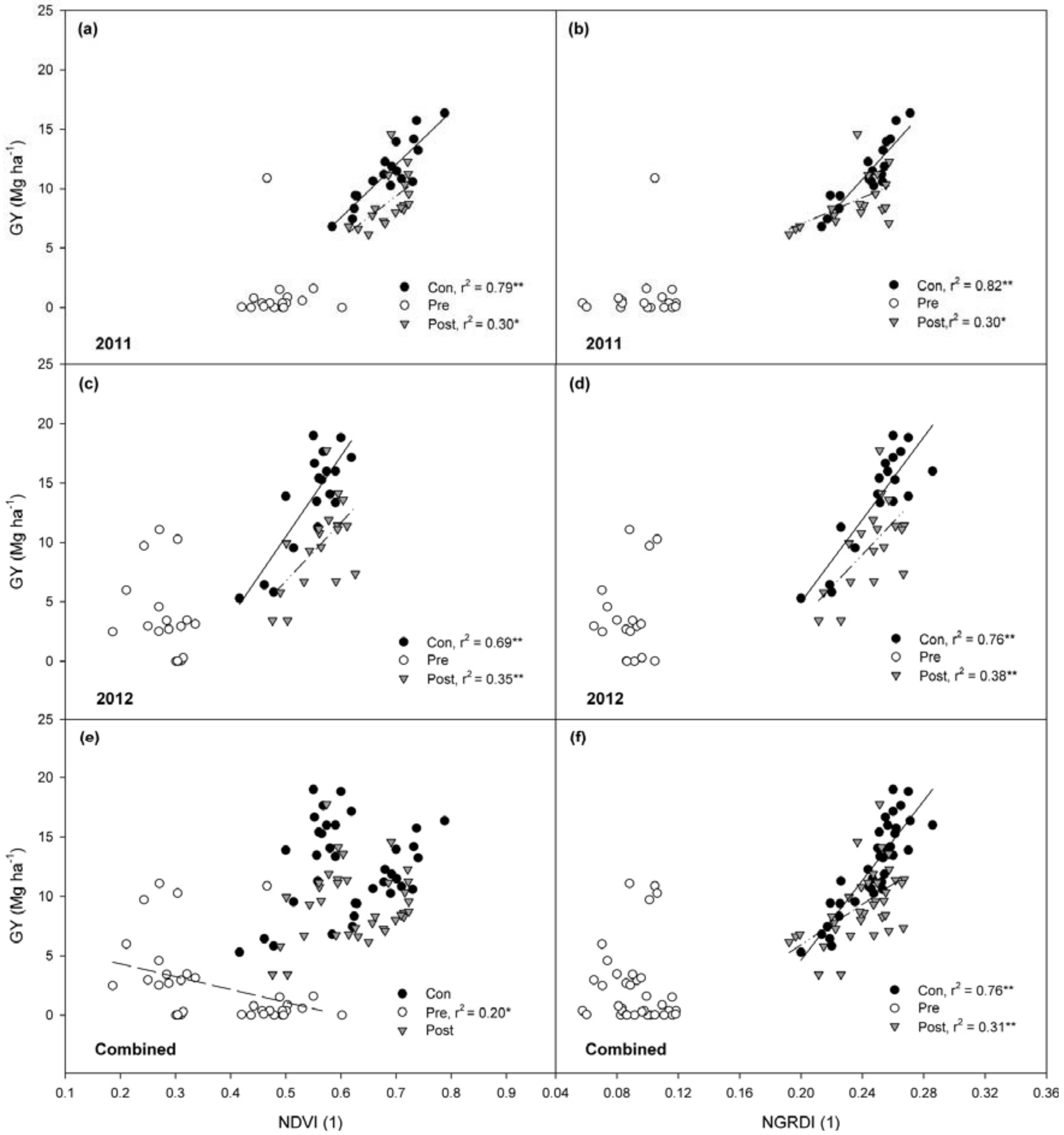
The correlations of the normalized difference vegetation index (NDVI, a, c and e) and the normalized green red difference index (NGRDI, b, d and f) to aerial biomass (AB, Mg ha^{-1}) at harvest in the three studied heat treatments: control treatment at ambient air temperature (Con, closed circles), pre-flowering heat treatment (Pre, open circles), and post-flowering heat treatment (Post, grey triangles). For each heat treatment, each value represents the individual experimental value of a plot within a heat treatment (*n*=18 for each growing season and *n*=36 when combining both seasons). The fitted line is only included in the significant relationships: r^2 and probability is shown: *, $P<0.05$; **, $P<0.01$.

Fig. 3. Relationship of the NDVI (2) and NGRDI (2) to AB.



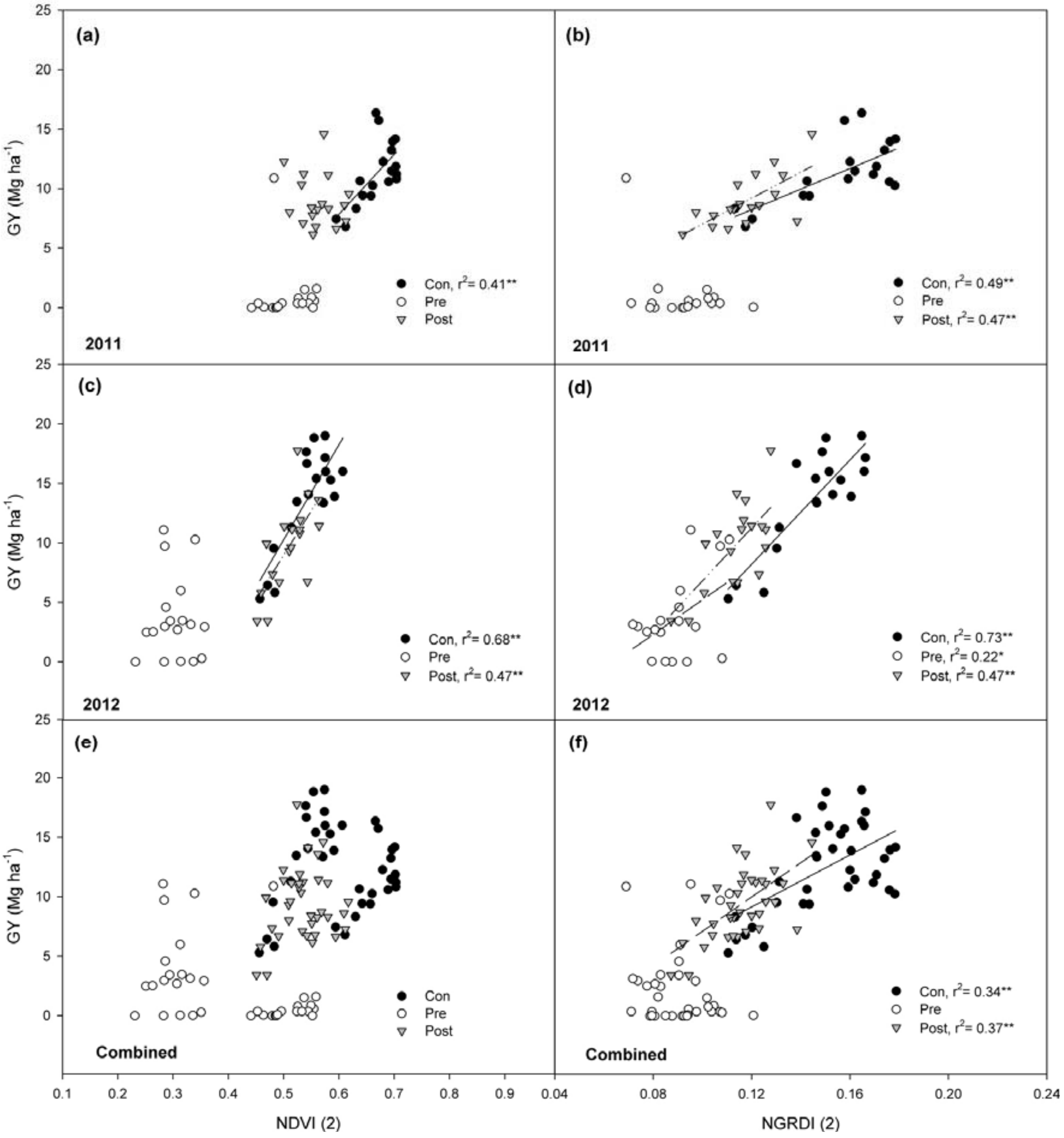
The correlations of the normalized difference vegetation index (NDVI, a, c and e) and the normalized green red difference index (NGRDI, b, d and f) to aerial biomass (AB, Mg ha^{-1}) at harvest in the three studied heat treatments: control treatment at ambient air temperature (Con, closed circles), pre-flowering heat treatment (Pre, open circles), and post-flowering heat treatment (Post, grey triangles). For each heat treatment, each value represents the individual experimental value of a plot within a heat treatment (*n*=18 for each growing season and *n*=36 when combining both seasons). The fitted line is only included in the significant relationships: r^2 and probability is shown: *, *P*<0.05; **, *P*<0.01.

Fig. 4. Relationship of the NDVI (1) and NGRDI (1) to GY.



The correlations of the normalized difference vegetation index (NDVI, a, c and e) and the normalized green red difference index (NGRDI, b, d and f) versus grain yield (GY, Mg ha^{-1}) at harvest in the three studied heat treatments: control treatment at ambient air temperature (Con, closed circles), pre-flowering heat treatment (Pre, open circles), and post-flowering heat treatment (Post, grey triangles). For each heat treatment, each value represents the individual experimental value of a plot within a heat treatment (*n*=18 for each growing season and *n*=36 when combining both seasons). The fitted line is only included in the significant relationships: r^2 and probability is shown: *, $P<0.05$; **, $P<0.01$.

Fig. 5. Relationship of the NDVI (2) and NGRDI (2) to GY.



The correlations of the normalized difference vegetation index (NDVI, a, c and e) and the normalized green red difference index (NGRDI, b, d and f) to grain yield (GY, Mg ha^{-1}) at harvest in the three studied heat treatments: control treatment at ambient air temperature (Con, closed circles), pre-flowering heat treatment (Pre, open circles), and post-flowering heat treatment (Post, grey triangles). For each heat treatment, each value represents the individual experimental value of a plot within a heat treatment (*n*=18 for each growing season and *n*=36 when combining both seasons). The fitted line is only included in the significant relationships: r^2 and probability is shown: *, *P*<0.05; **, *P*<0.01.

Table 1. Climatic conditions in the 2011 and 2012 growing seasons at Algerri, Lleida (Spain).

(a) 2011

Month	**T_{Av} (Cº)**	**RH_{Av} (%)**	**Rad_T (W/m^2)**	**P_T (mm)**	**ETP_T (mm)**
Mar	9.87	69.23	15.15	61.40	67.51
Apr	15.49	61.83	21.43	25.50	109.48
May	18.94	57.06	25.49	52.30	145.01
Jun	20.91	57.13	26.68	31.80	150.38
Jul	22.45	54.48	27.54	20.70	166.05
Aug	24.68	54.03	25.62	0.30	155.48
Sep	21.99	55.53	19.22	5.80	116.47
Total				197.80	910.38

(b) 2012

Month	**T_{Av} (Cº)**	**RH_{Av} (%)**	**Rad_T (W/m^2)**	**P_T (mm)**	**ETP_T (mm)**
Mar	11.19	52.48	18.55	45.70	87.76
Apr	12.05	62.00	20.41	69.80	95.04
May	18.47	57.00	26.77	37.40	149.07
Jun	23.52	47.67	28.58	23.20	170.79
Jul	23.39	53.19	27.69	17.80	167.04
Aug	25.85	50.87	25.89	18.10	159.34
Sep	20.09	58.10	17.65	49.50	103.52
Total				261.50	932.56

Monthly averaged air temperatures (T_{Av}), relative humidity (RH_{Av}), total monthly accumulated irradiance (Rad_T), precipitation (P_T), and the potential evapotranspiration (ETP_T).

Table 2. The effect of the growing seasons and heat treatments on the growth parameters.

T	AB	GY	HI	NGP	HGW	Chl (1)	Chl (2)
Con	24.95 ± 0.86 c	12.47 ± 0.62 c	49.49 ± 1.60 b	484.7 ± 18.39 b	29.87 ± 0.68 c	48.16 ± 0.89 b	46.69 ± 1.21 b
Pre	15.57 ± 0.61 a	2.33 ± 0.55 a	12.9 ± 2.67 a	100.43 ± 20.89 a	17.63 ± 2.01 a	43.13 ± 1.13 a	43.69 ± 1.18 ab
Post	20.42 ± 0.81 b	9.35 ± 0.51 b	45.4 ± 1.44 b	435.07 ± 22.53 b	25.54 ± 0.73 b	46.1 ± 1.04 ab	40.34 ± 1.34 a
ANOVA							
GS	NS	**	***	***	NS	NS	NS
T	***	***	***	***	***	*	**
GS x T	NS	NS	NS	NS	**	NS	NS

Traits included are: aerial biomass (AB, Mg ha^{-1}), grain yield (GY, Mg ha^{-1}), harvest index (HI, %), number of grains per plant (NGP), hundred grain weight (HGW, g) and chlorophyll content (Chl, SPAD units). For Chl, the number inside the parentheses following the acronym refers to the stage when the measures were taken: 1 refers to 9 days after silking (Pre-flowering termination) and 2 to 32 days after silking (Post-flowering termination). Data shown are the means and the standard error of means (± SEM). Means followed by different letters were significantly different ($P<0.05$) by Duncan's test. The heat treatments are: Con, control treatment at ambient air temperature; Pre, heat treatment applied at pre-flowering; and Post, heat treatment applied at post-flowering; GS, growing season; T, heat treatment; and GS x T, growing season by heat treatment interaction. Probabilities (ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) are shown.

Table 3. The effect of the growing seasons and heat treatments on the water status and vegetation indices.

T	δ^{13}C	NWI-1 (2)	NWI-2 (2)	NDVI (1)	NDVI (2)	NGRDI (1)	NGRDI (2)
Con	-11.66 ± 0.02 a	-0.14 ± 0.01 a	-0.17 ± 0.01 a	0.62 ± 0.01 b	0.60 ± 0.01 c	0.25 ± 0.00 b	0.15 ± 0.00 c
Pre	-10.66 ± 0.57 b	-0.14 ± 0.01 a	-0.17 ± 0.01 a	0.38 ± 0.02 a	0.40 ± 0.02 a	0.09 ± 0.00 a	0.09 ± 0.00 a
Post	-11.19 ± 0.03 ab	-0.13 ± 0.01 a	-0.16 ± 0.01 a	0.62 ± 0.01 b	0.54 ± 0.01 b	0.24 ± 0.00 b	0.12 ± 0.00 b
ANOVA							
GS	*	***	***	***	***	NS	NS
T	*	NS	NS	***	***	***	***
GS x T	*	NS	NS	***	***	NS	NS

Traits included are: carbon isotope composition of mature grains (δ^{13}C, ‰) the normalized water index 1 (NWI-1), the normalized water index 2 (NWI-2), the normalized difference vegetation index (NDVI) and the normalized green red difference index (NGRDI). For NWI-1, NWI-2, the NDVI and the NGRDI, the number inside the parentheses following the acronym refers to the stage when the images were taken: 1 refers to 9 days after silking (Pre-flowering termination) and 2 to 32 days after silking (Post-flowering termination). Data shown are the means and the standard error of means (± SEM). Means followed by different letters were significantly different ($P<0.05$) by Duncan's test. The heat treatments are: Con, control treatment at ambient air temperature; Pre, heat treatment applied at pre-flowering; and Post, heat treatment applied at post-flowering; GS, growing season; T, heat treatment; and GS x T, growing season by heat treatment interaction. Probabilities (ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) are shown.

Table 4. Multiple linear regression (stepwise) explaining variations in AB and GY.

T	Variable chosen	Final r^2	Final stepwise model
Con	NGRDI (1)	0.70***	AB = -30.32 + 223.94 NGRDI (1)
Pre	NGRDI (2)	0.17*	AB = 4.12 + 128.17 NGRDI (2)
Post	NGRDI (2)	0.33***	AB = -5.72 + 226.26 NGRDI (2)
	NGRDI (2), NGRDI (1)	0.41***	AB = -18.13 + 153.25 NGRDI (2) + 86.74 NGRDI (1)
Con	NGRDI (1)	0.76***	GY = -28.90 + 167.64 NGRDI (1)
Pre	Na	Na	Na
Post	NGRDI (2)	0.35***	GY = -7.31 + 144.27 NGRDI (2)

The combined data of the two studied growing seasons in the different studied heat treatments (T) were used in the analysis. Studied dependent variables are the aerial biomass (AB) and grain yield (GY). Independent variables included in the analysis are: (1) the water indices, the normalized water index 1 (NWI-1) and the normalized water index 2 (NWI-2) measured at 32 days after silking; (2) the vegetation indices, the normalized difference vegetation index (NDVI) and the normalized green red difference index (NGRDI) measured at 9 days and 32 days after silking; and (3) the carbon isotope composition of mature grains at harvest ($\delta^{13}C$). For the NDVI and the NGRDI, the number inside the parentheses following the acronym refers to the stage when the measures were taken: 1 refers to 9 days after silking (Pre-flowering termination) and 2 to 32 days after silking (Post-flowering termination). Con, refers to control treatment at ambient air temperature; Pre, refers to heat treatment applied at pre-flowering; Post, refers to heat treatment applied at post-flowering; and Na, refers to non-available data. AB and GY variability accounted by the variables included in the model is shown from r^2. For the calculations, the values of the entire individual experimental plot for the two years within a specific heat treatment (*n*=36) were used. Probabilities are shown: **, *P*<0.01; ***, *P*<0.001.

Table 5. Coefficients of determination of the studied vegetation indices with chlorophyll content measured at the same phonological stage.

T	Variables	Chl	
		(a) 2011	**(b) 2012**
Con	**NDVI (1)**	0.36	0.92**
	NDVI (2)	0.68*	0.76*
	NGRDI (1)	0.49*	0.84**
	NGRDI (2)	0.60*	0.78*
Pre	**NDVI (1)**	0.16	0.40
	NDVI (2)	-0.03	0.36
	NGRDI (1)	0.47	0.67*
	NGRDI (2)	0.41	0.27
Post	**NDVI (1)**	0.30	0.41
	NDVI (2)	-0.25	0.52
	NGRDI (1)	0.28	0.39
	NGRDI (2)	0.62*	0.66*

Probabilities (*, $P<0.05$; **, $P<0.01$) are shown. Bold figures indicate significant correlations. Chlorophyll content (Chl), normalized difference vegetation index (NDVI) and the normalized green red difference index (NGRDI). Con, control treatment at ambient air temperature; Pre, heat treatment applied at pre-flowering; and Post, heat treatment applied at post-flowering. For the calculations, the values of the mean over three replicates (means of six different N treatments) for each year within a specific heat treatment (n=6) were used. The negative sign before the coefficient refers to the negative relationships.

Appendix A. Supplementary data

Table 1. The effect of the heat treatments on AB, GY, SB, vegetation indices and water indices in the studied growing seasons

GS	T	AB	GY	SB[a]	NDVI (1)	NDVI (2)	NGRDI (1)	NGRDI (2)	NWI-1 (1)	NWI-2 (1)
2011	**Con**	25.12 ± 0.97 c	11.33 ± 0.62 c	13.79 ± 0.49 b	0.68 ± 0.01 b	0.67 ± 0.01 c	0.25 ± 0.00 b	0.16 ± 0.01 c	Na	Na
	Pre	14.65 ± 0.73 a	1.01 ± 0.59 a	13.63 ± 0.47 b	0.49 ± 0.01 a	0.51 ± 0.01 a	0.10 ± 0.00 a	0.10 ± 0.00 a	Na	Na
	Post	20.67 ± 0.83 b	8.95 ± 0.52 b	11.72 ± 0.44 a	0.69 ± 0.01 b	0.56 ± 0.01 b	0.25 ± 0.01 b	0.12 ± 0.00 b	Na	Na
2012	**Con**	24.77 ± 1.44 c	13.62 ± 1.01 c	11.15 ± 0.80 ab	0.55 ± 0.01 b	0.54 ± 0.01 c	0.24 ± 0.01 b	0.14 ± 0.00 c	-0.12 ± 0.00 a	-0.14 ± 0.01 a
	Pre	16.48 ± 0.96 a	3.65 ± 0.83 a	12.84 ± 0.73 b	0.28 ± 0.01 a	0.30 ± 0.01 a	0.09 ± 0.00 a	0.08 ± 0.00 a	-0.11 ± 0.01 a	-0.14 ± 0.01 a
	Post	20.16 ± 1.41 b	9.75 ± 0.87 b	10.41 ± 0.88 a	0.56 ± 0.01 b	0.51 ± 0.01 b	0.23 ± 0.00 b	0.11 ± 0.00 b	-0.12 ± 0.00 a	-0.15 ± 0.01 a

[a] SB = AB-GY

Traits included are: aerial biomass (AB, Mg ha^{-1}), grain yield (GY, Mg ha^{-1}), stover biomass (SB, Mg ha^{-1}) the normalized difference vegetation index (NDVI), the normalized green red difference index (NGRDI), the normalized water index 1 (NWI-1), the normalized water index 2 (NWI-2). For NDVI, NGRDI, NWI-1 and NWI-2 the number inside parentheses following the acronym refers to the stage when the images were taken: 1 refers to 9 days after silking (Pre-flowering termination) and 2 to 32 days after silking (Post-flowering termination). Data shown are the means and the standard error of means (± SEM). Means followed by different letters were significantly different ($P<0.05$) by Duncan's test. The heat treatments are: Con, control treatment at ambient air temperature; Pre, heat applied at pre-flowering; Post, heat applied at post-flowering; GS, growing season; T, heat treatment; and Na, refers to non-available data.

Table 2. Coefficients of determination of the water indices and the grain carbon isotope composition with AB and GY

(a)- 2011 (Pioneer PR31N28)

Con	AB	GY
NWI-1 (1)	Na	Na
NWI-2 (2)	Na	Na
NWI-1 (2)	-0.05	0.00
NWI-2 (2)	-0.03	-0.01
$\delta^{13}C$	-0.05	-0.04

Pre	AB	GY
NWI-1 (1)	Na	Na
NWI-2 (2)	Na	Na
NWI-1 (2)	-0.04	-0.08
NWI-2 (2)	-0.05	-0.20
$\delta^{13}C$	-0.02	-0.04

Post	AB	GY
NWI-1 (1)	Na	Na
NWI-2 (2)	Na	Na
NWI-1 (2)	-0.06	0.00
NWI-2 (2)	-0.12	-0.03
$\delta^{13}C$	-0.18	**-0.24***

(b)- 2012 (Pioneer PR33Y72)

Con	AB	GY
NWI-1 (1)	0.00	-0.02
NWI-2 (2)	0.01	0.01
NWI-1 (2)	0.00	0.07
NWI-2 (2)	0.00	0.03
$\delta^{13}C$	-0.01	0.00

Pre	AB	GY
NWI-1 (1)	-0.04	-0.01
NWI-2 (2)	-0.05	-0.03
NWI-1 (2)	-0.08	-0.07
NWI-2 (2)	-0.12	-0.06
$\delta^{13}C$	-0.11	-0.23

Post	AB	GY
NWI-1 (1)	0.01	0.08
NWI-2 (2)	0.00	-0.05
NWI-1 (2)	0.00	0.11
NWI-2 (2)	0.00	0.06
$\delta^{13}C$	-0.17	-0.14

Probabilities (*, $P<0.05$; **, $P<0.01$) are shown. Bold figures indicate significant correlations. Aerial biomass (AB), grain yield (GY), normalized water index 1 (NWI-1), normalized water index 2 (NWI-2) and carbon isotope composition of mature grains ($\delta^{13}C$). For NWI-1 and NWI-2, the number inside parentheses following the acronym refers to the stage when the images were taken: 1 refers to 9 days after silking (Pre-flowering termination) and 2 to 32 days after silking (Post-flowering termination). Con, refers to ambient air temperature; Pre, refers to the pre-flowering heat treatment; Post, refers to post-flowering heat treatment; and Na, refers to non-available data. The negative sign before the coefficient refers to the negative relationships.

Chapter 6

Discussion

Cereals are the basis of the normal diet in most Mediterranean countries and it is estimated that they account for 35-50% of the regional populations' dietary energy consumption (Hervieu et al., 2006; Abis, 2012). Agriculture defines the success of a plant as productivity per unit of land area. Water deficit, which is frequently accompanied by low nitrogen availability, is the main constraint limiting cereal productivity in the Mediterranean regions (Loss and Siddique, 1994; Oweis et al., 2000; Araus et al., 2002; Passioura, 2002; Cabrera-Bosquet et al., 2009*a*). Crop management and breeding may improve the performance of cereals under such stress conditions. However, the lack of efficient tools to monitor the performance of agronomical practices or to undertake appropriate phenotyping in breeding programs limits the efficiency of both avenues.

This study has focused on the use of stable isotopes, root traits and the use of proximal (remote) sensing techniques as novel phenotyping traits that can address the abiotic stresses in Mediterranean environments.

1. Effect of growth conditions on aerial biomass and grain yield

Decreases in aerial biomass (AB) (Chapters 2, Table 2; Chapter 3, Table 1; Chapter 4, Table 2) as well as in grain yield (GY) and its different components (Chapter 4, Table 2) and the increases in the time-integrated water use efficiency (WUE_{AB}) (Chapter 2, Table 3; Chapter 3, Table 2) are well known responses of plants to water stress (Zhang, 1995; Gregory et al. 1997; Tambussi et al. 2007; Carvalho, 2009; Yousfi et al. 2009; Araus et al., 2013). The high nitrogen regime (Chapter 3, Table 1) increased the AB and its different components and similar results have been reported by Cabrera-Bosquet et al. (2007) in a durum wheat pot experiment and by Kondo et al. (2004) in field grown rice.

The ratio of aerial to root dry biomass is a parameter that evaluates the distribution of dry matter between shoots and roots (Wang et al., 2014). It is generally reported to decrease under water deficit (Tinker and Nye, 2000; Ayad et al., 2010; Elazab et al. 2012; Carvalho et al., 2014) or under nitrogen deficiency (Rufty et al., 1988; Dreccer et al., 2000; Reich, 2000; Miller and Cramer, 2005). The results in durum wheat reported in Chapter 2 (Table 2) and Chapter 3 (Table 1) revealed that the decrease in the ratio of aerial to root dry biomass was mainly due to the negative effect of low water supply on the AB, while the root dry biomass was not significantly affected, or was even slightly enhanced, by the low water supply. In the case of the N regime, the results of Chapter 3 (Table 1) revealed that the decrease in the ratio of aerial to root dry biomass under low nitrogen conditions was the consequence of a decrease in AB together with an increase in the root biomass of a similar magnitude.

The heat stress applied in Chapter 5 sharply decreased AB and GY as well as their components in maize (Table 2). This sharp decrease appeared to be associated not only with a loss of biomass and photosynthetic capacity due to the accelerated leaf senescence (Wahid et al., 2007; Prasad and Staggenborg, 2008; Farooq et al., 2011; Rattalino Edreira and Otegui, 2012, 2013) but also with other limiting factors that are not directly related to assimilate availability per plant. Heat stress can also reduce GY due to a decline in the harvest index (Rattalino Edreira and Otegui, 2012) and this response usually occurs around flowering and is linked to a reduction in the number of grains set (Prasad et al., 1999; Wahid et al., 2007). In the present study the silks of the pre-flowering heat-treated plants were hand pollinated with pollen from the control (non heat-treated) plants to ensure adequate pollination (Cicchino et al., 2010; Rattalino Edreira et al., 2011), and thus avoiding the expected reduced pollen viability reported after heat stress (Schoper et al., 1987). However, the grain number per ear, the harvest index and the resultant GY did not improve. Actually, the pre-flowering heat treatment was applied during the critical period of the plant cycle (from 7 days after silking to 9 days after silking), which is characterized by the establishment of the number of grains and the potential grain size (Maddonni et al., 1998). Thus, the negative effects of the heat stress in reducing silk receptivity and/or increasing grain abortion may be the main reasons involved in the low grain number per ear, and thus poor GY (Samuel et al., 1986; Prasad et al., 1999; Wahid et al., 2007: Rattalino Edreira and Otegui, 2012, 2013).

Overall, significant genotypic differences in AB and GY were detected as follows: in Chapter 2 (Table 2) only under water stress conditions, where the durum wheat genotype KS194 had significantly lower AB than the rest; in Chapter 3 (Table 2) where almost all the old durum wheat genotypes had higher AB than the modern genotypes, regardless of the water and nitrogen regimes, with the old genotypes Jerez 37 and Foment de Artes having the highest AB in all water and nitrogen regime combinations, while the modern genotypes had no significant difference in AB regardless of the growing conditions (Figure S2); in Chapter 4 (Table 2) the combined analysis of variance (ANOVA) shows that no significant differences were detected between genotypes in AB. However, the modern durum wheat genotype Don Pedro had significantly lower AB under low water supply (rainfed) and significantly lower GY under both low water supply and high water supply (supplemental irrigation) than the other modern genotypes (data not shown); and in Chapter 5 the AB was not significantly different across the two consecutive years while the GY significantly differed between growing seasons, with 2012 yielding less than 2011 (Table 2 and Figures 2, 3, 4 and 5). This difference might be attributed only to growing season effects, together with the fact that different hybrids were grown each year.

2. Stable isotopes

a. *Carbon isotope composition (*$\delta^{13}C$)

In C_3 plants the carbon isotope composition ($\delta^{13}C$; frequently expressed as a discrimination against surrounding air, $\Delta^{13}C$) measured in plant tissues is negatively correlated with the ratio of intercellular CO_2 leaf concentration to ambient CO_2 (C_i/C_a) and positively correlated with the ratio of net assimilation to water transpired (A/E). Therefore, $\delta^{13}C$ is positively related to water use efficiency (*WUE*) (Farquhar and Richards, 1984; Monneveux et al., 2006). Genetic variability for $\delta^{13}C$ has been reported in durum and bread wheat (e.g. Condon et al., 1987, 1993, 2002, 2004; Rebetzke et al., 2002; Araus et al., 2003*a*, 2007, 2013; Royo et al. 2008). Correlations between $\delta^{13}C$ and GY and/or AB are normally high, and are either negative or positive according to the plant tissue sampled and particularly due to the environmental conditions tested (Condon and Richards, 1992; Voltas et al., 1999; Araus et al., 2003*a*).

A negative relationship between $\delta^{13}C$ and GY (Chapter 4, Table 5) and/or AB (Chapter 2, Figure 4; Chapter 4, Table 5) across and within water regimes has been frequently reported for durum wheat plants grown in Mediterranean environments under different water regimes with a similar nitrogen level (Araus et al*.,* 1998; 2003*a*; Merah et al*.,* 2001; Monneveux et al*.,* 2006). In Mediterranean agro-environments, characterized by precipitation after planting and moderate to medium drought during the reproductive part of the crop, the efficient use of water (*EUW*) by the crop rather than the *WUE* is the factor affecting productivity (Blum, 2009). Genotypes exhibiting a higher crop water status, and thus a higher *EUW*, are those with a lower *WUE* and therefore lower $\delta^{13}C$.

As a consequence, such genotypes with a lower δ^{13}C of mature grains (or of any other plant part developed at the end of the crop) may reflect better growing conditions and therefore will exhibit higher GY (Araus et al., 2013). In fact, negative phenotypic and genotypic correlations between δ^{13}C and both GY and AB have been reported (Araus et al., 1998; 2003*a*, 2013; Merah et al., 2001; Monneveux et al., 2006), meaning that genotypes that are able to maintain higher water use (even if it is at the expense of a lower *WUE*) are the most productive (Araus *et al.*, 2008, 2013).

The results of gas exchange and stable isotopes supported the idea that a better water status is the reason why the relationship of δ^{13}C with GY and/or AB is negative. The high water supply increased the following: the AB (Chapter 2, Table 2; Chapter 3, Table 2; Chapter 4, table 2); the GY (Chapter 4, Table 2); the gas exchange traits including stomatal conductance (g_s), transpiration (E), and the CO_2 assimilation rate (A_{Sat}); and the C_i/C_a (Chapter 2 Table 3; Chapter 3, Table 2). In contrast, the high water supply decreased the $\delta^{13}C$ and $\delta^{18}O$ of the grains (Chapter 4, Table 3) (Araus et al., 2013), spikes (Chapter 2, Table 4) (Cabrera-Bosquet et al., 2009*a*), flag leaves (Chapter 2 table 4; Chapter 3, Table 2; Chapter 4, Table 3) and roots (Chapter 2, Table 4) (Elazab et al., 2012), together with the instantaneous water use efficiency (WUE_{Inst}) (Chapter 2, Table 3; Chapter 3, Table 2) (Cabrera-Bosquet et al., 2009*a*; Elazab et al., 2012).

In Chapter 3, a positive relationship was obtained between $\delta^{13}C$ of the flag leaves and AB within each water regime (Table 4). The high nitrogen regime increased the AB but decreased the root biomass and root length (Table 1). This resulted in a negative effect on the water status, decreasing the g_s, the transpiration on a leaf area basis and to a lesser extent the C_i/C_a. As a consequence the WUE_{Inst}, the WUE_{AB} and $\delta^{13}C$ of the flag leaves increased (Table 2). In the same sense, no matter which water regime or group of genotypes was studied, when the two N regimes were combined the AB correlated positively with $\delta^{13}C$ of the flag leaves, WUE_{AB} and WUE_{Inst} and all these parameters correlated negatively with g_s (Table 4). It has been report by Cabrera-Bosquet et al. (2007) in a durum wheat pot experiment and by Kondo et al. (2004) in field grown rice that although high nitrogen fertilization increases the AB it also induces water deficit, leading to a decrease in g_s, whereas the $\delta^{13}C$ increased. Thus, the positive relationship obtained in this study between $\delta^{13}C$ of the flag leaves and AB within each water regime revealed that the nitrogen regime, in addition to the water regime, affected the predictive nature of $\delta^{13}C$ as an indirect trait for breeding under drought.

Overall, high grain $\delta^{13}C$ (and therefore *WUE*) is a 'conservative' trait in terms of water use and crop growth rate, and thus in the absence of soil water deficit, high $\delta^{13}C$ genotypes tend to grow slower than low $\delta^{13}C$ genotypes, resulting in lower AB and GY (Condon and Richards, 1993; Condon et al., 2004). High $\delta^{13}C$ has been used as a selection trait for high *WUE* (therefore GY) at the seedling stage in commercial wheat varieties for the summer-dominant rainfall environments of Australia where crop yield relies on the water accumulated before planting (Rebetzke *et al.*, 2002; Condon *et al.*, 2004; Richards et al., 2011). However, these high $\delta^{13}C$ genotypes have almost no increased GY in either the winter dominant rainfall environments (Mediterranean environments) or in the environments with highly variable rainfall of Australia (Condon et al., 2004).

Besides the environmental conditions previously mentioned, the relationship between $\delta^{13}C$ and GY may also be altered due to the fact that frequently these studies have used sets of genotypes that have variations not only in $\delta^{13}C$, but in heading and/or anthesis dates as well. Phenology could strongly influence GY and $\delta^{13}C$, being responsible for the negative relationships between $\delta^{13}C$ and GY (Araus et al., 2002, 2003*a*; Condon et al., 2004). Therefore, when studying the relationship between $\delta^{13}C$ and GY it is necessary to test genotypes with a similar phenology (Araus et al. 1998, 2003*a*; Rebetzke et al., 2002; Condon et al., 2004; Richards et al., 2011).

The results of Chapter 5 showed that even for a C_4 crop like maize the grain $\delta^{13}C$ was able to detect differences in growing conditions between heat treatments (Table 3). However, a significantly negative (even if weak) correlation of grain $\delta^{13}C$ with GY was only observed in the 2011 season (Supplementary Table 2) which seems to be the opposite to what would be expected for a C_4 species under moderate/mild water status (Cabrera-Bosquet at al. 2009*b*; Araus et al. 2010). Moreover, the $\delta^{13}C$ from the control was the most negative (Table 3), which does not support the idea of a poorer water status in the heat treatments where greater levels of stress are expected to result in a more negative $\delta^{13}C$ signal in mature grains than the non stressed conditions (Henderson et al., 1999; Cabrera-Bosquet et al., 2009*b*; Araus et al., 2010). In fact, the less negative $\delta^{13}C$ in the heat treatments may be the consequence of grains being filled from assimilates produced before grain filling, when evapotranspirative demand is smaller and less prone to some degree of atmospheric water stress.

b. *Oxygen isotope*

The oxygen isotope composition (δ^{18}O) of plant tissues is known to reflect the evaporative conditions throughout the crop cycle (Barbour et al., 2000) and thus it has been proposed as a proxy method for measuring transpiration as well as an indicator of genotypic differences in stomatal conductance (g_s) (Barbour et al., 2000; Ferrio et al., 2007; Cabrera-Bosquet et al., 2011; Elazab et al., 2012; Araus et al., 2013). Leaf δ^{18}O has been negatively correlated in wheat with the transpiration rate (T), g_s and GY (Barbour et al., 2000; Barbour and Farquhar, 2000; Ferrio et al., 2007; Cabrera-Bosquet et al., 2009*a*). In maize, Cabrera-Bosquet et al. (2009*b*) found negative correlations between the δ^{18}O of grains and GY under high water and moderate water stress conditions, whereas under severe low water conditions the correlations were positive. However, the correlation of δ^{18}O analysed in mature grains of wheat with GY is frequently weak or absent (Ferrio et al., 2007; Araus et al., 2013).

The results of Chapter 2 in wheat revealed the importance of the δ^{18}O of the flag leaves in predicting AB (Figure 4b), and g_s (Figure 3b) under high water conditions and the cumulative plant transpiration (T_{cum}) (Figure 2c) under low water conditions. However, the results of Chapter 3 revealed that within each genotype (old or modern) and watering regime (low water and high water), the $\delta^{18}O$ of the flag leaves was not the best physiological trait to inform on the plant water status because it lacked the ability to show any significant correlation with the time-integrated measures of AB, $T_{cum,}$ $\delta^{13}C$ of the flag leaves or the instantaneous g_s (Table 4). The only exception was the old genotypes under low water, where a significant negative correlation was detected between $\delta^{18}O$ of the flag leaves and $T_{cum,}$ which may reveal the negative effect of a high nitrogen regime on the plant water status (increasing the transpirative area which may further decrease transpiration). Furthermore, the combined effect of N fertilizer and water regime on the $\delta^{18}O$ of plant material has not been assessed before, despite the fact that the amount of N fertilization may affect the photosynthetic (Lawlor, 2001) and transpiration rates (Claus et al., 1993) in wheat and other cereals. It was also observed that $\delta^{18}O$ increased significantly in the water-limited treatments (Chapter 2, Table 4; Chapter 3, Table 2), while no differences in $\delta^{18}O$ were found between N treatments (Chapter 3, Table 2) (Cabrera-Bosquet et al., 2007).

Overall, previous studies (Ferrio et al*.,* 2007; Cabrera-Bosquet et al*.,* 2009*b*; Elazab et al*.,* 2012; Araus et al*.,* 2013) have already raised doubts about the use of $\delta^{18}O$ to evaluate wheat genotypic performance under different water regimes due to a number of miscellaneous factors (e.g. the $\delta^{18}O$ of the source water, exchange with water during assimilate formation and metabolic fractionation during assimilate transport), which can distort the usefulness of $\delta^{18}O$ as an environmental indicator. The current studies with wheat (Chapters 3 and 4) add concern about the use of δ^{18}O as a phenotypic trait for breeding, even when differences in the water regime significantly affect the δ^{18}O signature.

3. Root traits

The plant functions as a complete system with a balance between the shoot and root in the capture of resources and the exchange of assimilates over the long term (Lawlor et al., 2001). Plant roots are the key organs in the plant, responsible for the absorption of water and nutrients (Shen et al., 2013). Root growth is known to vary according to soil conditions such as availability of water (Asseng et al., 1998; Elazab et al., 2012) and N (Comfort et al., 1988; Carvalho, 2009; Shen et al., 2013; Wang et al., 2014). Concerning root traits, their response to drought stress is still a challenging subject for research. The laborious work required for the study of the root system has prevented the adoption of root characteristics as phenotyping traits by breeding programs. Therefore, it is necessary to develop precise enough but easy-to-handle techniques for estimating directly or indirectly (e.g. through stable isotopes) root traits associated with water use and crop yield.

a. Effect of growth conditions on root traits

Root dry matter in durum wheat was not significantly affected by the water regime conditions of the first study with lysimeters (Chapter 2, Table 2). According to several authors (Sharp and Davies, 1979; Sharp et al., 1990; Elazab et al., 2012; Wang et al., 2014) root growth is less affected or is even enhanced by water deficit in comparison to the shoot. Under water stress, osmotic adjustment occurs in root tips and thus prolongs root cell expansion, which allows the roots to grow even further under moderate water deficits, whereas shoot growth is stopped (Sharp et al., 1990). By contrast, severe water deficits are reported to inhibit root growth (Sharp et al., 2004). Although the root dry matter was not affected by the water regimes applied, the root weight density (RWD) of the high water regime (Table 5) tended to increase in the lower soil layers. This could be due to a higher availability of resources (water and nutrients) in the deeper soil layers (Gregory et al., 1978; Dwyer et al., 1988; Carvalho, 2009).

In the study reported in Chapter 3, the root dry matter was slightly decreased by the low water supply (Table 1), whereas the RWD was not affected (Table 3). A previous study by Gajri et al. (1989) reported that water stress restricted root growth, while Elazab et al. (2012) reported that root growth was not affected or was even slightly enhanced in response to water deficit and that the RWD increased in almost all soil layers.

Both root length and root length density (RLD) responded positively to the high water regime in almost all soil layers (Chapter 2, Tables 2, 5 and Supplementary Table S2; Chapter 3, Tables 1 and 3). This is in agreement with previous studies in wheat (Barraclough, 1989; Nagesh, 2006; Elazab et al., 2012). The increase in root length could be due to the fact that plants under high water conditions produce a large number of adventitious roots (Elazab et al., 2012). The adventitious roots are known to constitute 86–99% of the entire root system, occupy the upper soil layer (0-40 cm) and are unable to grow in deep soil layers like the seminal roots (Kang et al., 1994; Manske and Vlek, 2002; Elazab et al., 2012). In fact, the high water regime increased the RLD of the upper layer by 15 and 30% compared with the low water regime for Chapter 2, (Table 5) and Chapter 3 (Table 3), respectively. Drying of the soil surface may inhibit development of the adventitious root system (Gregory, 1994; Blum, 2005). In addition, the number of adventitious roots depends mainly on the tillering ability of the plant (Manske and Vlek, 2002) and the low water regime significantly decreased the number of tillers and subsequently the number of spikes per plant (Chapter, 2 Table 2; Chapter 3, Table 1).

Besides water availability, root growth can also vary according to the availability of N (Herrera et al., 2007). Both root biomass and root length increased under the low nitrogen regime (Chapter 3, Table 1 and Supplementary Figure S1b and c). These results are in agreement with earlier studies (Comfort et al., 1988; Karrou and Maranville, 1994; Wang et al., 2009, 2014; Shen et al., 2013) reporting that high N fertilization negatively affects root growth (decreasing both root biomass and length).

The specific root length (SRL) (Chapter 2, Table 5; Chapter 3, Table 3) increased in response to an increase in water supply. Contrary to these findings, an increase in SRL in response to water stress was reported by Nagesh (2006) in a winter wheat lysimeter experiment and by Song et al. (2010) in spring wheat grown in pots. Furthermore, Carvalho et al. (2014) in a lysimeter experiment with durum wheat and barley observed an increase in SRL in the low water regime in some years, but in other years the water regime did not affect the SRL. Overall, studies on the effect of different water regimes on the SRL have reported contradictory results (Ryser, 1998; Manske and Vlek, 2002; Carvalho, 2009; Elazab et al., 2012; Carvalho et al., 2014).

Whereas in Chapter 2 the SRL of the middle and lower soil layers was higher than in the upper soil layers regardless of the water regime (Table 5), in Chapter 3 the SRL in the upper soil layers was higher than the middle and lower soil layers under the high water regime (Table 3). The reason behind such a difference in response could be related to the artificial substrate (perlite) used in both lysimeter experiments, which is characterized by a low water holding capacity. While in the experiment of Chapter 2 the upper half of the lysimeters was filled with fine sand perlite (B10) aggregates, the lower part was filled with coarse perlite sand aggregates (A13), where A13 and B10 (from EUROPERL, Passau, Germany) are different commercial sizes of perlite sand aggregates. Thus, as a response to the low soil water holding capacity of the substrate filling the lower part of the lysimeters, plants increased their root length and consequently the root surface area, therefore facilitating water absorption in the lower soil layer particles while the fine sand perlite aggregates in the upper soil layers have higher water holding capacity and thus the plants need to invest less root biomass and root length in response to water availability. In the experiment of Chapter 3, the whole lysimeter was filled with A13 coarse perlite sand aggregates. Therefore the plants increased their root length and thus their surface area so as to facilitate water absorption in the upper soil layers, while in the lower soil layers the plants did not increase their SRL because water availability in these layers was not limiting due to the infiltration of the soil water from the upper to the lower soil layers.

Reports about the effect of N fertilization on the SRL are scarce and the results are inconsistent (Ebrahim, 2008; Carvalho, 2009). Under the high nitrogen regime of Chapter 3 (Table 3) the high SRL in the middle and lower soil layers compared to the upper soil layers could be due to the mobility of N into deeper soil layers due to leaching (Herrera et al., 2007; York et al., 2013). While high N availability is reported to induce reductions in root biomass (Comfort et al., 1988; Wang et al., 2009; Shen et al., 2013), it also increases root branching and thus increases the production of thinner roots, with an associated low biomass allocation to roots and an increase in SRL as a consequence (Belford et al., 1987; Herrera et al., 2007; Bonifas and Lindquist, 2009).

b. Relationship of root traits with AB

The ability of a plant to extract water and nutrients has a large effect on the AB (Ayad et al*.,* 2010). Generally, it is well established that plants with large deep roots are more tolerant to drought than shallow-rooted plants (Kramer and Boyer*,* 1995) because a larger root system can access more water from the deep soil layers (Kramer, 1969; Ma et al*.,* 2010; Qi et al*.,* 2012; Chimungu et al., 2014; Wang et al., 2014). Therefore, breeding strategies for drought have included selection of progeny with large root systems (Hurd, 1974; Ma et al., 2010).

The results of Chapters 2 and 3 reinforce the importance of a large root system in capturing more resources, where root biomass, length, RWD and RLD exhibited significant positive correlations with T_{cum} (Chapter 3, Tables 4 and 5) and $\delta^{13}C$ and $\delta^{18}O$ (Chapter 2, Figure 6; Chapter 3, Tables 4 and 5).

The results of Chapter 2 (Figure 5 and Supplementary Figure 3) revealed the importance of a larger root system (represented by RWD and RLD) for higher AB production, especially under low water regimes.

However, developing and further maintaining a large root system (i.e. with a high root biomass) is very resource consuming for the plant in terms of assimilates required (Passioura, 1983) and respiration costs (McCree, 1986; Ma et al., 2010). Therefore, several studies have proposed that a larger root system might not necessarily be an advantage for higher AB and/or GY production under water stress (Bruce et al., 2002; Lynch et al., 2014).

Indeed, a plant root system that is able to acquire a limiting soil resource at a reduced metabolic cost would increase the agronomical *WUE*, formulated either as AB or GY per unit of water transpired (Passioura, 1983; Palta and Gregory, 1997; Fan et al*.,* 2008; Carvalho, 2009; Song et al*.,* 2009; Zhang et al., 2009; Ma et al*.,* 2010; Lynch et *al.*, 2014). The results of Chapter 3 support the idea that a large root system might not be necessary to improve performance under a low water regime because AB correlated negatively with root biomass, length (Table 4) and root distribution (RWD and RLD, respectively) across soil layers (Table 5). Also, root biomass and root length showed significant negative correlations with the ratio of aerial to root biomass (irrespective of the water regime) and with WUE_{AB} (only under the low water regime), whereas these two traits correlated positively with each other under both water regimes (Table 4). Moreover, under a low water regime, RWD and RLD showed negative correlations with WUE_{AB} throughout the soil profile (Table 5). Indeed, extensive root growth early in the growing season may also be counterproductive by exhausting soil water reserves before the plant is able to complete its life cycle (Richards and Passioura, 1989; Lynch et al., 2014). It could be concluded from this study that the high nitrogen regime increased the AB but in turn accelerated soil moisture depletion (Ayad et al*.,* 2010), therefore generating some degree of water stress even under what is considered a high water regime with a frequent replenishment of evapotranspired water. In fact, a large transpiring area under what is regarded a high water regime may contribute to the generation of drought; this is the case, for example, of the typical midday decrease in stomatal conductance (Chaves, 1991; Lawlor, 2002). Consequently, a larger root system is not necessarily a favourable trait under water stress conditions because it could sometimes result in rapid soil moisture consumption (Richards, 2004; Ma et al*.,* 2009).

Overall, it could be concluded from the results of Chapters 2 and 3 that the optimal root system for high AB production also requires attention to the costs of water capture in terms of carbon resources (metabolic costs for root construction and maintenance) alongside the root system's ability to capture water.

In Chapter 2, the SRL did not show any significant correlation with AB (i.e. was not efficient in detecting genotypic variability) within the low or the high water regimes. However, when both regimes were combined the SRL correlated positively with AB, which suggested that SRL can still detect variability in environmental conditions (Figure 5 and Supplementary Figure S4). Also, in Chapter 3 the SRL under the low water regime correlated positively with AB (Table 5). Previous studies (Fitter, 2002; Løes and Gahoonia, 2004; Bonifas and Lindquist, 2009; Carvalho, 2009) reported the importance of a high SRL in increasing the root surface area, and thus the access to resources within a given soil volume, to increase AB and GY. However, a high SRL limits transport, storage and support capacity of the roots and it is associated with a shorter root life span compared with roots with a low SRL (MaCully and Canny, 1988; Fitter, 1996; Cornelissen et al., 2003).

In fact SRL is a complex trait because it combines information for root diameter and/or root tissue density (Ryser and Lambers, 1995; Carvalho et al., 2009), and thus a high SRL could be due to a decreasing root diameter and/or root tissue density (Ryser, 1998; Hajek et al., 2013). It could be concluded that in Chapter 2 and 3 the wheat plants increased their SRL by decreasing the root diameter, which decreases the amount of water uptake earlier in the growth cycle, therefore preserving water for the reproduction stages (grain filling). In this sense, the wheat breeding strategies for the summer-dominant rainfall environments of Australia, where crops rely on the water accumulated in the soil prior to emergence have focused on designing wheat plants with a conservative strategy of water consumption, allowing the crop to complete its life cycle before the available soil water is exhausted. In these plants the xylem diameter of seminal roots was reduced to increase the axial resistance to water from the root system to the shoot (Richards and Passioura, 1989; Rebetzke et al., 2002; Condon et al., 2004). However, in this study it could not be confirmed whether increasing SRL is related to a decrease in root diameter and/or reduction in the root tissue density (Ryser, 1998; Hajek et al., 2013) because measures of root diameter and root tissue density were not performed in Chapters 2 and 3.

On the other hand, in most cases the AB under the high water regime did not show any significant correlation with root biomass, root length, RWD, RLD and SRL in both Chapter 2 (Figure 5 and Supplementary Figure S4) and Chapter 3 (Tables 4 and 5). It could be concluded that the expression of root traits occurs more clearly under resource limitation.

4. The use of proximal (remote) sensing

The assessment of AB is key for monitoring crop growth because it could reflect the effect of stresses on crop growth and senescence (Araus et al., 2008; Royo and Villegas, 2011). Larger green AB represents higher potential canopy photosynthesis and thus more yield. Therefore, the effect of water stress in limiting plant growth has a subsequent impact on reducing the photosynthetic potential at the crop level and thus GY. In that sense a number of studies have revealed that spectral reflectance and/or digital imaging by ground-based remote sensing approaches has the potential to provide precise, non destructive and instantaneous quantitative estimates of AB and GY (Raun et al., 2001; Aparicio et al., 2004).

a. Defining the best phonological stage for sensing

Defining the best crop stage for sensing when GY components are more susceptible to stress conditions is important for guiding breeding programs and management practices aimed at increasing GY under optimal and stress conditions (Raun et al., 2001, 2002; Teal et al., 2006; Gambín et al., 2008; Roberts et al., 2011).

For Chapter 4, the normalized green red difference index (NGRDI) measured at mid-tillering in durum wheat showed a positive correlation with AB under the rainfed treatment (Table 5), which suggests a role of early plant vigour in the final AB. Indeed, early vigour is a very important trait in rainfed Mediterranean conditions (Botwright et al., 2002; Casadesús and Villegas, 2014) because it prevents loss of water due to evaporation from the soil surface, and it ensures a higher canopy photosynthesis at early stages (Richards et al., 2011). However, these results contradict the studies by Aparicio et al. (2002, 2004) who stressed the importance of the normalized difference vegetation index (NDVI), as well as other vegetation indices derived from spectral reflectance in the VIS and NIR regions, measured at early growth stages.

Also, The NGRDI measured at anthesis was the only vegetation index positively correlated with GY in durum wheat regardless of the growing conditions (Table 5). The study contradicts previous work reporting grain filling as the best stage for vegetation indices to correlate with GY under high water conditions when the leaf area index (LAI) had already decreased to values around 2 (Aparicio et al., 2000; Gutiérrez Rodríguez et al., 2004). Casadesús et al. (2007) reported in durum wheat that NDVI and digital-image-derived indices measured at anthesis correlated positively with GY under severe water stress conditions (with LAI around 2), but failed to correlate under high water conditions. The results of this thesis agree with Aparicio et al. (2000) and Ferrio et al. (2005) who reported anthesis as the best crop stage for GY prediction under rainfed conditions.

In Chapter 5, both NGRDI and NDVI failed to predict the GY of the pre-flowering heat-treated plants. This could be due to the fact that heat stress before flowering has a direct effect on grain set, while heat stress after flowering affects GY mostly through an accelerated senescence of the crop canopy (Samuel et al., 1986; Rattalino Edreira and

Otegui, 2012, 2013). Therefore, the vegetation indices, which are designed to capture differences in the photosynthetic apparatus (canopy green area), were better at assessing the effect of post-flowering heat treatment on GY and even on AB than the effect of the pre-flowering heat treatment (Figures 2-5).

b. *Superiority of NGRDI over NDVI*

The study in Chapter 4 highlighted the superiority of NGRDI compared to NDVI measured at mid-tillering and at anthesis for predicting GY in durum wheat under different water regimes (Table 5). In the case of maize in Chapter 5, NGRDI also correlated better with AB and GY than the NDVI, regardless of the heat treatment and growing season (Figures 2-5).

The reason behind the superiority of NGRDI over NDVI may be due to differences in their formulation. Whereas the NDVI uses light reflected in the visible (VIS) (usually red) and near infrared (NIR) regions of the spectrum, the NGRDI is only formulated using the VIS region (Green and Red). Thus, at early growth stages (Chapter 4) the difference in reflectance between the NIR and VIS on bare soil is very small compared to the differences between the NIR and VIS spectra associated with green AB (Kipp et al., 2013). This may create problems when measuring NDVI at early stages of the crop cycle or under very severe stress conditions (which decreased the LAI) because there is a large amount of bare soil present (Aparicio et al., 2004; Kipp et al., 2013). However, the VIS region **(**Green and Red**)** used in the NGRDI calculation is characterized by high light absorption of the red and reflection of the green wavelengths due to the presence of chlorophyll. By contrast, NDVI uses spectral regions (red and NIR) that are absorbed to some extent by bare soil (Kipp et al., 2013). As a result, the NGRDI is able to assess AB better than the NDVI under conditions where bare soil is exposed at early growth stages.

While in the advanced growth stages at wheat anthesis (Chapter 4) and/or 9 and 32 days after silking in maize (Chapter 5) the NGRDI correlated with AB and GY better than the NDVI, because at these stages the crop leaf area reach its maximum and caused saturation of the reflectance spectra in the red and the NIR regions. This means that increases in leaf area do not result in a parallel increase in NDVI values (Aparicio et al., 2000; Gitleson et al., 2002; Ferrio et al., 2005; Casadesús et al., 2007). However, NGRDI does not saturate in the same way because it depends mainly on changes in pigment colour and therefore it is better at detecting differences in green AB and GY than the NDVI. Different authors have reported that vegetation indices derived from the VIS region are more sensitive for detecting differences in green AB at high canopy densities (i.e. when no bare soil is exposed) than the NDVI, in spite of the small changes in leaf colour associated with senescence (Kanemasu, 1974; Gitelson et al., 2002; Motohka et al., 2010). Moreover, vegetation indices that only use the VIS region depend on the pigment's (mostly chlorophyll) reflectance characteristics, which are basically the same in all plant species (Gitelson et al., 2002). Thus, the formulation of the NGRDI enhances the ability of the NGRDI to detect chlorophyll loss caused by water and/or heat stresses.

By contrast, NIR reflectance (used in NDVI formulation) is affected by other (miscellaneous) factors such as canopy structure, cell structure and soil moisture (Gitelson and Merzlyak, 1997; Gitelson et al., 2002). Also, the NIR reflectance is reported to not be sensitive to the presence or absence of the chlorophyll pigments (Knipling, 1970; Gitelson el al., 2002).

c. *Digital imaging as a promising monitoring and phenotyping technique*

Recent studies on different cereals under field conditions have reported the applicability of digital imaging-derived indices for estimating very diverse crop traits such as chlorophyll and nitrogen content, early vigour and the LAI, AB and GY (Gitelson et al., 2002; Mullan and Reynolds, 2010; Casadesús and Villegas, 2014). A number of studies have used digital RGB imaging to measure different colour parameters such as: greenness; intensity of green, red and blue; and derived normalized indices from the green, red and blue bands (Gitelson et al., 2002; Casadesús et al., 2007; Mullan and Reynolds, 2010; Kipp et al., 2013; Elazab et al., 2015).

Digital imaging is considered a high throughput, easy-to-use monitoring technique, and thus a large number of experimental plots could be phenotyped in a short time. Moreover, the low-cost of RGB imaging is related to the affordability of conventional digital cameras, together with the availability of open-access software for image processing, making this approach most appropriate for precision agriculture and crop phenotyping instead of using the more expensive multispectral cameras. However, the practical integration of digital imaging as a monitoring tool needs some considerations. Among them, the image quality from different commercial cameras can vary and may result in different information for the same canopy (Li et al., 2010). Also, the existence of exposure problems, due to changes in light intensity over time under field conditions, requires a radiometric calibration of digital numbers into reflectance, a task that is frequently not easy (Gates, 1980; Hunt et al., 2005; Kipp et al., 2013; Zhou et al., 2013). In Chapters 4 and 5 direct radiometric calibrations for the Green and Red channels of the digital RGB camera was not undertaken because the wavelengths of these bands were not supported by the manufacturer of the digital camera. However, it was suggested in Chapter 4 and 5 to use the NGRDI values of the calibration panels at the first sensing date or crop stage or year to standardize the NGRDI values of the same panels during later sensing dates or growth stages or years. In addition, measuring the AB at critical crop stages (mid-tillering, anthesis and grain filling, as in Chapter 4) and/or contrasting heat treatments (Control, pre-flowering and post-flowering, as in Chapter 5) that are characterized by clear differences in the AB magnitude and leaf characteristics (e.g. colour and orientation), could compensate for error effects that result from the use of the non radiometrically calibrated images.

5. The combined use of the different physiological/morphological traits for genotype selection

Lafitte et al (2003) suggested that selection may be for an index consisting of GY plus secondary traits, and the expected progress from selection using the secondary trait and yield together must be greater than the progress made by selecting GY alone.

Besides the choice of the most appropriate trait (or combination of traits) for predicting GY performance under different water regimes, it is also crucial to determine the key time(s) for their evaluation. Measuring these traits at more or less frequent intervals during the crop cycle together with measurements in the harvestable components would be unfeasible, or at best impractical (Araus and Cairns, 2014). Moreover, the relative importance of each trait in explaining plant performance and the appropriate time to measure them may change depending on the severity, time and duration of the stress and the phenological stage when it occurs. Beyond that, a mechanistic explanation of how the different physiological traits can predict GY performance is not always clear, but the existence of a statistical relationship between the trait measured and the GY is used as proof of concept for the adequacy of the trait.

Due to the difficulty of measuring root traits, the indirect screening for specific shoot traits has been used in field trials for high throughput phenotyping, especially during the grain filling period (Richards et al., 2010). For example, selection can be made for genotypes that maintain greener leaf area, or higher g_s, or lower canopy temperature, or lower stable isotopes (e.g. $\delta^{13}C$ and $\delta^{18}O$) because these traits are expected to be good indicators of which genotypes are able to access more soil water in the deep soil layers (Olivares-Villegas et al., 2007; Lopes and Reynolds, 2010; Richards et al., 2010; Elazab et al., 2012). However, the usefulness of these traits is frequently countered by their low heritability in drying soils (Richards et al., 2010). Moreover, studies that relate genotypic variability of $\delta^{13}C$ and $\delta^{18}O$ to root characteristics are scarce and the results contradictory (White et al., 1990; Lopes and Reynolds, 2010; Elazab et al., 2012).

In Chapter 2, the $\delta^{13}C$ and $\delta^{18}O$ of the flag leaves negatively correlated with RWD and RLD under the high water regime and was the same for SRL when both high and low water regimes were combined (Figure 6). These relationships could be explained by a higher AB and/or GY being expected under a favourable water status, where a deep root system (i.e. high RWD and RLD at deeper soil layers) can access more soil water deeper in the soil, or where roots with a high SRL could increase root resource interception within a given soil layer (Wasson et al., 2012; Lynch et al., 2014).

For Chapter 3, the negative correlations of root biomass and root length, as well as RWD and RLD throughout the soil layers with the $\delta^{13}C$ of flag leaves and to a lesser extent with the $\delta^{18}O$ of flag leaves (Tables 4 and 5) were not due to a better water status because the AB was positively correlated with the $\delta^{13}C$ of flag leaves (Table 4). Indeed, the negative correlations of the $\delta^{13}C$ of the flag leaves with different root traits were mainly due to the effect of the high nitrogen regime increasing the AB, WUE_{AB} and $\delta^{13}C$ of flag leaves, while decreasing root biomass, root length and root distribution (RWD and RLD) in the soil (Tables 1 and 3). On the other hand, the positive correlations between the SRL and $\delta^{13}C$ of flag leaves were due to the effect of the high nitrogen regime decreasing the root biomass (Comfort et al., 1988; Karrou and Maranville, 1994; Wang et al., 2009, 2014), while increasing root branching (i.e. producing thinner and longer roots) (Belford et al., 1987; Herrera et al., 2007). Overall, the interaction of the nitrogen and the water regime (Chapter 3) changed the expected relationship between the $\delta^{13}C$ and root structure when only the water regime is changed (Chapter 2). Thus, it could be concluded that when using indirect selection traits to assess root performance the interaction between different growth factors should be taken into account.

In Chapter 4, both the NGRDI at anthesis and the $\delta^{13}C$ of mature grains were chosen as the two significant variables of a stepwise model explaining almost 60% of the GY variability within each water treatment (Table 6). However, the order of both variables changed, with the NGRDI at anthesis and the $\delta^{13}C$ of mature grains being the first variables chosen under rainfed and supplemental irrigation, respectively. For a better understanding of the mechanisms that might predict GY performance under contrasting water conditions, a path-analysis was run to model the relations between the best physiological traits informing on growth and photosynthesis (NGRDI and $\delta^{13}C$ of mature grains) with the agronomical yield components and ultimately GY (Figure 2). The results of the suggested path-models revealed that under rainfed conditions the high water supply affects early plant vigour (assessed through NGRDI at mid-tillering) and had a positive effect on GY through generating a larger number of fertile ears and contributing to a higher photosynthetic area during the reproductive stage (further assessed through NGRDI at anthesis) (López-Catañeda et al., 1995; Richards et al., 2011). Thus, an improved water status during the reproductive stage had a positive effect on GY primarily through a larger photosynthetic area and a subsequent improvement in ear fertility (number of grains per ear) (Simane et al., 1993; García del Moral et al., 2003). Under supplemental irrigation the terminal (during grain filling) water stress (monitored through the $\delta^{13}C$ of mature grains) is the main factor determining GY performance (Araus et al., 2013), whereas the constitutive (i.e. in absence of water stress) differences in plant growth that exist at anthesis (represented by the NGRDI) have a minor role. Under such conditions, plants exhibiting better water conditions during the reproductive stage (lower $\delta^{13}C$ of mature grains) benefitted from a direct positive effect on grain filling (i.e. increasing thousand grains weight). An indirect effect through a larger photosynthetic area during the reproductive stage (higher NGRDI at anthesis) is also involved. A larger photosynthetic area was positively related to a larger number of fertile tillers (higher number of tillers per m^2), which affected GY positively.

General conclusions

1. The $\delta^{13}C$ is a powerful phenotyping tool that informs on the plant water status (g_s, T_{cum}), plant growth (A_{sat}, AB, GY) and water use efficiency (A_{sat}/E, A_{sat}/g_s, AB/T_{cum}). However, the interaction of different growing conditions (e.g. water and nitrogen regimes) may change the predictive nature of $\delta^{13}C$ as a phenotyping trait when breeding for drought resilience.
2. In contrast, the use of $\delta^{18}O$, especially from mature grains, as a predictor of plant water status (g_s, T_{cum}) and plant growth (AB, GY) is not encouraging; probably due to miscellaneous factors other than the crop water status that affects the final ^{18}O signature.
3. Higher root biomass, root length, RWD and RLD improve soil water capture, especially under low water regimes.
4. Selecting for root traits like high SRL could be valuable when breeding for low resource availability (such as water or N deficit alone or in combination), as it could optimize the capacity of the root system in terms of metabolic cost.
5. The digital imaging-derived index (NGRDI) was better than the NDVI measured with a spectroradiometer in detecting variability in the GY and AB of durum wheat growing in the field under different water regimes.
6. The NGRDI was better than the NDVI measured with a multispectral camera in detecting variability in the GY and AB of field maize under different control and post-flowering heat treatment conditions. However, both indices were unable to predict GY variations under pre-flowering heat treatment due to grain set establishment problems that could not be detected by vegetation indices, which are designed to monitor differences in green area.
7. The superior performance of the NGRDI compared with NDVI seems to be due to the use of the VIS wavelengths (green and red) in its formulation. This enhances the ability of the NGRDI to detect variations resulting from the rapid induction of leaf senescence (i.e. chlorophyll loss) caused by either drought or heat stress.
8. The combination of a digitally-derived vegetation index with $\delta^{13}C$ may predict a substantial percentage of the variability in durum wheat GY. Moreover, path-analysis of these factors (including agronomical components) determining grain yield, modelled how phenotypic traits may predict GY performance under different water availabilities in a Mediterranean environment.

Summary

Importancia de los cereales en la agricultura mediterránea

Los cereales son la base de la dieta normal en la mayoría de los países del Mediterráneo y se estima que representan del 35 % al 50 % del consumo de energía alimentaria por los habitantes de la región. Los cereales más consumidos en la cuenca Mediterránea son el trigo duro y el trigo blando los cuales tienen su origen en el Oriente Próximo. Entre los otros cereales presentes en la cuenca mediterránea, podemos destacar el arroz, el centeno, el mijo, la cebada, la avena y el maíz, que son más regionales y se cultivan en menor cantidad que los dos primeros.

El trigo duro (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.) es la única especie tetraploide de trigo que se cultiva ampliamente en la actualidad. Su gran contenido en proteína y gluten, así como su dureza, hace que la el trigo duro resulte una buena opción para la elaboración de una gran variedad de productos alimenticios. Los productos más comunes elaborados con trigo duro son la pasta, el cuscús y el búrgol. A nivel global, la producción mundial de trigo en 2013 fue de 715,9 millones de toneladas, de los que el 94% fue trigo blando (*Triticum aestivum* L.), mientras que el trigo duro se colocó segundo, con menos del 6% de la producción mundial de trigo, lo que representa alrededor de 37,7 millones de toneladas.

El maíz (*Zea mays* L.) es originario de Mesoamérica. Se cultiva en una gran variedad de entornos agroecológicos de todo el mundo, desde el nivel del mar hasta zonas templadas de montaña a más de 3000 m de altura. Se trata de un cereal muy importante, tanto para la alimentación del ganado como la nutrición humana, y además se utiliza para la producción de bioetanol y biocombustibles. En 2013, la producción mundial fue aproximadamente de 1018 millones de toneladas y los cinco principales países productores fueron Estados Unidos, China, Brasil, Argentina y Ucrania. España se encuentra en el puesto número 25 de la lista de países productores, con 4,93 millones de toneladas, la mayoría producidos en condiciones de riego.

Limitaciones para la producción de cereales en la cuenca mediterránea

La tendencia actual con respecto al crecimiento demográfico sugiere que es poco probable que la producción mundial de alimentos pueda satisfacer las necesidades futuras de la población, a menos que se acelere el avance genético en la mejora de cultivos y se mejoren las prácticas agronómicas y/o se produzcan cambios radicales en los patrones de consumo humano de alimentos. Se considera que una gran parte de la población de la región mediterránea se encuentra en condiciones de inseguridad alimentaria: de alrededor de 500 millones de personas, al menos 215 millones se encuentran en condiciones de "inseguridad alimentaria cualitativa y cuantitativa". La región mediterránea es una de las principales áreas críticas de insostenibilidad medioambiental que interactúa de forma negativa con la intensa actividad humana y la explotación agrícola. Se espera que el cambio climático global, debido principalmente a las emisiones de CO_2 por la quema de combustibles fósiles, junto con la liberación continuada de óxido nitroso (un gas de efecto invernadero) a la atmósfera como resultado de la desnitrificación de fertilizantes nitrogenados, genere un aumento de la temperatura del aire de 0,4 °C a 0,6 °C por década a lo largo del siglo XXI. En el caso de las regiones mediterráneas, las perspectivas para el futuro son, posiblemente, peores que para la media mundial, con una previsión de calentamiento global de 1 °C para el año 2025, lo cual se traduciría en inviernos menos fríos y veranos más calurosos. Un aumento más rápido de la temperatura incrementaría la frecuencia de veranos secos en todo el Mediterráneo. En el sur de Francia y el centro de España, se espera que los veranos secos comiencen tres semanas antes y finalicen dos semanas después, lo cual supondría un adelanto hacia la primavera en la temporada seca. Además, el aumento de la temperatura de la Tierra es suficiente para cambiar los patrones de lluvia y la capacidad productiva de los cultivos en muchas regiones de la misma. La escasez de agua supone un problema alarmante en la cuenca mediterránea debido al crecimiento demográfico y la creciente necesidad de agua para la agricultura y otras actividades humanas.

En esta tesis, nos hemos centrado en el uso de isótopos estables, características de las raíces y el uso de técnicas de detección proximal (remota) como nuevos caracteres fenotípicos que pueden detectar diferentes tipos de estrés abiótico en las regiones Mediterráneas.

Técnicas de monitoreo y fenotipeado actuales

Isótopos estables

En plantas C_3, la composición de isótopos de carbono ($\delta^{13}C$) medida en los tejidos de la planta se considera como uno de los caracteres secundarios más prometedores del trigo (y otros cereales C_3) en la mejora para resistencia a la sequía. Se sabe que el $\delta^{13}C$ se correlaciona negativamente con el C_i/C_a (relación de la concentración intercelular de CO_2 de la hoja y de CO_2 del ambiente) y positivamente con la *A/E* (relación entre la asimilación neta y el agua transpirada de la hoja). Por lo tanto, el $\delta^{13}C$ se relaciona positivamente con la Eficiencia en el Uso del Agua (*WUE*), que se considera como la biomasa producida por unidad de agua transpirada. Las correlaciones entre el $\delta^{13}C$ y el rendimiento de grano (GY) y/o la biomasa aérea (AB) pueden ser negativas o positivas de acuerdo con las condiciones medioambientales del ensayo. En la mejora de trigo comercial para las condiciones Mediterráneas de Australia, caracterizadas por precipitaciones estivales, y donde el rendimiento del cultivo depende del agua acumulada antes de la siembra, se ha utilizado un $\delta^{13}C$ elevado como un carácter de selección de una alta *WUE* . Sin embargo, en determinados casos, el $\delta^{13}C$ puede reflejar el agua utilizada de manera efectiva (*EWU*) por el cultivo y, por lo tanto, la relación entre el $\delta^{13}C$ y la AB o la GY puede ser negativa, lo que significa que los genotipos que son capaces de mantener un mayor índice de *EWU* (incluso a expensas de un menor WUE) son los más productivos.

En el caso de una planta C_4, como el maíz, las variaciones en el δ^{13}C en respuesta a las condiciones hídricas son mínimas en comparación con plantas C_3, como el trigo, pero todavía son adecuadas para su uso como indicador de las condiciones hídricas presentes durante el crecimiento.

Se sabe que la composición isotópica de oxígeno (δ^{18}O) de los tejidos vegetales refleja las condiciones de evaporación a lo largo del ciclo de cultivo y, por eso, se ha propuesto como método para inferir las diferencias genotípicas y causadas por el ambiente en transpiración y la conductancia estomática (g_s) en una gran variedad de plantas C_3 y C_4.

Características de las raíces

Las raíces son los órganos clave de la planta por donde se absorbe el agua y nutrientes. El crecimiento de la raíz varía de acuerdo a la textura y estructura del suelo y otros parámetros relacionados, tales como la disponibilidad de nitrógeno y agua. En cuanto a las características de las raíces, su respuesta al estrés por sequía continúa resultando un ámbito de investigación complejo. El laborioso trabajo que se requiere para el estudio del sistema radicular ha impedido la adopción de las características de la raíz como un carácter de fenotipeado rutinario en mejora de cultivos. Por lo tanto, es necesario desarrollar técnicas que sean lo suficientemente precisas y fáciles de manejar para estimar caracteres radiculares asociados con el uso del agua y el rendimiento de los cultivos.

La capacidad de las raíces para extraer el agua y los nutrientes de un volumen determinado de suelo depende de las características arquitectónicas de la raíz, tales como la longitud y la distribución del peso de las raíces en profundidad. De esta manera, la densidad de peso de la raíz (RWD) y la densidad de longitud de la raíz (RLD) se utilizan con frecuencia en estudios de raíces para describir el peso y la longitud de la raíz, respectivamente, en un volumen de suelo. En la actualidad, la RLD es el rasgo más comúnmente utilizado para describir la distribución de la raíz y se trata de un parámetro importante para modelar la absorción de agua y nutrientes. En general, los resultados de los experimentos de campo en trigo y cebada indican que una RLD de 1 cm cm^{-3} es un valor crítico para la extracción del 90 % del agua disponible en el suelo (la cantidad de agua del suelo entre la capacidad del campo y el punto de marchitamiento permanente), mientras que unos valores de RLD por encima de 1 cm cm^{-3} son excesivos y, en estos casos, se produce competencia entre las raíces.

La longitud específica de la raíz (SRL) se considera otro de los rasgos morfológicos más importantes y comúnmente medidos. La SRL es la relación entre la longitud de la raíz y la biomasa de la raíz. Un valor alto de SRL facilita la absorción de nutrientes en entornos con deficiencia de nutrientes y hace que las plantas sean más competitivas en la absorción de nutrientes del suelo. Sin embargo, las raíces con una SRL alta pueden tener una capacidad limitada de transporte, almacenamiento y suministro de agua, junto con una vida útil menor que las raíces con una SRL baja.

El uso de técnicas de detección proximales (remotas).

La evaluación de la AB es importante para supervisar el crecimiento del cultivo, ya que puede reflejar el efecto de las diferentes condiciones de estrés en el crecimiento y la senescencia del cultivo. Así, una serie de estudios han revelado que las técnicas de reflectancia espectral son capaces de facilitar estimaciones cuantitativas, instantáneas, no destructivas y precisas de la AB. El *Índice Normalizado de Diferencia de Vegetación (NDVI)*, que utiliza longitudes de onda visibles (VIS) e infrarrojas cercanas (NIR) en su formulación, se encuentra entre los índices de reflectancia espectral más utilizados y se relaciona con la biomasa fotosintéticamente activa. El NDVI se ha utilizado como indicador de la AB y la GY en trigo duro y maíz. Sin embargo, los valores de NDVI se saturan a elevadas densidades de biomasa verde (es decir, a índices de área foliar altos), lo que significa que esta técnica puede tener una precisión baja en los momentos clave del ciclo de cultivo (por ejemplo, en antesis), cuando hay gran densidad vegetal, especialmente si las condiciones agronómicas son favorables. Además, su precisión se ve disminuida en ciertas etapas del ciclo de la planta por cambios en la estructura de la cubierta o dosel del cultivo. Este es el caso por ejemplo de la etapa reproductiva en trigo cuando las espigas, que los órganos reproductores, emergen por encima del dosel. .

Durante los últimos años, las imágenes digitales rojo-verde-azul (RGB) se han usado como una alternativa para el desarrollo de índices de vegetación que podrían sustituir al NDVI basado en espectroradiometría. El precio, el tamaño y la facilidad de uso de las cámaras digitales convencionales las convierten en alternativas viables para evaluar la AB y la GY en los cereales. Diferentes estudios han utilizado imágenes RGB digitales para medir parámetros de color diferentes, tales como: verdor, intensidad de verde, rojo y azul e índices normalizados derivados de las bandas verdes, rojas y azules. Dicha información ha permitido estimar una gran variedad de parámetros de cultivo en trigo duro y blando, tales como el vigor inicial, el índice de área foliar, la senescencia foliar, la AB y la GY. Por otra parte, mientras que la adquisición de una gran cantidad de imágenes RGB digitales (fotografías) de los cultivos requiere un esfuerzo mínimo, el procesado de las imágenes con programas informáticos de edición fotográfica (ya sea de acceso libre o comercial) resulta rápido y permite determinar parámetros relacionados con la AB verde.

Objetivos del estudio

En esta tesis se estudian diversas metodologías s para el monitoreo y fenotipado del rendimiento de grano, la producción de biomasa y el estado hídrico y fotosintético de dos de los cultivos más importantes en el mundo (trigo y maíz).

Los objetivos específicos de la tesis son:

Para los Capítulos 2 y 3:

1. Comparar el efecto del régimen de agua y la variabilidad genotípica (Capítulo 2) y de la interacciones entre el régimen hídrico, la fertilización nitrogenada y la variabilidad genotípica (Capítulo 3) sobre:
    a. La biomasa aérea, el crecimiento y la arquitectura de la raíz.
    b. Diversos caracteres instantáneos e integrados en el tiempo de intercambio gaseoso.
    c. La composición de diversos isótopos estables ($\delta^{13}C$ y $\delta\ ^{18}O$) que nos darán una indicación del estado fotosintético e hídrico de la planta.
2. Evaluación de los isotopos estables de carbono y oxígeno como indicadores del estado hídrico y el crecimiento de la planta ($\delta^{13}C$ y δ^{18}).
3. Estudio del funcionamiento de diversos caracteres relacionados con la distribución y la morfología de la raíz como indicadores del estado hídrico y el crecimiento de la planta.
4. Estudiar las relaciones entre la firma de diversos isótopos estables y los caracteres de la raíz y cómo estas relaciones pueden ser útiles para seleccionar los mejores caracteres radiculares en las condiciones crecimiento estudiadas.

Para el Capítulo 4:

5. Investigar el efecto del régimen de agua y la variabilidad genotípica en trigo duro cultivado en condiciones de campos sobre:
    a. La biomasa aérea, producción del grano y los componentes agronómicos del rendimiento.
    b. La $\delta^{13}C$ y $\delta\ ^{18}O$ de hojas y de granos maduros.
6. La evaluación de $\delta^{13}C$ y $\delta\ ^{18}O$ como indicadores del estado hídrico de la planta y el crecimiento.
7. Comparar el uso potencial de dos técnicas diferentes de teledetección: La reflectancia espectral y la alternativa de bajo coste consistente en el uso de las imágenes digitales RGB (rojo-verde-azul) para determinar el rendimiento de la producción del grano.

8. Determinar la combinación de caracteres fenotípicos que pueden predecir el rendimiento en grano a través de los diversos componentes agronómicos de la producción.

Para el Capítulo 5:

9. Comparar el rendimiento de dos técnicas diferentes de teledetección remota: el uso de cámaras multiespectrales y el uso de las imágenes digitales RGB (rojo-verde-azul) para detectar los efectos del estrés térmico en condiciones del campo sobre la biomasa aérea y producción del grano en maíz.

Capítulo 2

En este capítulo se estudiaron el crecimiento de las plantas de trigo duro, las características de las raíces y la composición isotópica de carbono ($\delta^{13}C$) y oxígeno ($\delta^{18}O$). Lisímetros de 150 cm de alto se ubicaron bajo cubierta en los Campos Experimentales de la Universidad de Barcelona y en ellos se cultivaron cuatro líneas endogámicas recombinantes (seleccionadas por su buena adaptación agronómica) de trigo duro en dos condiciones de riego: buen riego y estrés hídrico hasta el estadio de mitad del llenado del grano. Se midieron los parámetros de intercambio de gases en la hoja bandera justo antes de finalizar el experimento. Posteriormente, se evaluaron la materia seca aérea (AB, por sus siglas en inglés), la densidad del aparato radicular en términos de peso (RWD) y de longitud (RLD) y la longitud específica de la raíz (SRL, todos ellos por sus siglas en inglés), y se analizaron la $\delta^{13}C$ y la $\delta^{18}O$ de las raíces, la hoja bandera y la espiga.

Capítulo 3

En este experimento se cultivaron genotipos antiguos y modernos de trigo duro en lisímetros de 150 cm de alto, con dos regímenes de agua y nitrógeno contrastados, y se estudió el efecto de estos tratamientos sobre la biomasa aérea (AB, por sus siglas en inglés), el crecimiento, la distribución y la morfología de las raíces, las tasas de fotosíntesis y transpiración y la eficiencia en el uso del agua. Igual que en el primer ensayo, los lisímetros se ubicaron bajo cubierta en los Campos Experimentales de la Universidad de Barcelona.

Capítulo 4

En este estudio, se cultivaron cinco genotipos modernos de trigo duro en régimen de secano (RF, por sus siglas en inglés) y de riego suplementario (SI, por sus siglas en inglés). Se estudiaron dos tipos de características fisiológicas: (1) índices de vegetación: *Índice Normalizado de Diferencia de Vegetación (NDVI*, por sus siglas en inglés) y el Índice Normalizado de Diferencia de color Rojo-Verde (NGRDI, por sus siglas en inglés); y (2) la composición en isótopos estables de carbono y de oxígeno ($\delta^{13}C$ y $\delta^{18}O$, respectivamente) de diferentes órganos de la planta. Los objetivos de este estudio fueron: (1) comparar el rendimiento de las plantas de trigo duro bajo diferentes regímenes de riego evaluando el crecimiento y el estado hídrico de las plantas; y (2) comprender cómo dichas características pueden predecir el rendimiento de grano (GY, por sus siglas en inglés). El ensayo se realizó en los campos experimentales del INIA (Instituto Nacional de Innovación Agraria), Aranjuez, Madrid.

Capítulo 5

Dicho trabajo se realizó en maíz cultivado en condiciones de campo durante dos temporadas consecutivas en Algerri, Lleida, España. Se aplicaron dos tratamientos térmicos antes y después de la floración (pre y post-floración, respectivamente), además del tratamiento de control a temperatura ambiente. Se midieron dos índices de vegetación. Por un lado el NDVI, mediante una cámara multiespectral, y por el otro el NGRDI empleando una cámara digital RGB. Las imágenes fueron tomadas desde una avioneta tripulada. El objetivo de este estudio ha sido comparar la potencialidad de dos técnicas diferentes de teledetección para estudiar los efectos del estrés térmico en condiciones de campo en sobre la biomasa aérea (AB) y el rendimiento de grano (GY) de maíz.

Resultados

Resultados del Capítulo 2

Bajo condiciones de estrés hídrico se redujo la conductancia estomática, la transpiración acumulada de la planta y la AB, mientras que se incrementó la $\delta^{13}C$ y la $\delta^{18}O$. Existían diferencias debidas al genotipo en los parámetros de intercambio de gases, morfología de raíces y firmas isotópicas. La AB se correlacionó positivamente con la RLD, con independencia del régimen hídrico, mientras que se correlacionó negativamente con el δ^{13}C y el δ^{18}O, pero únicamente en condiciones del buen riego. Por otra parte, la RWD y la RLD se correlacionaron negativamente tanto con el δ^{13}C como con el δ^{18}O en condiciones del buen riego, pero no se observó un patrón claro en condiciones del estrés hídrico.

Resultados del Capítulo 3

Los tratamientos con un nivel alto de agua y nitrógeno aumentaron de forma significativa la AB. Por el contrario el peso y longitud de la raíz y la densidad de longitud de la raíz aumentaron y disminuyeron, respectivamente, en respuesta a un mayor aporte de agua y nitrógeno. Un nivel elevado de riego no afectó a la densidad de peso de la raíz, mientras que un nivel alto de nitrógeno disminuyó este parámetro. El régimen de nivel alto de nitrógeno aumentó de forma significativa la composición isotópica de carbono de la hoja bandera ($\delta^{13}C_{FL}$) y disminuyó la fotosíntesis neta, la conductancia estomática y la transpiración de la hoja bandera, mientras que se observó el efecto opuesto al aumentar el aporte de agua. Además, el riego elevado disminuyó significativamente la composición isotópica de oxígeno de la hoja bandera ($\delta^{18}O_{FL}$) e incrementó la transpiración acumulada de la planta, mientras que un aumento en el abonado nitrogenado no afectó de forma significativa a ninguno de esos parámetros. Independientemente del régimen hídrico, la AB se correlacionó positivamente tanto con el $\delta^{13}C_{FL}$ como con la eficiencia del uso del agua integrado en el tiempo (WUE_{AB}, por sus siglas en inglés) y negativamente con los parámetros de intercambio de gases.

Resultados del Capítulo 4

El NGRDI medido en antesis y la δ^{13}C en los granos maduros fueron los caracteres mejor correlacionados (positiva y negativamente, respectivamente) con el GY. Ambos caracteres en combinación explicaron como mínimo, el 50 % de la variabilidad en el GY dentro de cada régimen hídrico. Los modelos de "sendero" producidos para las condiciones de RF y SI destacaron el papel importante del NGRDI y de la δ^{13}C en la predicción del GY.

Resultados del Capítulo 5

Ninguno de los dos índices vegetativos pudo predecir el GY de las plantas sometidas a un tratamiento por calor anterior a la floración, debido a que el efecto del estrés por calor afectó sobre todo al el desarrollo de los granos, lo que no se pudo detectar por los índices de vegetación, ya que éstos están diseñados para captar diferencias en dosel verde del cultivo. Por el contrario, tanto el NGRDI como el NDVI se correlacionaron positivamente con el GY y la AB tanto en el tratamiento térmico posterior a la floración como en el control. Sin embargo, en ambos tratamientos, el NGRDI estuvo mejor correlacinado con la AB y el GY que el NDVI. Además, el NGRDI parece comportarse de manera más independiente que el NDVI respecto a variaciones interanuales en el estado del cultivo, ya que el NGRDI fue capaz de predecir la AB y el GY mejor que el NDVI cuando se combinaron los datos de los dos años consecutivos.

Discusión

Isotopos estables

Los resultados del intercambio de gases y de los isótopos estables apoyaron la idea de que un buen estado hídrico en los genotipos o los ambientes más productivos es el responsable de que la relación de $\delta^{13}C$ con el GY y la AB sea negativa. Un alto nivel de irrigación aumentó la AB (Capítulo 2, Tabla 2; Capítulo 3, Tabla 2; Capítulo 4, Tabla 2), el GY (Capítulo 4, Tabla 2), los parámetros de intercambio de los gases como la conductancia estomática (g_s), la transpiración (E), la asimilación neta (A_{sat}), y el cociente concentración intercelular de CO_2/concentración de CO_2 ambiental (C_i/C_a) (Capítulo 2, Tabla 3; Capítulo 3, Tabla 2), así como $\delta^{13}C$ y $\delta^{18}O$ de los granos (Capítulo 4, Tabla 3) (Araus y col., 2013), espigas (Capítulo 2, Tabla 4) (Cabrera-Bosquet y col. , 2009), hojas bandera (Capítulo 2, Tabla 4; Capítulo 3, Tabla 2; Capítulo 4, Tabla 3) y las raíces (Capítulo 2, Tabla 4) (Elazab y col., 2012), mientras que la eficiencia instantánea del uso del agua (WUE_{Inst}) (Capítulo 2, Tabla 3; Capítulo 3, Tabla 2) disminuía (Cabrera-Bosquet y col., 2009; Elazab y col., 2012).

Al contrario, en el Capítulo 3, la correlación obtenida entre el $\delta^{13}C$ y la biomasa aérea dentro de cada régimen del agua (tabla 4) fue positiva. Un elevado nivel de nitrógeno (HN) aumentó la biomasa aérea pero disminuyó la biomasa y la longitud de la raíz (Tabla 1). Esto dio lugar a un efecto negativo sobre el estado hídrico, disminuyendo el g_s, y la tasa de transpiración foliar por unidad de área,y en menor medida el C_i/C_a. Como consecuencia la WUE_{Inst}, WUE_{AB} y $\delta^{13}C$ aumentaron (Tabla 2). En este sentido, con independencia del régimen hídrico o para cualquiera de los grupos de genotipos estudiados, cuando se combinaron los dos regímenes de N, la biomasa aérea correlacionaba positivamente con $\delta^{13}C_{FL,}$ WUE_{AB} y WUE_{Inst} y todos estos parámetros correlacionaban negativamente con g_s (Tabla 4). Según Cabrera-Bosquet y col. (2007) en un experimento en macetas en trigo duro y según Kondo y col. (2004) en arroz cultivado en condiciones de campo una alta fertilización de nitrógeno aumenta la biomasa aérea, pero también induce un déficit hídrico, que conlleva una disminución de g_s mientras que la $\delta^{13}C$ aumenta. Así, la correlación positiva obtenida en este estudio entre el $\delta^{13}C_{FL}$ y la biomasa aérea dentro de cada régimen del agua reveló que la interacción entre los regímenes hídrico y de nitrógeno afectó el comportamiento del $\delta^{13}C$ como carácter indirecto para la mejora genética en condiciones de sequía.

El resultado del Capítulo 5 demostró que la δ^{13}C de los granos podía detectar diferencias en condiciones del crecimiento entre los tratamientos térmicos (tabla 3). Sin embargo, una correlación negativa significativa pero débil fue observada entre el δ^{13}C y GY solamente en el año 2011 (Tabla Suplementaria 2). Sin embargo la δ^{13}C del control era más negativa (Tabla 3) que las observaba bajo condiciones de estrés, lo que no apoya la existencia de un peor estado hídrico en los tratamientos térmicos, (Araus y col., 2010; Cabrera-Bosquet y col., 2009b; Henderson y col., 1999).

Los resultados del Capítulo 2 revelaron la importancia del δ^{18}O de la hoja bandera para predecir la AB (figura 4b) y la g_s (Figura 3b) en condiciones hídricas no limitantes y la T_{cum} (Figura 2c) en condiciones de déficit hídrico. Mientras que otros estudios (Capítulos 3 y 4) revelaban dudas en el uso de δ^{18}O como carácter fenotípico para mejora genética, e studios anteriores (Ferrio y col., 2007; Cabrera-Bosquet y col., 2009b; Elazab y col. , 2012; Araus y col., 2013) han sembrado dudas sobre el uso de $\delta^{18}O$ para evaluar el rendimiento de los genotipos de trigo bajo diversos regímenes de agua debido a un número de factores misceláneos (e.g. la $\delta^{18}O$ del agua que capta la planta, el intercambio de ^{18}O con agua durante la formación de asimilados y el fraccionamiento metabólico durante el transporte de los asimilados) que pueden afectar negativamente la utilidad del $\delta^{18}O$ como indicador del estado hídrico de la planta.

Características de las raíces

Los resultados de los Capítulos 2 y 3 refuerzan la importancia de un sistema radicular extenso para capturar más recursos del suelo. En este sentido, la biomasa y la longitud de la raíz y su distribución (RWD y RLD, respectivamente) a través del perfil del suelo, correlacionaron positivamente con la T_{cum} (Capítulo 3, Tablas 4 y 5) y la $\delta^{13}C$ y $\delta^{18}O$ (Capítulo 2, Figura 6; Capítulo 3, Tablas 4 y 5). Los resultados del Capítulo 2 (CFigura 5 y CFigura suplementaria 3) revelaron la importancia de un sistema radicular más extenso (representado por RWD y RLD) para alcanzar una AB mayor, especialmente en condiciones de déficit hídrico.

Sin embargo, el desarrollo y manteniendo de un sistema radicular extenso (es decir, una elevada biomasa de raíz) consume una gran cantidad de asimilados producidos por la planta (Passioura, 1983) a la vez que puede comportar un elevado coste respiratorio (McCree, 1986; Ma y col., 2010). En este sentido algunos estudios han sugerido que un aumento en el tamaño del sistema radicular no comporta necesariamente una ventaja que se traduzca en una AB y GY más elevados bajo condiciones del estrés hídrico (Bruce y col., 2002; Lynch y col., 2014).

Los resultados del Capítulo 3 apoyan la idea de que un sistema radicular extenso no necesariamente mejora el rendimiento de la planta bajo condiciones de estrés hídrico, porque el AB correlacionó negativamente con la biomasa y la longitud de la raíz (Tabla 4) y su distribución (RWD y RLD, respectivamente) en las diferentes capas del suelo (Tabla 5). También, la biomasa de la raíz y la longitud de la raíz mostraron correlaciones negativas con el cociente entre la biomasa aérea y la biomasa de la raíz (para cualquiera de las condiciones hídricas de crecimiento) y con la WUE_{AB} (solamente bajo condiciones de estrés hídrico), mientras que estos dos parámetros correlacionaron positivamente entre sí en condiciones de estrés hídrico (Tabla 4). Por otra parte, en condiciones de estrés hídrico, RWD y RLD mostraron correlaciones negativas con WUE_{AB} a través todo el perfil del suelo (Tabla 5). De hecho, un gran crecimiento de la raíz en los estadios de desarrollo tempranos de la planta puede también ser negativo al agotar las reservas de agua del suelo antes de que la planta pueda terminar su ciclo vital (Richards y Passioura, 1989; Lynch y col., 2014). A partir de los resultados de los Capítulos 2 y 3 podríamos concluir que la selección del sistema óptimo de la raíz para una elevada producción del AB requiere considerar los costes asociados a la captura del agua en términos de recursos del carbono (costes metabólicos para la construcción y el mantenimiento de la raíz en relación a la capacidad del sistema radicular para capturar agua.

Detección remota

En el Capítulo 4, se destaca la superioridad de NGRDI comparado con el NDVI medido en estadios tempranos y avanzados para determinar el GY en trigo duro bajo diversos regímenes de agua (tabla 5). En el caso del maíz (Capítulo 5), NGRDI también correlacionó mejor con la AB y el GY que el NDVI, con independencia del tratamiento térmico y el año de crecimiento (Figuras. 2-5). La razón de la superioridad del NGRDI con respecto al NDVI puede ser debido a las diferencias en su formulación. Mientras que el NDVI utiliza la luz reflejada en las regiones visibles (VIS) (generalmente rojo) e infrarroja cercanas (NIR) del espectro, el NGRDI se formula solamente usando la región del VIS (verde y rojo). Por el contrario, el NDVI usa la reflectancia del NIR que está afectada por factores (misceláneos) tales como estructura del dosel vegetal, la anatomía foliar, estructura celular y la humedad del suelo (Gitelson y col., 2002; Gitelson y Merzlyak, 1997). También, la reflectancia del NIR no es sensible a la presencia o a la ausencia de los pigmentos de la clorofila (Gitelson y col., 2002; Knipling, 1970).

Conclusiones generales

- el δ^{13}C es un carácter de fenotipeado eficaz que informa del estado hídrico de la planta (g_s, T_{cum}), crecimiento vegetal (A_{sat}, AB, GY) y eficacia del uso del agua (A_{sat}/E, A_{sat}/g_s, AB/T_{cum}). Sin embargo, la interacción de diferentes condiciones de crecimiento (e.g. regímenes del agua y del nitrógeno) pueden cambiar la naturaleza del $\delta^{13}C$ como carácter de fenotipeado para condiciones de resistencia de la sequía.
- En cambio, el uso del δ^{18}O, especialmente en granos maduros, como predictor del estado hídrico de la planta (g_s, T_{cum}) y el crecimiento vegetal (AB, GY) no es prometedor; probablemente debido a los factores misceláneos que afectan a la firma isotópica del^{18}O en la planta.
- Un aumento en la biomasa y longitud de la raíz, RWD y RLD mejoran la captación del agua del suelo, especialmente en condiciones de estrés hídrico.
- El índice de vegetación derivado de las imágenes digitales NGRDI funcionó mejor que el NDVI medido con un espectroradiómetro para la detección de

variabilidad en el GY y la AB del trigo duro en condiciones de campo bajo distintos regímenes hídricos.

- El NGRDI también funcionó mejor que el NDVI medido con una cámara multiespectral, a la hora de detectar variabilidad en GY y AB en condiciones de campo tanto, en ausencia de estrés térmico como en respuesta al tratamiento térmico post-floración. Sin embargo, ambos índices no fueron capaces de predecir variaciones del GY en respuesta a un estrés térmico pre-floración debido a que el efecto de la temperatura sobre el cuajado del grano no se podría detectar por los índices de vegetación diseñados para monitorear diferencias en área verde.

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