# Leaf photosynthesis traits and associations with biomass and drought tolerance in amphidiploid and ancestral wheat genotypes

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## Abstract

Future progress in wheat grain yield will depend on increasing photosynthesis capacity and biomass. Wheat wild relatives represent sources of novel genetic variation for photosynthesis traits. Our objective was to quantify variation in leaf photosynthesis traits, biomass and grain yield in a panel of diverse germplasm comprising: i) elite winter bread wheat (*Triticum aestivum* L.) lines, ii) wheat amphidiploid lines and iii) accessions of two wheat wild relatives *Th. bessarabicum* and *Aegilops speltoides* under well watered and drought conditions. A field experiment was carried out in one season examining 94 bread winter wheat Savannah × Rialto (S ×R) doubled-haploid (DH) lines under irrigated and unirrigated conditions; and two glasshouse experiments were carried out examining: i) six bread wheat S × R DH lines, ii) seven amphidiploid lines (durum wheat (*Triticum turgidum* subsp. *Durum*) × *Thinopyrum bessarabicum* crosses) and iii) three accessions each of *Th. bessarabicum* and *Aegilops speltoides* under well-watered and drought conditions. In the field experiment, drought reduced grain yield by 22% (P<0.001). Flag-leaf photosynthetic rate ($A_{max}$),~~ and~~ stomatal conductance ($g_s$) and grain $\Delta^{13C}$ were each positively associated with grain yield in the unirrigated treatment (*P*<0.05). This implied that the basis of the higher grain yield amongst genotypes under drought was high stomatal conductance.– In the glasshouse experiments, biomass plant$^{-1}$ was higher in *Ae. speltoides* accessions (54.7 g plant$^{-1}$) and the amphidiploid lines (50.8 g plant$^{-1}$) than the bread wheat S × R lines (40.9 g plant$^{-1}$) (*P*<0.001) under drought. Flag-leaf $A_{max}$ was higher in the *Th. bessarabicum* accessions and the amphidiploid lines than the bread wheat S × R lines under both irrigation and drought conditions (*P*<0.05). There was a positive association between flag-leaf specific weight (FLSW) and flag-leaf $A_{max}$ across the 19 genotypes in both irrigated and unirrigated conditions in the glasshouse experiments; there was no association between flag leaf area and flag-leaf specific weight. Our results indicated that *Th. bessarabicum* accessions and amphidiploid lines (durum wheat × *Th. bessarabicum*) offer novel variation for high expression of leaf photosynthesis rate for introgression into bread wheat genotypes to support genetic gains in biomass.

**Keywords**: wheat, photosynthesis rate, *Triticum aestivum*, amphidiploids, *Aegliops speltoides*, *Thinopyrum bessarabicum*.

## 1. Introduction

Worldwide, drought limits agricultural productivity more than any other single factor (Mahmood et al., 2019). Bread wheat (*Triticum aestivum* L.) provides, on average, one-fifth of the total calorific input of the world's population. Therefore, new cultivars with higher yield potential must be developed to maintain food security (Hassan et al., 2019) but also with enhanced drought resistance to combat the increased frequency and severity of drought now arising from climate change (IPCC, 2022).

Genetic improvement of wheat depends on utilizing variation within a broad pool of functional genetic resources. Because of reduced genetic diversity during domestication, wild relatives of wheat and lines derived from them represent sources of novel genetic variation for resource-use efficiency traits (Dubcovsky and Dvorak, 2007; Reynolds et al., 2007). Moreover, wheat wild relatives are generally grown in areas with harsh climatic conditions where dry conditions prevail and are well adapted to these conditions. Amphidiploid lines developed from crosses between wheat and related species from the genera *Triticum, Aegilops* and *Thinopyrum* have been reported to have high expression of traits related to abiotic stress tolerance (Nemeth et al., 2015; Nehe et al., 2022).

Any genetic gains in biomass production will require an increase in photosynthetic carbon fixation (Murchie et al., 2009; Parry et al., 2011). Historically several studies did not show an association between genetic variation in flag-leaf light-saturated photosynthetic rate ($A_{max}$) and grain yield in wheat (Richards, 2000; Calderini et al., 1995). However, associations between leaf photosynthetic rate or associated traits (e.g. stomatal conductance and C isotope discrimination) and grain yield progress have been observed in the last decades, e.g. in eight spring wheat cultivars in Mexico (Fischer et al., 1998), in 18 winter wheat cultivars in China (Jiang et al., 2003) and in 18 facultative wheat cultivars in China (Zheng et al., 2011). Driever et al. (2014), however, reported that for 64 cultivars grown in the UK flag-leaf photosynthesis in the pre-anthesis phase was not well correlated with above-ground biomass or grain yield. Genetic variation in flag-leaf $A_{max}$ has been associated with flag-leaf N content (Austin et al., 1982) and chlorophyll content (Gaju et al.,~~,~~ 2016) in wheat and flag-leaf leaf Rubisco content in rice (Hubbart et al., 2007).

Grain yield under drought was positively associated with flag-leaf stomatal conductance amongst 17 UK winter wheat cultivars, and negatively associated with flag-leaf transpiration efficiency (Foulkes et al., 2016). The ability of plants to transpire more water and maintain leaf photosynthesis rate during grain filling appears to be a key factor in determining higher grain yield under UK drought conditions droughts (Aravinda-Kumar et al., 2011). Similar findings were reported previously under Mediterranean-type droughts (Araus et al., 2001, 2003; Blum, 2009). Enhanced leaf photosynthetic capacity should benefit yields under these types of drought where significant amounts of rainfall occur within the season. Improved drought performance has also been associated with stay-green traits. For example, genetic variation in the stay green trait quantified by Normalized Difference Vegetation Index (NDVI) was positively associated with grain yield in wheat synthetic-derivatives, landraces and elite cultivars in UK rainfed conditions (Gaju et al., 2016) and in synthetic-derived wheat under drought in NW Mexico (Lopes and Reynolds, 2012). Genetic variation in flag-leaf green area duration (stay-green) has also been associated with grain yield under drought in wheat (Gorny and Garczynski, 2002; Verma et al.,2004; Foulkes et al., 2007; Christopher et al., 2008), barley (Mahmood, 2020), sorghum (Borrell and Hammer, 2000) and maize (Campos et al., 2004).

This study aimed to quantify novel genetic variation for leaf and canopy traits~~stomatal aperture traits and~~ and associations with grain yield, above-ground biomass under well-watered and water-stressed conditions. The potential of *Thinopyrum bessarbicum* accessions and derivatives as source of high expression for these traits was assessed compared to modern hexaploid bread wheat and the diploid wheat ancestor *Aegilops speloitdes* for the first time.~~ by~~ This was done by screening three groups of germplasm: i) elite bread wheat lines of a Savannah x Rialto doubled-haploid (DH) population, ii) wheat amphidiploid lines (durum wheat *Triticum turgidum* subsp. *durum* × *Thinopyrun bessarabicum* crosses) and iii) wheat wild relative accessions of *Aegilops speltoides* and *Thinopyrum bessarabicum*.

## 2. Materials and methods

### *2.1. Field experiments*

#### *Experimental design and plot management*

A doubled-haploid (DH) population of 94 lines, derived from the $F_1$ between UK winter wheat cultivars Savannah and Rialto, was characterised in one field experiment in 2012-13 (referred hereafter as 2013). Both parents (Rialto and Savannah) are semi-dwarf (Rht-D1b) UK winter wheat and hard endosperm cultivars. Rialto is suitable for some bread-making processes, and first released in 1995. Savannah is a feed wheat cultivar with high yield potential and was first released in 1998. The experiment used a randomised block, split-plot design, in which two irrigation treatments (fully irrigated and unirrigated) were randomised on main-plots, and the DH lines and the two parents were randomised on sub-plots (1.62 m x 6 m) with two replicates. There were 12 rows per sub-plot with a row spacing of 13.5 cm.

The field experiment was located at the University of Nottingham Farm, Leicestershire, UK (52° 50' N, 1° 15' W, 50 m above sea level). The soil type was a sandy loam (63% sand, 18% silt, 19% clay) to 80 cm over kyper marl clay Dunnington Heath Series. A total of 180 kg N ha$^{-1}$ of N fertilizer as ammonium nitrate was applied. In the irrigated main-plots, water was applied using a linear overhead irrigator to maintain soil moisture deficit to 50% available water capacity (180 mm). 168 mm of water was applied from early booting to late grain-filling stage (20 mm in May, 43 mm in June and 105 mm in July). The previous crop was winter oats, the sowing date was 10 October 2012 and the seed rate was 350 seeds m$^{-2}$. The metrological data for air temperature, rainfall and solar radiation were collected from the nearby meteorological station at Sutton Bonington Campus within 0.5 km of the experiment. Fungicides, herbicides and pesticides were applied as required to minimize the effects of diseases, weeds and pests.

#### ~~*Crop measurements*~~

~~Flag-leaf gas-exchange and grain C isotope discrimination measurements were carried out for a subset of six DH lines from the S x R DH population (selected based on high/low flag-leaf transpiration efficiency according to grain carbon isotope discrimination ($\Delta^{13}C$) analysis from previous field experiments (Foulkes, personal communication)) and the two parents. All other measurements were carried out for all 94 DH lines and the two parents.~~

#### *Grain yield, biomass, anthesis date and plant height*

Date of anthesis (GS61) was recorded in all sub-plots, as when more than 50 % of the shoots were at anthesis GS61 (Zadoks et al., 1974). One to two days before combine harvest, 75 fertile shoots per sub-plot were hand-harvested randomly within the sub-plot at ground level. In the laboratory, shoots were separated into fertile (those with an ear) and infertile shoots and counted. The fertile shoots were separated into ears and straw. After threshing the ears using a Wintersteiger KG threshing machine (Wintersteiger, Austria), the dry weight of all plant components (grain, chaff and straw) was separately recorded after drying for 48 h at 80ºC. The sub-plots were machine-harvested on an area of at least 5 $m^2$, and grain yield then adjusted to 85% DM. Above-ground dry matter per $m^2$ (AGDM) was calculated by dividing the machine-harvested grain yield by the harvest index (ratio of grain dry weight to above-ground dry weight) calculated from the measurements on the 75-shoot sample. The plant height was measured 1-2 days before harvest from ground level to the tip of the ear for three randomly selected fertile shoots per sub-plot.

### *Flag-leaf gas exchange*

Flag-leaf gas-exchange and grain C isotope discrimination measurements were carried out for a subset of six DH lines from the S x R DH population (selected based on high/low flag-leaf transpiration efficiency according to grain carbon isotope discrimination ($\Delta^{13}C$) analysis from previous field experiments (Foulkes, personal communication)) and the two parents. All other measurements were carried out for all 94 DH lines and the two parents.

Gas-exchange measurements of light-saturated photosynthetic rate ($A_{max}$) and stomatal conductance ($g_s$) were taken on flag leaves for the subset of six S × R DH lines and the two parents under unirrigated conditions on four dates between GS61 to GS61+21 days (19 and 27 June and 4 and 15 July) using a LiCor 6400-XT Photosynthesis system (LiCor NE, USA). Measurements were taken for three flag-leaves per sub-plot when the leaf surface was well illuminated from 11.00 to 14.00. Conditions in the leaf chamber were set as: cuvette temperature 20ºC, flow rate 500 µmol $s^{-1}$, $CO_2$ concentration 400 µmol $mol^{-1}$ and artificial light supply (PAR) 2000 µmol $m^{-2}$ $s^{-1}$ (PQuantum 500 µm, 10% blue).

### *NDVI and flag-leaf senescence*

The Normalized Difference Vegetative Index (NDVI) was measured using a Cropscan, Inc. spectral radiometer MSR16R model with 16 sensor bands in the region 450-1750 nm. Measurements were taken at approximately GS61+3 weeks, GS61+4 weeks and GS61+5 weeks for all 94 DH lines and the two parents in the unirrigated treatment in two replicates. The spectroradiometer sensor was held approximately 50 cm above the crop and readings were taken between 11.00h and 15.00h when the sky was clear and there was sufficient radiation (Pask et al., 2012). NDVI was then calculated as Eq. 1 (Gutiérrez-Rodríguez et al., 2004).

$$\mathrm{NDVI} = (R900-R680)/ (R900+R680)) \qquad \text{Equation 1}$$

Flag-leaf senescence was measured for all sub-plots from anthesis (GS61) to full senescence every 3-4 days using a visual senescence score chart ranging from 0 - 10 (0; fully green and 10; fully senesced), as described by Gaju et al. (2011). Visual assessments were carried out for the 94 DH lines and the two parents in the irrigated and unirrigated treatments in two replicates, and values fitted against thermal time (GS61; base temperature 0°C) applying a logistic regression equation:

$$Y = A + C / (1 + e^{(-B \times (X - M))}) \qquad \text{Equation 2}$$

where Y is the visual senescence score; X is thermal time from GS61 (base temp. 0°C); M is thermal time for the point of inflection,~~and~~ B is the slope at the point of inflection, taken as the rate of senescence, and A+C is the upper asymptote. The onset of leaf senescence ($\mathrm{SEN_{ONSET}}$) was taken as the thermal time (base temp. 0°C) post-anthesis (GS61) at leaf visual senescence score 2 and end of leaf senescence ($\mathrm{SEN_{END}}$) as thermal time at score 9.5. Values were calculated for each sub-plot and the fitted values subjected to ANOVA.

### *Carbon isotope discrimination*

Carbon isotope discrimination ($\Delta^{13}C$) of grain was measured for the subset of six S x R DH lines in the unirrigated treatment using a mass spectrometer for $\Delta^{13}C$ determination. Hand-harvested grains were oven dried for 48 h at 80 °C and milled to a fine powder using an Ultra Centrifugal Mill ZM 200 (Retsch, Germany). The milled samples (1 mg) were then weighed out in tin cups and analysed through an online system composed of an elemental analyser (EA), a TripleTrap and a mass spectrometer (Carlo Erba 2100, Milan, Italy) to determine carbon isotope composition (Aravinda Kumar et al., 2011). The EA interfaced with an isotope ratio mass spectrometer (IRMS; Thermo-Finnigan Deltaplus Advantage, Bremen, Germany) to analyse $^{13}C$:$^{12}C$ ratio (R) of plant material. Results were expressed as $\delta^{13}C$ composition values, using a secondary standard calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB), and the analytical precision was ~0.1‰ (Eq. 3).

As described by Farquhar et al. (1989), the value of $^{13}C/^{12}C$ ratio (R) was expressed as carbon isotope composition (δ13C), calculated as:

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

where the standard used for calibration was Vienna Pee Dee Belemnite calcium carbonate (VPDB), and the analytical precision was ~0.1‰.

Then, $\delta^{13}C$ was converted to values of discrimination (Δ) against $^{13}C$ ($\Delta^{13}C$) which is calculated as:

$$\Delta^{13}C = (\delta a - \delta p)/\ (1 + \delta p) \qquad \text{Equation 4}$$

where 'a' refers to the C isotope composition of air and 'p' to plant, and the carbon isotope composition of air was taken as -8‰.

### *2.2. Glasshouse experiments*

#### *Experimental design and treatments*

Two glasshouse experiments were conducted, one in each of 2012-13 (referred hereafter as 2013) and 2013-14 (referred hereafter as 2014) at the University of Nottingham, Sutton Bonington Campus, UK (52° 50' N, 1° 15' W). Nineteen wheat genotypes were examined comprising three groups of germplasm: (i) the two parents (Savannah and Rialto) and a subset of four DH lines from the S x R winter bread wheat population, (ii) seven amphidiploid lines derived from crosses between durum wheat cultivars and *Thinopyrum bessaribicum* (8388607) [cv Karim x *Th.*

*bessarabicum*, cv Stewart x *Th. bessarabicum*, cv Langdon x *Th. bessarabicum*, cv Macoun x *Th. bessarabicum*, cv Creso x *Th. bessarabicum*, cv Neodur x *Th. bessarabicum* and cv Azaziah x *Th. bessarabicum*] obtained from Germplasm Resource Unit at John Innes Centre (King et al., 1997); and (iii) three accessions of each wheat wild relative *Th. bessarabicum* (531711-4 (1), P208/552-1 (2) and 531712-50 (3)) and *Aegilops speltoides* (2140008 (1), 2140018 (2) and 487238 (3)) obtained from Germplasm Resource Unit at John Innes Centre.

A 'split-plot' randomized block design was used with two irrigation treatments (well-watered and water-stressed) and three replicates. Plants were grown in PVC columns (15 cm diam. × 50 cm). Seeds were sown on 13 December 2012 and 20 November 2013 and were initially vernalized in modular trays at 6 $^{o}$C and a 12 hour photoperiod in a controlled-environment room; seeds were vernalized for 62 days in 2013 and 77 days in 2014. Seedlings were transplanted into the columns (one plant per column) in a glasshouse. Columns were filled with soil medium (50% sand and 50% loam soil) and the column bottom was closed with MyPex polypropylene to allow drainage. The gravimetric soil water content (WC %) (Eq. 5) and soil bulk density (BD) (Eq. 6) at field capacity (FC) of the soil medium were measured as described by Rowell (2014):

$$\text{WC \%} = \frac{\text{Wet soil (g)} - \text{Oven dried soil (g)}}{\text{Wet soil (g)}} \times 100 \qquad \text{Equation 5}$$

$$\text{BD (g cm}^{-3}) = \frac{\text{Oven dried soil (g)}}{\text{Soil volume (cm}^{3})} \qquad \text{Equation 6}$$

The average of soil bulk density was 1.16 g cm$^{-3}$ in 2013 and 1.33 g cm$^{-3}$ in 2014. The average gravimetric water content (WC %) at field capacity was 17.4% in 2013 and 14.2% in 2014. Available water was considered to be half of the water content percentage at field capacity (Or and Wraith, 2002) and was 1.28 l per column in 2013 and 0.99 l per column in 2014.

In the well-watered treatment, plants were irrigated weekly to return columns to 90% of available water at field capacity (AWFC). In the water stressed treatment, water stress was applied by returning the soil water content of the columns to 50% AWFC from GS39 to GS61; and then 25% AWFC from GS61 to physiological maturity based on estimated evapotranspiration (gravimetric analysis). Columns were weighed weekly using a digital scale (model STW-60 KE).

Plants were initially irrigated with VITAX nutrient solution (Vitax Ltd, UK) diluted at a ratio of 1:200 at transplanting to avoid any nutrient deficiencies. Plants were sprayed with fungicide and insecticide as necessary to control fungal pathogens and aphids. The glasshouse had vents to control the temperature as close as possible to the outside temperature. There was supplementary heating to avoid frost during the experiments. The daily minimum and maximum temperature and humidity were recorded using a Tinytag Ultra 2 data logger (Gemini Data Loggers Ltd, UK) in both years.

*Plant measurements*

Dates of anthesis (GS61) and physiological maturity (GS89; taken as stem 90% yellow) on the main shoot were recorded in all plants. At harvest, individual plants were harvested at soil level and the ear and straw weights were recorded after drying at 80°C for 48 h for the fertile shoots (those with an ear); the dry weight of the main shoot was recorded separately from the other fertile shoots. The weight of the infertile shoots was recorded after drying at 80°C for 48 h. After hand-threshing the ears, the grain weight was recorded after drying for 48 h at 80°C separately for the main shoot and the remaining fertile shoots. Plant height was measured 1-2 days before harvest from soil level to the tip of the ear on the main shoot.

In both years, flag-leaf photosynthetic rate, stomatal conductance and transpiration efficiency were measured for all genotypes in the well-watered and water stressed treatments (in two replicates). Measurements were taken approximately every two weeks from booting (GS41) to mid-to-late grain filling (GS61+28 days) in 2013 (3, 20 May, 6 and 11 June) and in 2014 (1, 21 May, 10 and 19 June), using a LiCor 6400-XT Photosynthesis system (LiCor NE, USA). Measurements were taken for three flag-leaves per plant (main shoot and tillers 1 and 2), as described for the field measurements of $A_{max}$ and $g_s$.

In 2014, at GS61 the length and width of the flag leaf for the main shoot, the first tiller and the second tiller of each plant was recorded. Flag-leaf area was estimated by multiplying the product of the length and width (at the widest point) by a factor of 0.83 (Bryson et al., 1997). At GS61, the three flag-leaves were weighed separately from the other straw components after drying for 48 h at 80°C. The flag-leaf specific weight (FLSW) was then estimated as the ratio of the flag leaf weight at harvest to the leaf area at GS61 (Equation 5):

$$\mathrm{FLSW}\ (\mathrm{g\ m^{-2}}) = \frac{\text{leaf dry weight (g)}}{(\text{leaf length (m)} \times \text{leaf width (m)} \times 0.83)}$$ Equation ~~5~~7

*2.3. Statistical analysis*

For both field and glasshouse data, GenStat 19th Edition (VSN_International, Hemel Hempstead UK) was used for statistical analysis of variance (ANOVA) by applying a split-plot design where replicates were regarded as random effects and cultivar as fixed effects. The least significant difference (LSD) was calculated to compare means between specific treatments. The data were tested for normality using Shapiro-Wilk test prior ANOVA analysis, and non-normally distributed data were transformed by square root to improve the normality of the trait distribution, if necessary. For grain yield and biomass in the field experiments, a Restricted Maximum Likelihood (REML) variance-component model was used to analyse effects of season, irrigation and genotype and their interactions; this is an auto-regressive model fitted to the spatial correlations across the field. Pearson's correlation coefficient and linear regressions were calculated using mean data for replicates using Genstat version 19 (VSN_International, Hemel Hempstead UK). The GraphPad Prism 6.00 software package (GraphPad Software, 2015) was used for the logistic regression curve fitting analysis to calculate the flag-leaf senescence timing parameters using the mean data for replicates.

## 3. Results

### *3.1. Field experiment: S × DH population*

*Rainfall pattern*

Rainfall in the field season was overall close to the long-term mean- but was drier than average in April and May leading to mild drought conditions in the unirrigated treatment during the stem extension and early grain filling phases (Table 1).

Table 1. Monthly rainfall data (mm) for the Sutton Bonington site in 2012-13 and the long-term mean (LTM, 1964 - 2013)

| | Oct. | Nov. | Dec. | Jan. | Feb. | Mar. | Apr. | May | June | July |
|---|---|---|---|---|---|---|---|---|---|---|
| (2012-13) mm | 58 | 90 | 104 | 31 | 28 | 45 | 6 | 73 | 48 | 81 |
| LTM (1964-2013) mm | 62 | 56 | 58 | 53 | 39 | 42 | 46 | 45 | 60 | 59 |

*Grain yield, biomass and association with stay green traits*

Restricted water availability in the unirrigated treatment reduced grain yield by 3.0 t ha$^{-1}$ (-22.6%) compared to irrigated conditions ($P$<0.01). Above-ground DM was reduced from 2040 to

1673 g m$^{-2}$ (-18.0%). There was no effect of irrigation on anthesis date, but a slight reduction in plant height from 72.0 to 69.8 cm under drought ($P< 0.05$). There was an irrigation × genotype interaction for grain yield, but not for AGDM, plant height or anthesis date. There was also an interaction for HI, with the decrease under drought ranging amongst DH lines for 0.001 to 0.070 ($P< 0.05$). Grain yield reductions under drought ranged amongst DH lines from 1.72 to 4.03 t ha$^{-1}$ ($P<0.05$; Table ~~1~~2). Under drought, canopy NDVI during late grain filling (GS61+28d) was positively associated with grain yield ($R^2=0.29$; $P<0.001$), AGDM ($R^2=0.24$; $P<0.001$) and 1,000 grain weight ($R^2=0.07$; $P<0.01$; Fig. 1) (similar associations were observed for NDVI at GS61+21d and GS61+35d; data not shown).

~~Onset of flag-leaf senescence was positively correlated with grain yield and above-ground DM amongst the 94 DH lines under both irrigated and unirrigated conditions (P< 0.01, Fig. 2). However, the end of flag-leaf senescence was only associated with GY under unirrigated conditions (Table 32). Anthesis date was not associated with flag-leaf senescence timing under irrigated conditions, but under drought earlier anthesis date was associated with later senescence.~~

**Table ~~1~~2.** Mean, maximum, minimum for grain yield (85% DM), above-ground DM, plant height and anthesis date in irrigated (Irr) and unirrigated (Unirr) treatments for 94 Rialto x Savannah DH lines and values for the two parents in 2013; P values and standard error of the difference of the means (SED) for irrigation, genotype and interaction.

| | **Grain yield (85% DM, t ha$^{-1}$)** | | **Above-ground DM (g m$^{-2}$)** | | **Plant height (cm)** | | **Anthesis date (DAS)** | |
|---|---|---|---|---|---|---|---|---|
| | **Irr** | **Unirr** | **Irr** | **Unirr** | **Irr** | **Unirr** | **Irr** | **Unirr** |
| Mean | 13.3 | 10.3 | 2040 | 1673 | 72.0 | 69.8 | 250.8 | 250.8 |
| Max. | 14.5 | 11.4 | 2246 | 1853 | 86.0 | 85.8 | 253.5 | 254.2 |
| Min. | 11.9 | 9.2 | 1828 | 1462 | 58.1 | 54.4 | 248.8 | 248.9 |
| Rialto | 13.5 | 10.4 | 2127 | 1668 | 69.1 | 69.0 | 250.0 | 251.0 |
| Savannah | 13.6 | 11.3 | 2052 | 1740 | 72.1 | 71.0 | 251.0 | 251.6 |
| ***SED (df)*** | | | | | | | | |
| Irrigation (1) | 0.08$^{***}$ | | 15.6$^{***}$ | | 0.28$^{***}$ | | 0.07$^{ns}$ | |
| Genotype (95) | 0.35$^{***}$ | | 59.0$^{***}$ | | 1.72$^{***}$ | | 0.45$^{***}$ | |
| Irri. × Gen (95) | 0. 50$^{*}$ | | 84.0$^{ns}$ | | 2.44$^{ns}$ | | 0.63$^{ns}$ | |

$^{***}$denotes $P<0.001$; $^{**}P<0.01$ and $^{*}P<0.05$ significance levels; $^{ns}$ = not significant.

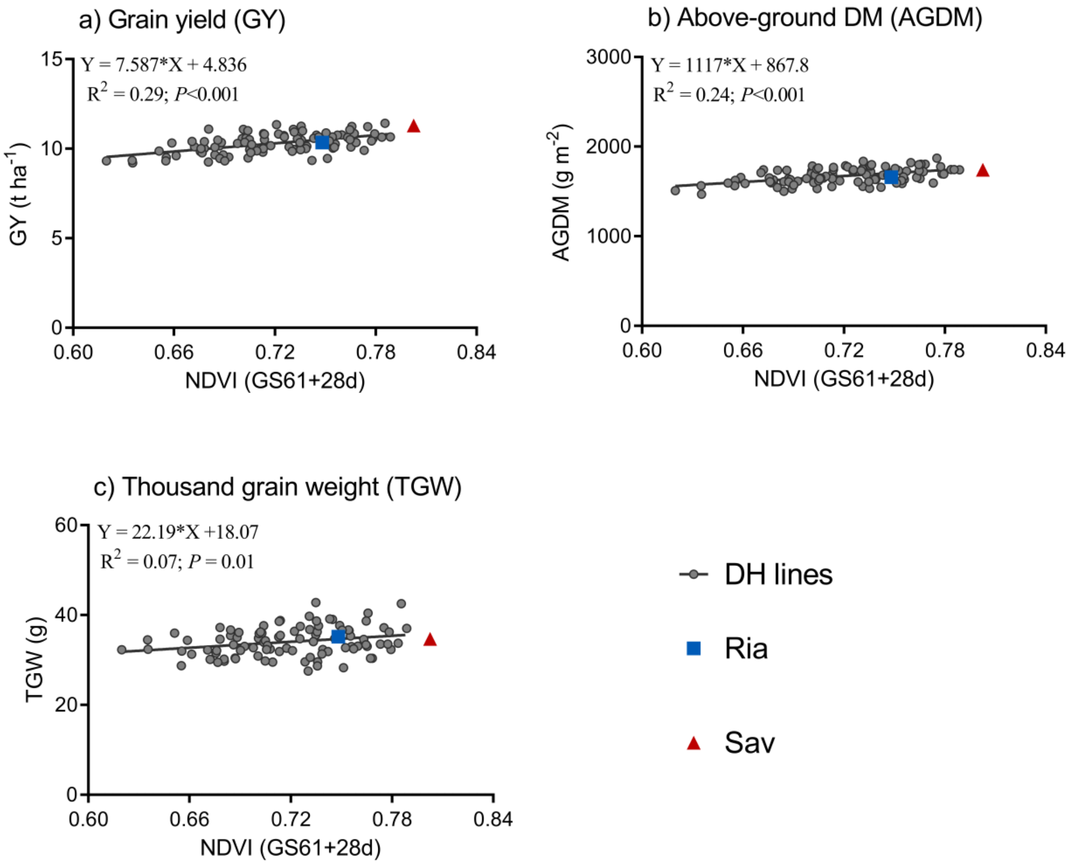


**Figure 1.** Linear regression of Normalized Difference Vegetation Index (NDVI) at GS61+28d) on (a) grain yield (85% DM), (b) above-ground dry matter (AGDM) and (c) thousand grain weight (TGW; 100% DM), under unirrigated conditions for 94 Rialto x Savannah DH lines. Rialto (■) and Savannah (▲) are also shown.

Onset of flag-leaf senescence was positively correlated with grain yield and above-ground DM amongst the 94 DH lines under both irrigated and unirrigated conditions (P< 0.01, Fig. 2). However, the end of flag-leaf senescence was only associated with GY under unirrigated conditions. Anthesis date was not associated with flag-leaf senescence timing under irrigated conditions, but under drought earlier anthesis date was associated with later senescence (Table 3).

**Table 3~~2~~.** The phenotypic correlation (r) of onset ($SEN_{ONSET}$) and end ($SEN_{END}$) of flag-leaf senescence with grain yield (GY; 85% DM), above-ground dry matter (AGDM) and anthesis date (GS61, DAS) among 94 Rialto x Savannah DH lines in 2013 under irrigated and unirrigated conditions.

| | Grain yield (85% DM, t $ha^{-1}$) | | Above-ground DM (g $m^{-2}$) | | Anthesis date (DAS; day) | |
|---|---|---|---|---|---|---|
| | Irr. | Unirr. | Irr. | Unirr. | Irr. | Unirr. |
| $SEN_{ONSET}$ (°Cd) | $0.40^{***}$ | $0.46^{***}$ | $0.32^{**}$ | $0.29^{**}$ | $-0.10^{ns}$ | $-0.55^{***}$ |
| $SEN_{END}$ (°Cd) | $-0.19^{ns}$ | $0.22^{*}$ | $0.00^{ns}$ | $0.11^{ns}$ | $0.0003^{ns}$ | $0.42^{***}$ |

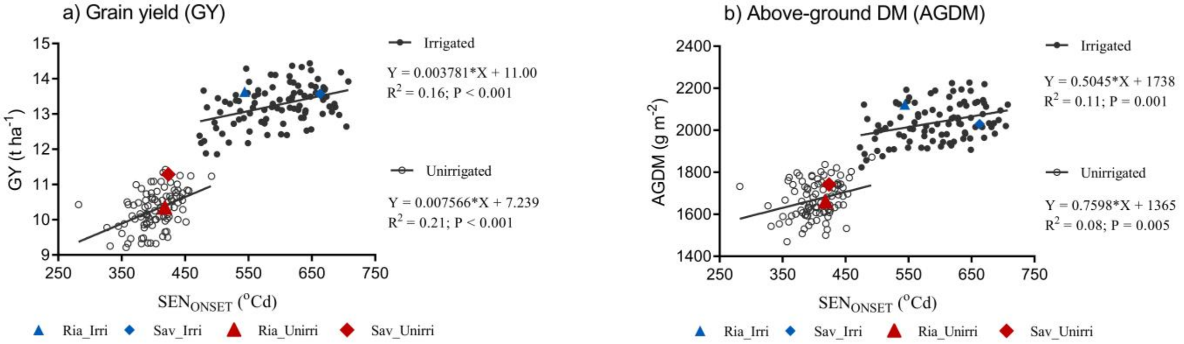


**Figure 2.** Linear regression of onset of flag-leaf senescence ($SEN_{ONSET}$) on a) grain yield (85% DM) and b) above-ground dry matter (AGDM) under irrigated and unirrigated conditions for 94 Rialto x Savannah DH lines. Rialto (■) and Savannah (▲) are also shown.

### 3.1.2. Flag-leaf stomatal aperture traits and associations with grain yield

For the subset of six S x R DH lines and the two parents under unirrigated conditions, flag-leaf $A_{max}$ at anthesis varied in the range 30.4 - 33.2 μmol $m^{-2}$ $s^{-1}$ (P = 0.09) and post-anthesis flag-leaf $A_{max}$ in the range 15.1 - 21.0 μmol $m^{-2}$ $s^{-1}$ (*P*< 0.05; Table ~~3~~4). At-anthesis, $A_{max}$ was positively associated with grain yield ($R^2$=0.55; P<0.001~~; Fig. 3~~), but there was no association post-anthesis. In addition, post-anthesis $A_{max}$ showed a positive association with grain $\Delta^{13}C$ (Figs. 3a, b, c and d).

There were genetic differences in post-anthesis flag-leaf stomatal conductance ($g_s$) in the range 0.17-0.26 mmol m$^{-2}$ ($P< 0.05$), but no differences at-anthesis under unirrigated conditions (Table 4). There was a positive linear relationship between $g_s$ at anthesis and grain yield ($R^2=0.59$; $P=0.03$). A positive linear relationship was also found amongst the eight genotypes between post-anthesis $g_s$ and grain $\Delta^{13}C$ ($R^2=0.63$; $P=0.02$~~; Fig. 4c~~3g). There was no association between post-anthesis $g_s$ and grain yield (Figs. 3e, f, g and h~~P=0.29~~).

**Table ~~3~~4.** At-anthesis and post-anthesis flag-leaf photosynthetic rate ($A_{max}$) and stomatal conductance ($g_s$) in unirrigated treatment for six Rialto × Savannah DH lines and Savannah and Rialto in 2013, and standard errors of the differences of the means (SED) and degrees of freedom (df).

| Genotypes | Flag-leaf $A_{max}$ (µmol m$^{-2}$ s$^{-1}$) | | Flag-leaf $g_s$ (mol $H_2O$) m$^{-2}$ s$^{-1}$) | |
|---|---|---|---|---|
| | At-anthesis | Post-anthesis | At-anthesis | Post-anthesis |
| Line 1 | 30.4 | 19.2 | 0.42 | 0.21 |
| Line 20 | 31.5 | 18.9 | 0.45 | 0.21 |
| Line 25 | 32.7 | 17.8 | 0.47 | 0.21 |
| Line 63 | 33.7 | 21.0 | 0.48 | 0.24 |
| Line 64 | 32.5 | 19.2 | 0.49 | 0.21 |
| Line 88 | 31.7 | 15.1 | 0.46 | 0.17 |
| Rialto | 31.6 | 17.1 | 0.49 | 0.21 |
| Savannah | 33.4 | 20.5 | 0.56 | 0.26 |
| *Mean* | 32.2 | 18.6 | 0.48 | 0.22 |
| *SED* ~~(df~~*Genotype*~~)~~ | | | | |
| ~~*Gen*~~ (*df*7) | 0.89$^{(0.09)}$ | 1.34$^{*}$ | 0.046$^{ns}$ | 0.013$^{**}$ |

N.B: $^{***}$denotes $P<0.001$; $^{**}P<0.01$ and $^{*}P<0.05$ significance levels; $^{ns}$ = not significant

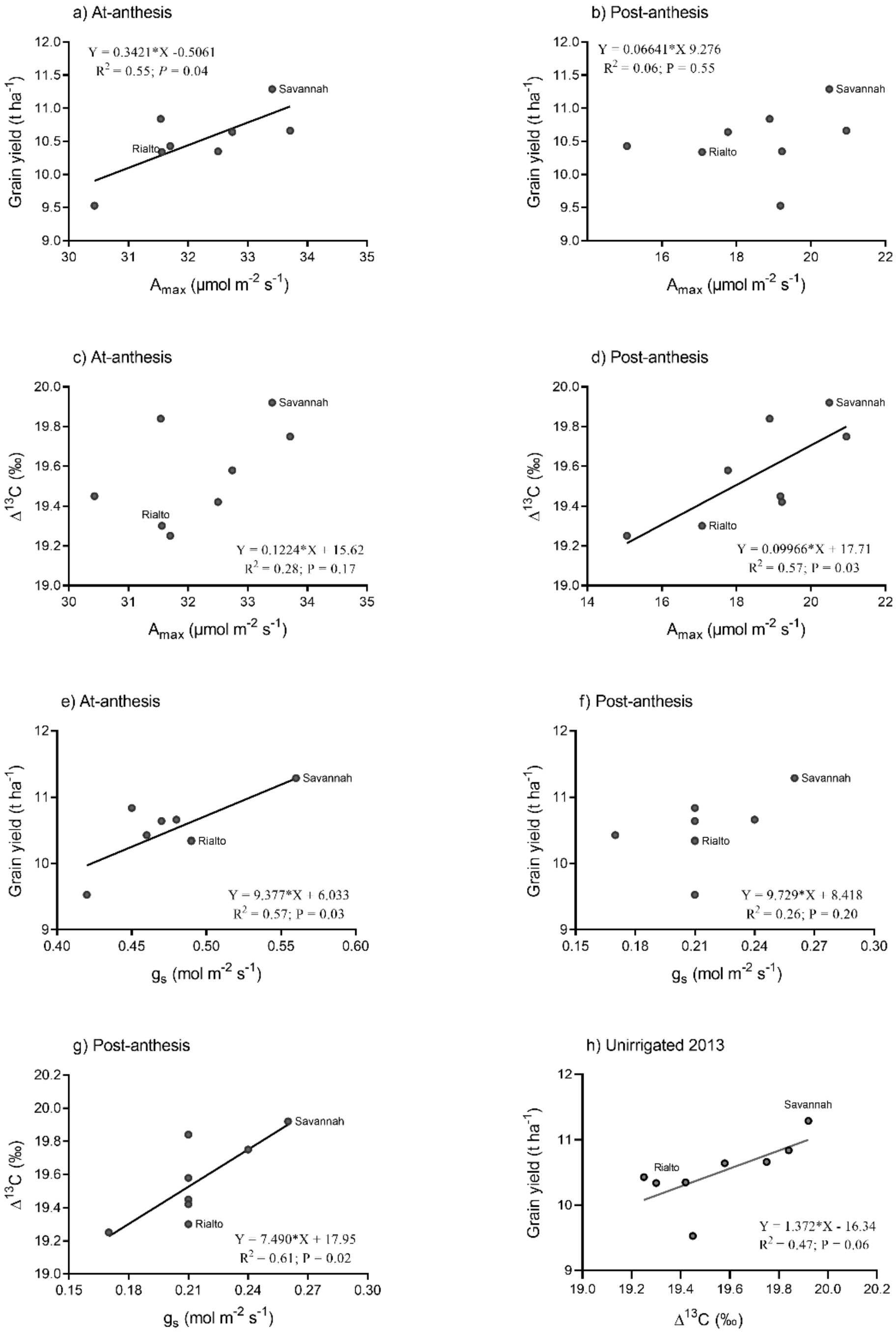
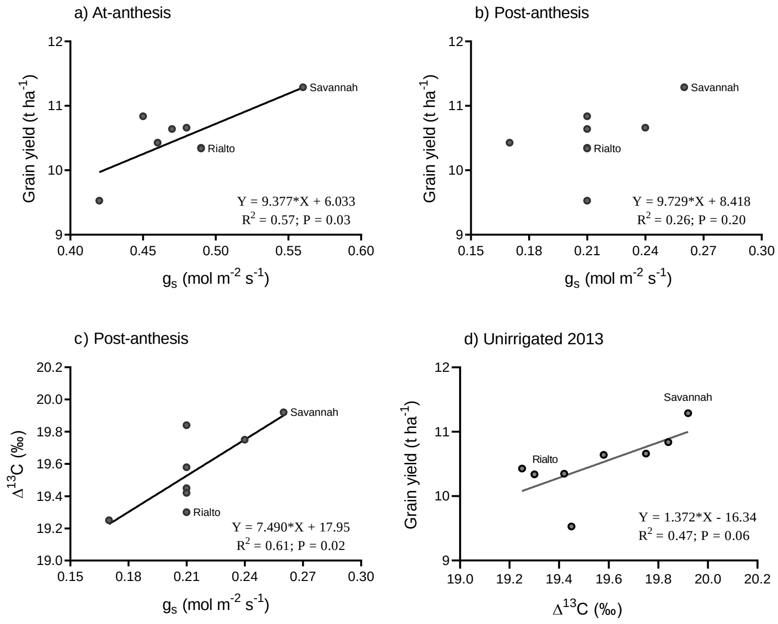
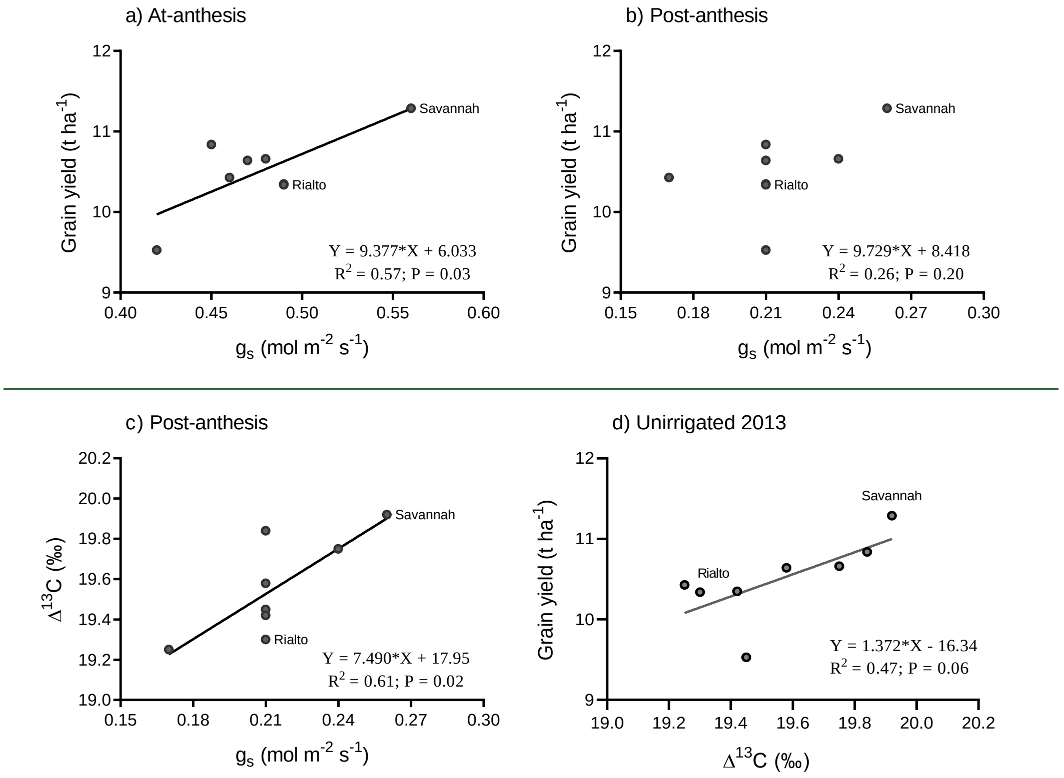


**Figure 3.** Linear regression of grain yield (85% DM) on flag-leaf photosynthetic rate ($A_{max}$) at (a) anthesis and (b) post-anthesis; and of grain C isotope discrimination ($\Delta^{13}C$) on flag-leaf photosynthetic rate ($A_{max}$) at (c) anthesis and (d) post-anthesis under unirrigated conditions; and linear regression of grain yield (85% DM) on flag-leaf stomatal conductance ($g_s$) at (e) at-anthesis (19 June) and (f) post-anthesis (mean 27 June and 4 and 15 July), and of post-anthesis grain $\Delta^{13}C$ (‰) on flag-leaf ($g_s$) (g) and of grain yield (85% DM) on grain $\Delta^{13}C$ (h) under unirrigated conditions for six Rialto x Savannah DH lines and Savannah and Rialto.



~~**Figure 3a).** Linear regression of grain yield (85% DM) on flag leaf photosynthetic rate ($A_{max}$) at (a) anthesis and (b) post-anthesis; and grain C isotope discrimination ($\Delta^{13}C$) on flag leaf photosynthetic rate ($A_{max}$) at (c) anthesis and (d) post-anthesis under unirrigated conditions for six Rialto x Savannah DH lines and Savannah and Rialto;~~



~~**Figure 4.** Linear regression of (a) at-anthesis (19 June) and (b) post-anthesis (mean 27 June and 4 and 15 July) of grain yield (85% DM) on flag-leaf stomatal conductance ($g_s$), (c) post-anthesis grain $\Delta^{13}$C (‰) on flag-leaf ($g_s$) and (d) grain yield (85% DM) on grain $\Delta^{13}$C under unirrigated conditions for six Rialto x Savannah DH lines and Savannah and Rialto.~~

### *3.2. Glasshouse experiments*

#### *Flag-leaf stomatal aperture traits*

Flag-leaf $A_{max}$ was measured in the glasshouse experiments for a subset of four S × R DH lines and the Rialto and Savannah parents, the seven amphidiploid lines (durum wheat x *Th. bessarabicum*) and three accessions each of *Th. bessarabicum* and *Ae. speltoides*. Post-anthesis flag-leaf $A_{max}$ measured in the glasshouse was positively associated with the field expression of post-anthesis $A_{max}$ amongst the four S x R DH lines and Rialto and Savannah ($P < 0.05$; Fig. ~~5~~4a). Similarly, a positive association was observed for flag-leaf post-anthesis $g_s$ between glasshouse and field measurements (P=0.05; Fig. ~~5~~4b).

Averaging across years under irrigated conditions, pre-anthesis flag-leaf $A_{max}$ was greater in the *Th. bessarabicum* accessions (range 21.2-26.9 µmol m$^{-2}$ s$^{-1}$) than in the amphidiploid lines (range 15.1-20.0 µmol m$^{-2}$ s$^{-1}$) or the bread wheat genotypes in the S × R DH subset (17.1-18.9 µmol m$^{-2}$

s$^{-1}$); $A_{max}$ for the *Ae. speltoides* accessions was lower than the three other groups in the range 13.8-16.7 µmol m$^{-2}$ s$^{-1}$ (Table ~~4~~5). Overall pre-anthesis $A_{max}$ was lower in the unirrigated treatment at 16.2 µmol m$^{-2}$ s$^{-1}$ than the irrigated treatment at 18.6 µmol m$^{-2}$ s$^{-1}$ ($P$<0.001). The irrigation × genotype interaction was not significant, and genotypes ranked similarly under irrigated and unirrigated conditions. Post-anthesis flag-leaf $A_{max}$ for *Th. bessarabicum* accessions was again higher (18.2-28.8 µmol m$^{-2}$ s$^{-1}$) than for the amphidiploid lines (17.2-20.2 µmol m$^{-2}$ s$^{-1}$) or the S × R genotypes (16.8-22.6 µmol m$^{-2}$ s$^{-1}$), with lowest values again observed for the *Ae. speltoides* accessions (13.4-16.7 µmol m$^{-2}$ s$^{-1}$) ($P$<0.001). Post-anthesis $A_{max}$ was reduced in the unirrigated treatment compared to the irrigated treatment at (19.1 versus 15.5 µmol m$^{-2}$ s$^{-1}$); the irrigation × genotype interaction was not statistically significant.

With regard to individual genotypes, amphidiploid line *Th. bessarabicum* × cv. Karim had higher pre-anthesis $A_{max}$ (19.6 µmol m$^{-2}$ s$^{-1}$ ) than the highest bread wheat genotype S × R DH line 63 (15.1 µmol m$^{-2}$ s$^{-1}$) under drought (P< 0.05), and was also higher than the two parents Savannah (16.9 µmol m$^{-2}$ s$^{-1}$) and Rialto (12.2 µmol m$^{-2}$ s$^{-1}$). This genotype was also the highest-ranking genotype for pre-anthesis $A_{max}$ under irrigation and drought out of the 13 bread wheat and amphidiploid genotypes.

For stomatal conductance generally similar genetic differences were observed between the groups as for $A_{max}$ under drought, with a positive association between $A_{max}$ and $g_s$ across all 19 genotypes in the three groups both pre-anthesis ($R^2 = 0.31$, $P< 0.05$) and post-anthesis ($R^2 = 0.53$, $P< 0.001$). However, the *Th. bessarbicum* accessions did not show higher pre-anthesis $g_s$ than the bread wheat genotypes as they did for pre-anthesis $A_{max}$. Under well-watered conditions, there was no overall association amongst genoptypes between $g_s$ and $A_{max}$ either pre-anthesis or post-anthesis. The bread wheat (0.52-0.64 mol m$^{-2}$ s$^{-1}$) and amphidiploid (0.39-0.69 mol m$^{-2}$ s$^{-1}$) genotypes had slightly higher post-anthesis $g_s$ than the *Th. bessarabicum* accessions (0.35-0.47 mol m$^{-2}$ s$^{-1}$). In the pre-anthesis period *Th. bessarabicum* accessions (0.42-0.49 mol m$^{-2}$ s$^{-1}$) showed higher $g_s$ than bread wheat genotypes (0.32-0.47 mol m$^{-2}$ s$^{-1}$ ) but had a similar range to the amphidiploid group (0.39-0.52 mol m$^{-2}$ s$^{-1}$). With regard to individual genotypes pre-anthesis $g_s$ was significantly higher for cv. Karim × *Th. bessarabicum* than any of the bread wheat genotypes under drought, similar to the high expression for $A_{max}$ observed for this amphidiploid line.

There were genotype differences in transpiration efficiency in both treatments both pre- and post-anthesis ($P$< 0.001; Table ~~5~~6). The amphidiploid lines overall showed a tendency for higher

TE than the bread wheat genotypes pre-anthesis under drought (4.58 vs 3.53 μmol mol$^{-1}$). Amphidiploid line cv. Macoun x *Th. bessarabicum* had higher TE (5.00 μmol mol$^{-1}$) than any of the bread wheat genotypes (range 2.70-4.14 μmol mol$^{-1}$) ($P< 0.05$). There was also a trend for the amphidiploids to show higher TE than the bread wheat genotypes post-anthesis under both drought (3.46 vs 2.97 μmol mol$^{-1}$) and well-watered conditions (3.87 vs 3.40 μmol mol$^{-1}$). There was a linear negative relationship between TE and $g_s$ post-anthesis under both drought ($R^2$ 0.27, $P< 0.10$) and well-watered conditions ($R^2=0.38$, $P< 0.01$), although there was no association between TE and $g_s$ for the pre-anthesis measurements.

**Table 45.** Pre-anthesis and post-anthesis flag-leaf photosynthetic rate ($A_{max}$) and stomatal conductance ($g_s$) for 19 genotypes (amphidiploid lines, R x S DH lines and wheat wild relatives) for the cross-year mean of 2013 and 2014 under well-watered (WW) and drought (DR) treatments, and standard errors of the differences of the means (SED) and degrees of freedom (df).

| | $A_{max}$ (μmol m$^{-2}$ s$^{-1}$) | | | | $g_s$ (mol m$^{-2}$ s$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-anthesis | | Post-anthesis | | Pre-anthesis | | Post-anthesis | |
| | WW | DR | WW | DR | WW | DR | WW | DR |
| *S × R DH pop* | | | | | | | | |
| Rialto | 17.2 | 16.94 | 20.61 | 15.03 | 0.35 | 0.31 | 0.58 | 0.28 |
| Savannah | 18.0 | 12.15 | 17.64 | 17.49 | 0.47 | 0.33 | 0.64 | 0.56 |
| Line 20 | 18.9 | 15.11 | 19.21 | 15.21 | 0.44 | 0.34 | 0.57 | 0.42 |
| Line 25 | 17.1 | 14.06 | 19.69 | 15.47 | 0.32 | 0.33 | 0.55 | 0.42 |
| Line 63 | 18.2 | 15.11 | 16.84 | 15.94 | 0.39 | 0.30 | 0.52 | 0.42 |
| Line 88 | 17.6 | 13.52 | 20.13 | 14.73 | 0.41 | 0.27 | 0.55 | 0.28 |
| *Amphidiploids* | | | | | | | | |
| *Th. bess.* x Creso | 20.0 | 17.16 | 20.12 | 15.74 | 0.47 | 0.34 | 0.49 | 0.28 |
| *Th. bess.* x Karim | 19.8 | 19.58 | 19.64 | 16.62 | 0.50 | 0.45 | 0.62 | 0.41 |
| *Th. bess.* x Langdon | 16.5 | 17.24 | 19.82 | 15.59 | 0.39 | 0.38 | 0.46 | 0.44 |
| *Th. bess.* x Macoun | 19.1 | 17.11 | 17.97 | 12.97 | 0.41 | 0.30 | 0.39 | 0.22 |
| *Th. bess.* x Neodur | 19.5 | 19.27 | 20.21 | 12.93 | 0.48 | 0.37 | 0.42 | 0.22 |
| *Th. bess.* x Stewart | 19.4 | 17.83 | 19.85 | 17.77 | 0.39 | 0.33 | 0.49 | 0.31 |
| *Th. bess.* x Azaziah | 15.1 | 14.93 | 17.18 | 14.57 | 0.52 | 0.38 | 0.69 | 0.37 |
| *Ancestral wheats* | | | | | | | | |
| *Th. bess.* 1 | 26.9 | 19.45 | 28.82 | 23.03 | 0.42 | 0.26 | 0.38 | 0.47 |
| *Th. bess.* 2 | 21.2 | 19.85 | 19.99 | 19.12 | 0.49 | 0.40 | 0.47 | 0.52 |
| *Th. bess.* 3 | 24.0 | 18.54 | 18.21 | 19.17 | 0.45 | 0.30 | 0.35 | 0.39 |
| *Ae. spelt.* 1 | 14.7 | 13.20 | 15.91 | 11.42 | 0.35 | 0.28 | 0.34 | 0.19 |
| *Ae. spelt.* 2 | 16.7 | 14.00 | 16.72 | 12.08 | 0.39 | 0.23 | 0.33 | 0.21 |
| *Ae. spelt.* 3 | 13.8 | 11.74 | 13.37 | 9.78 | 0.41 | 0.20 | 0.31 | 0.26 |
| Mean | 18.6 | 16.15 | 19.05 | 15.51 | 0.42 | 0.32 | 0.48 | 0.35 |
| SED (df) | | | | | | | | |
| Year (2) | $0.143^{***}$ | | $1.046^{*}$ | | $0.047^{ns}$ | | $0.109^{ns}$ | |
| Irrigation (2) | $0.510^{*}$ | | $0.268^{**}$ | | $0.010^{**}$ | | $0.021^{*}$ | |
| Genotype (72) | $1.613^{***}$ | | $1.188^{***}$ | | $0.046^{**}$ | | $0.056^{***}$ | |
| Irri. × Gen (72). | $2.279^{ns}$ | | $1.657^{ns}$ | | $0.064^{ns}$ | | $0.080^{*}$ | |
| Year × Gen. (72) | $2.225^{***}$ | | $1.941^{***}$ | | $0.079^{*}$ | | $0.133^{***}$ | |

$^{***}P<0.001$; $^{**}P<0.01$ and $^{*}P<0.05$ significance level; $^{ns}$ not significant.

Table ~~5~~6. Pre-anthesis and post-anthesis transpiration efficiency (TE) for 19 genotypes (amphidiploid lines, R x S DH lines and wheat wild relatives) for the cross-year mean of 2013 and 2014 under well-watered (WW) and drought (DR) treatments, and standard errors of the differences of the means (SED) and degrees of freedom (df)

| | Pre-anthesis TE | | Post-anthesis TE | |
|---|---|---|---|---|
| | WW | DR | DR | WW |
| | µmol mol$^{-1}$ | µmol mol$^{-1}$ | µmol mol$^{-1}$ | µmol mol$^{-1}$ |
| *Amphidiploids* | | | | |
| *cv. Cresso x Th. bessaribicum* | 4.02 | 4.32 | 3.92 | 4.10 |
| *cv. Karim x Th. bessarabicum* | 4.13 | 4.14 | 3.13 | 3.84 |
| *cv. Langdon x Th. bessarabicum* | 4.23 | 4.33 | 3.44 | 3.39 |
| *cv.* Macoun *x Th. bessarabicum* | 4.22 | 5.00 | 3.61 | 4.11 |
| *cv.* Neodur *x Th. bessarabicum* | 4.01 | 4.21 | 3.81 | 4.00 |
| *cv.* Stewart *x Th. bessarabicum* | 4.43 | 4.71 | 3.75 | 4.27 |
| *cv.* Azaziah *x Th. bessarabicum* | 2.93 | 3.71 | 2.56 | 3.35 |
| S x R DH pop | | | | |
| Rialto | 3.81 | 4.14 | 3.20 | 4.18 |
| Savannah | 3.77 | 2.70 | 2.80 | 2.97 |
| Line 20 | 3.85 | 3.52 | 2.96 | 3.12 |
| Line 25 | 4.32 | 3.40 | 3.06 | 3.06 |
| Line 63 | 3.65 | 3.70 | 2.72 | 3.29 |
| Line 88 | 3.80 | 4.04 | 3.09 | 3.82 |
| *Ancestral wheats* | | | | |
| *Th. bessarabicum* (1) | 4.59 | 4.98 | 5.97 | 4.02 |
| *Th. bessarabicum* (2) | 3.17 | 3.50 | 2.99 | 2.90 |
| *Th. bessarabicum* (3) | 4.10 | 4.05 | 4.42 | 3.37 |
| *Ae. speltoides* (1) | 2.77 | 3.20 | 3.42 | 3.27 |
| *Ae. speltoides* (2) | 3.07 | 4.14 | 3.65 | 3.44 |
| *Ae. speltoides* (3) | 2.55 | 4.32 | 3.70 | 3.45 |
| | | | | |
| *Mean* | 3.76 | 4.01 | 3.48 | 3.58 |
| *SED (df)* | | | | |
| *Year (2)* | 0.278* | | 0.517ns | |
| *Irrigation (2)* | 0.230ns | | 0.012* | |
| *Genotype (72)* | 0.359*** | | 0.348*** | |
| *Irr x Gen. (72)* | 0.546ns | | 0.479* | |
| *Year x Gen (72)* | 0.567*** | | 0.705*** | |

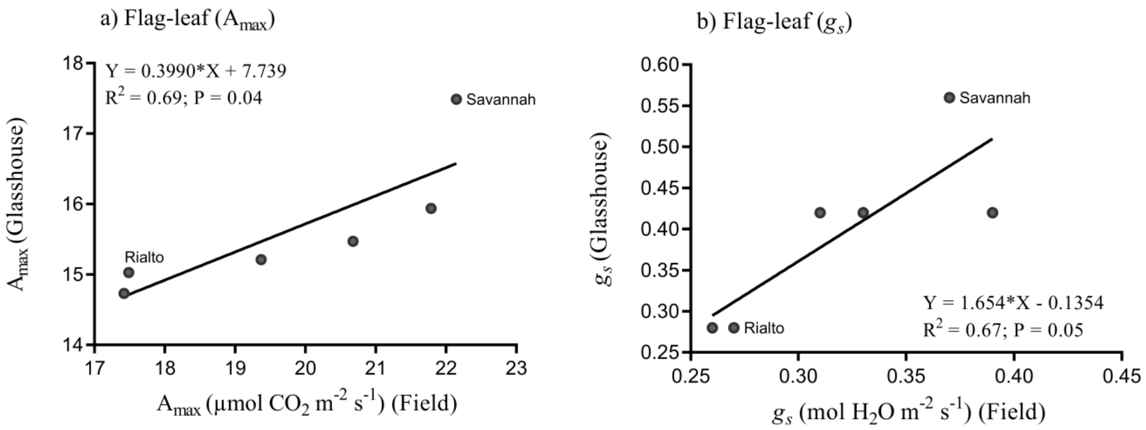


**Figure ~~5~~4.** Linear regressions of (a) glasshouse post-anthesis flag-leaf photosynthetic rate ($A_{max}$) in drought treatment (cross year mean) on field post-anthesis $A_{max}$ in the rain fed treatment and (b) glasshouse post-anthesis flag-leaf stomatal conductance ($g_s$) in drought treatment (cross year mean) on field post-anthesis $g_a$ in the rain-fed treatment for four Rialto x Savannah DH lines and Savannah and Rialto.

Flag-leaf specific weight and flag-leaf area were measured in 2014. *Th. bessarabicum* and *Ae. speltoides* accessions had smaller flag leaves than the other genotype groups (Fig. 5~~6~~). There was a strong positive association between flag-leaf specific weight (FLSW) and flag-leaf $A_{max}$ across the 19 genotypes in both irrigated ($R^2$=0.55, $P$<0.01) and unirrigated conditions ($R^2$=0.58; $P$<0.01; Fig. ~~7~~6a). The positive relationship between FLSW and $A_{max}$ was mainly driven by high values for FLSW and $A_{max}$ for *Th. bessarabicum* accessions and low values for the *Ae. speltoides* accessions. Excluding these two genotype groups, however, there was still a significant positive linear relationship between FLSW and $A_{max}$ amongst the remaining genotypes under unirrigated conditions ($R^2$=0.57, P<0.01), although not under irrigated conditions ($R^2$=0.14. P=0.21). There was no association between FLSW and flag-leaf area amongst the genotypes (Fig. 6~~7~~b). Averaging across years, positive linear relationships were found between pre-anthesis flag-leaf $A_{max}$ and pre-anthesis flag-leaf SPAD amongst genotypes under both well-watered and droughted conditions ($R^2$=0.79; $P$<0.001 and $R^2$=0.74; $P$<0.001, respectively; Fig. ~~8~~7a).

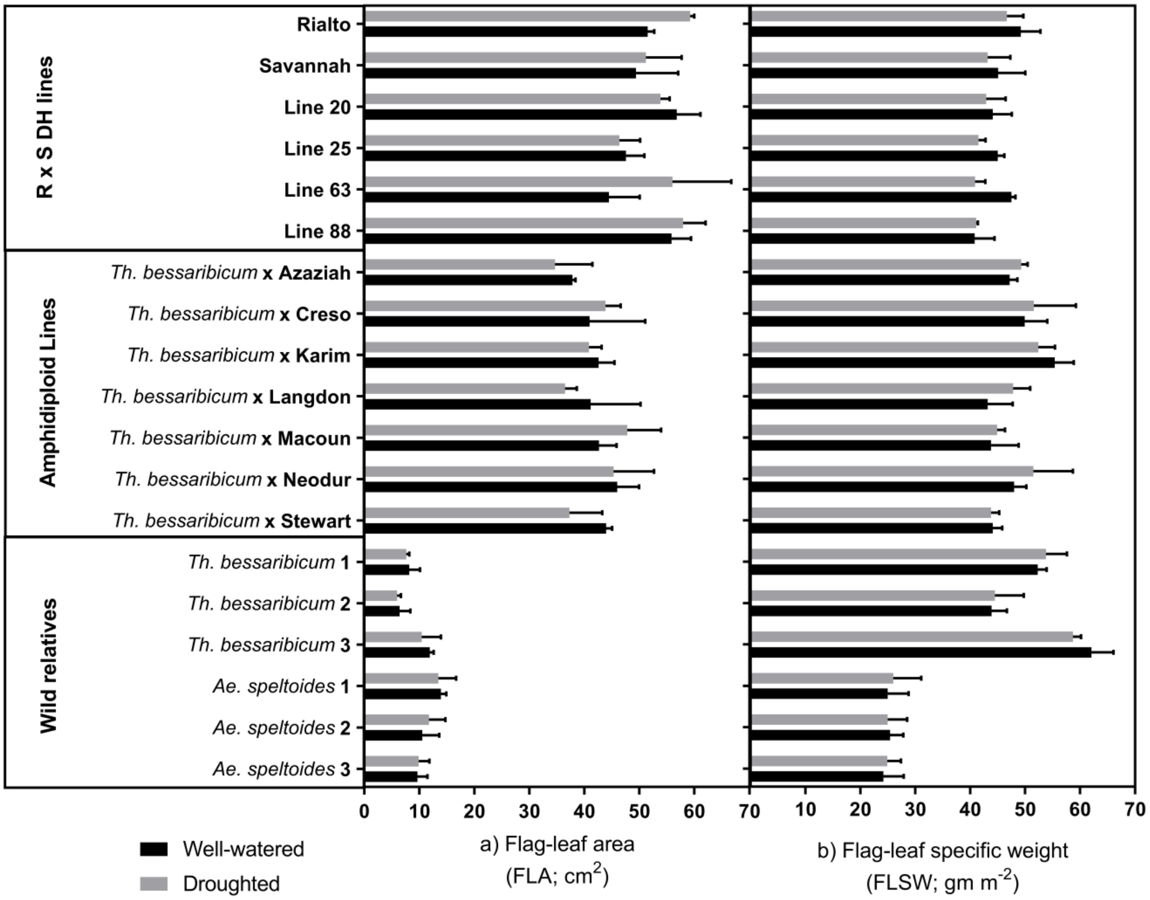


**Figure ~~6~~5.** Flag-leaf area (FLA; at anthesis) and flag-leaf specific weight (FLSW; at anthesis) for 19 genotypes (amphidiploid lines, R x S DH lines and wheat wild relatives) in 2014 under well-watered and droughted treatments, and standard deviation (SD) of the differences.

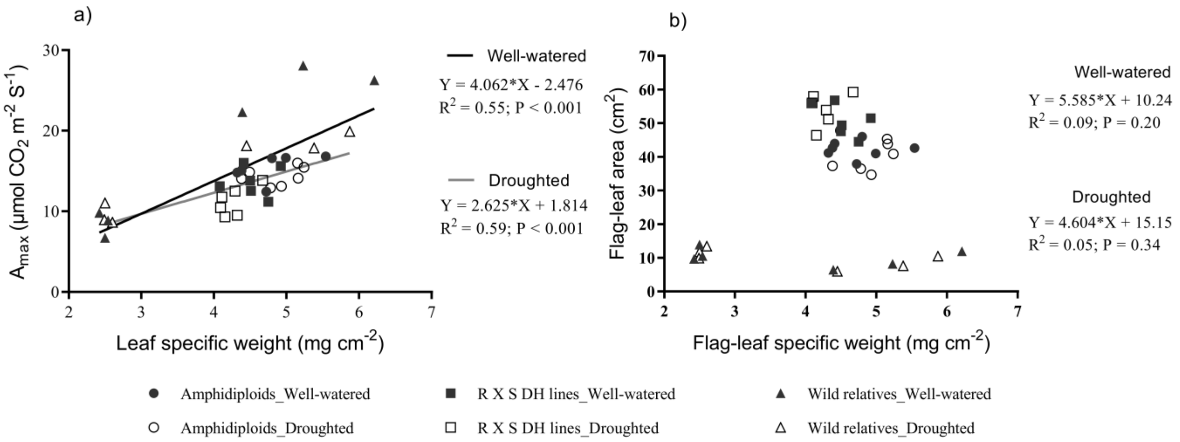


**Figure ~~7~~6.** Linear regressions of flag-leaf specific weight (mg cm$^{-2}$) (FLSW; at anthesis) on (a) flag-leaf $A_{max}$ (µmol m$^{-2}$ s$^{-1}$) and (b) flag-leaf area (cm$^{2}$) (FLA; at anthesis) for 19 genotypes (amphidiploid lines, R x S DH lines and wheat wild relatives) in 2014 under well-watered and droughted treatments.

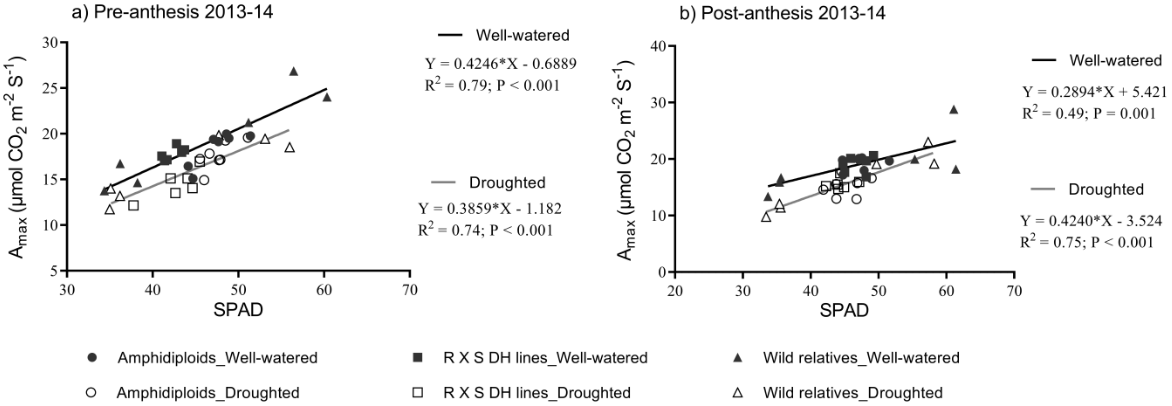


**Figure ~~8~~7.** Linear regressions of (a) pre-anthesis flag-leaf $A_{max}$ (µmol m$^{-2}$ s$^{-1}$) on pre-anthesis flag-leaf SPAD and (b) post-anthesis flag-leaf $A_{max}$ (µmol m$^{-2}$ s$^{-1}$) on post-anthesis flag-leaf SPAD for 19 genotypes (R x S DH lines, amphidiploids and wheat wild relatives) in 2013-14 under well-watered and droughted treatments.

### *Grain yield and biomass per plant*

Under irrigated conditions, grain yield per plant and per shoot was highest in the adapted S × R bread wheat DH genotypes compared to other genotypes as expected mainly associated with higher harvest index (grain yield / above-ground DM). Above-ground DM plant$^{-1}$ was higher in *Ae. speltoides* accessions (54.7 g plant$^{-1}$) and the amphidiploid lines (50.8 g plant$^{-1}$) than the S × R DH lines (40.9 g plant$^{-1}$) ($P$<0.001); and was much less in the *Th. bessarabicum* accessions at 12.4 g plant$^{-1}$ (Table ~~6~~7). The greater biomass plant$^{-1}$ was partly related to higher shoots plant$^{-1}$ for the amphidiploid lines and *Ae. speltoides* accessions compared to the bread wheat genotypes (Table ~~6~~7). The reduction in biomass plant$^{-1}$ under drought was less for the S × R DH genotypes (-15%) than the amphidiploid lines (-23%) or the *Th. bessarabicum* (-34%) or *Ae. speltoides* (-34%) accessions ($P$<0.05).

Under irrigated conditions, increased plant height was associated with increased biomass plant$^{-1}$ ($R^2$=0.29; P< 0.01); the positive association was even stronger omitting the three *Th. bessarabicum* accessions ($R^2$=0.72, P< 0.001; Table ~~7~~8). Similar associations between plant height and biomass were observed under drought. Anthesis date was latest in the *Th. bessarabicum* accessions (184-188 days after sowing, DAS) and earliest in the amphidiploid lines (156-164 DAS), with intermediate dates for the S × R DH genotypes and *Ae*. *speltoides* accessions. The *Th. bessarabicum* accessions with the latest anthesis dates had the lowest biomass plant$^{-1}$, driving an overall association between later anthesis date and lower biomass plant$^{-1}$ across the genotypes. However, excluding these three accessions there was no overall association between anthesis date and biomass plant$^{-1}$ amongst the remaining 16 genotypes.

**Table ~~6~~7.** Grain yield per plant (GY) and main shoot ($GY_m$), above-ground DM per plant (AGDM) and main shoot ($AGDM_m$) for 19 genotypes (amphidiploid lines, S × R DH lines and wheat wild relatives) for cross-year mean (2013-2014), and standard errors of the differences of the means (SED) and degrees of freedom (df) under well-watered (WW) and drought (DR) treatments.

| | GY (g plant$^{-1}$) 2013-4 | | $GY_m$ (g shoot$^{-1}$) 2014 | | AGDM (g plant$^{-1}$) 2013-4 | | $AGDM_m$ (g shoot$^{-1}$) 2014 | |
|---|---|---|---|---|---|---|---|---|
| | WW | DR | WW | DR | WW | DR | WW | DR |
| *S × R DH pop* | | | | | | | | |
| Rialto | 20.54 | 15.32 | 2.62 | 2.67 | 44.74 | 39.07 | 5.87 | 6.00 |
| Savannah | 14.15 | 11.26 | 2.07 | 2.04 | 34.04 | 24.74 | 4.35 | 4.23 |
| Line 20 | 19.83 | 15.27 | 2.39 | 2.32 | 42.55 | 34.90 | 5.18 | 5.05 |
| Line 25 | 16.69 | 15.30 | 2.31 | 1.90 | 38.87 | 34.94 | 5.28 | 4.44 |
| Line 63 | 22.61 | 16.86 | 2.06 | 1.83 | 42.74 | 36.44 | 4.47 | 4.10 |
| Line 88 | 19.20 | 16.45 | 2.18 | 2.21 | 42.46 | 39.10 | 4.70 | 5.02 |
| *Amphidiploids* | | | | | | | | |
| *Th. bess.* x Creso | 10.02 | 7.67 | 0.88 | 0.86 | 53.12 | 42.62 | 4.04 | 4.14 |
| *Th. bess.* x Karim | 11.58 | 10.57 | 1.88 | 1.95 | 37.16 | 32.22 | 5.69 | 5.59 |
| *Th. bess.* x Langdon | 8.33 | 3.87 | 1.27 | 0.40 | 53.86 | 41.90 | 4.63 | 3.04 |
| *Th. bess.* x Macoun | 7.89 | 5.61 | 0.70 | 0.86 | 49.30 | 39.74 | 3.51 | 3.59 |
| *Th. bess.* x Neodur | 12.41 | 8.20 | 1.26 | 1.36 | 64.25 | 45.72 | 4.25 | 5.22 |
| *Th. bess.* x Stewart | 8.49 | 4.81 | 1.40 | 0.71 | 55.93 | 41.67 | 4.86 | 3.41 |
| *Th. bess.* x Azaziah | 8.20 | 5.99 | 1.18 | 1.00 | 42.18 | 29.87 | 3.85 | 4.31 |
| *Anecestral wheats* | | | | | | | | |
| *Th. bess.* 1 | 0.72 | 0.46 | 0.14 | 0.15 | 12.75 | 8.30 | 1.37 | 1.42 |
| *Th. bess.* 2 | 0.11 | 0.10 | 0.01 | 0.01 | 8.04 | 6.47 | 0.78 | 0.78 |
| *Th. bess.* 3 | 1.20 | 0.66 | 0.24 | 0.23 | 16.39 | 9.57 | 2.21 | 2.20 |
| *Ae. spelt.* 1 | 6.42 | 2.85 | 0.18 | 0.16 | 57.66 | 33.64 | 1.03 | 1.12 |
| *Ae. spelt.* 2 | 2.55 | 2.29 | 0.09 | 0.11 | 56.34 | 39.27 | 0.83 | 0.97 |
| *Ae. spelt.* 3 | 2.57 | 3.45 | 0.15 | 0.13 | 50.06 | 37.96 | 0.83 | 0.84 |
| Mean | 10.18 | 7.74 | 1.21 | 1.10 | 42.23 | 32.53 | 3.57 | 3.45 |
| SED (df) | | | | | | | | |
| Year | 0.495** (4) | | - | | 0.692$^{***}$ (4) | | - | |
| Irrigation | 0.306*** (4) | | 0.155ns (2) | | 0.885$^{***}$ (4) | | 0.364$^{ns}$ (2) | |
| Genotype | 0.728*** (144) | | 0.199*** (72) | | 1.793$^{***}$ (144) | | 0.473$^{***}$ (72) | |
| Irri. x Gen. | 1.048*** (144) | | 0.314ns (72) | | 2.622$^{***}$ (144) | | 0.745$^{ns}$ (72) | |
| Year x Gen. | 1.118*** (144) | | - | | 2.563$^{***}$ (144) | | - | |

$^{***}$*P*<0.001; $^{**}$*P*<0.01 and $^{*}$*P*<0.05 significance levels; $^{ns}$ = not significant.

**Table ~~7~~8.** Anthesis days (Days after sowing, DAS) and plant height for 19 genotypes (amphidiploid lines, R x S DH lines and wheat relatives) for the cross-year mean (2013-2014), and standard errors of the differences of the means (SED) and degrees of freedom (df) under well-watered (WW) and drought (DR) treatments.

| | GS61 (DAS) | | Plant height (cm) | |
|---|---|---|---|---|
| | WW | DR | WW | DR |
| *S × R DH pop* | | | | |
| Rialto | 181 | 175 | 62.6 | 62.9 |
| Savannah | 179 | 182 | 55.3 | 54.5 |
| Line 20 | 178 | 174 | 56.8 | 55.3 |
| Line 25 | 183 | 174 | 57.7 | 59.9 |
| Line 63 | 179 | 175 | 52.3 | 52.7 |
| Line 88 | 180 | 176 | 67.5 | 65.8 |
| *Amphidiploids* | | | | |
| *Th. bess.* x Creso | 170 | 171 | 91.5 | 79.0 |
| *Th. bess.* x Karim | 166 | 166 | 69.1 | 71.0 |
| *Th. bess.* x Langdon | 173 | 172 | 112.2 | 106.7 |
| *Th. bess.* x Macoun | 171 | 172 | 103.5 | 95.8 |
| *Th. bess.* x Neodur | 172 | 172 | 129.1 | 120.6 |
| *Th. bess.* x Stewart | 177 | 172 | 126.0 | 118.2 |
| *Th. bess.* x Azaziah | 170 | 168 | 100.7 | 100.7 |
| *Ancestral wheats* | | | | |
| *Th. bess.* 1 | 196 | 196 | 84.1 | 83.6 |
| *Th. bess.* 2 | 197 | 198 | 75.6 | 70.6 |
| *Th. bess.* 3 | 190 | 196 | 92.3 | 92.0 |
| *Ae. spelt.* 1 | 182 | 180 | 111.7 | 106.3 |
| *Ae. spelt.* 2 | 185 | 181 | 99.3 | 101.1 |
| *Ae. speltoides* 3 | 180 | 181 | 97.9 | 93.0 |
| *Mean* | 179 | 178 | 86.6 | 83.7 |
| *SED (df)* | | | | |
| *Year* | $0.67^{***}$ *(4)* | | $3.16^{*}$ *(4)* | |
| *Irrigation* | $0.66^{ns}$ *(4)* | | $0.59^{**}$ *(4)* | |
| *Genotype* | $1.42^{***}$ *(144)* | | $2.87^{***}$ *(144)* | |
| *Irri. x Gen.* | $2.06^{***}$ *(144)* | | $3.99^{ns}$ *(144)* | |
| *Year x Gen.* | $2.06^{***}$ *(144)* | | $5.06^{***}$ *(144)* | |

## 4. Discussion

### *4.1. Responses of S × R DH lines to drought in the field*

In the UK, drought typically occurs late in the season, and reduces yield in the range of 1-3 t $ha^{-1}$ (Foulkes *et al.*, 2001, 2002). In the present study, drought reduced grain yield by 22%, associated with drought-stress conditions from mid-grain filling to maturity. This yield reduction was similar to those (*c.* 20-30%) reported by Foulkes *et al.* (2002) in UK winter wheat. The irrigation × genotype interaction was partly explained by genotypes with higher yield under irrigation tending to lose more yield under drought with a positive association between yield loss and yield under irrigated conditions. Similar relationships between yield potential and yield losses under drought have been frequently reported (Fischer and Maurer, 1978; Foulkes *et al.*, 2007).

In this study, there was a positive correlation between grain $\Delta^{13}C$ and grain yield for the sub-set of eight genotypes under drought, indicating a negative correlation between transpiration efficiency and grain yield. This implied that the basis of the higher grain yield amongst genotypes under drought was high stomatal conductance (Morgan *et al.*, 1993). The genetic range in $\Delta^{13}C$ values was close to those reported by Aravinda Kumar *et al.* (2011) of 19.2 to 20.5‰ for a Beaver x Soissons winter wheat population in the UK. Positive relationships amongst genotypes between grain $\Delta^{13}C$ and grain yield were reported previously in UK rain-fed conditions (Aravinda Kumar *et al.*, 2011; Foulkes et al., 2016) and in Mediterranean environments with a dependence on within-season rainfall (Araus *et al.*, 1998; Merah *et al.*, 2001; Tsialtas *et al.*, 2001; Monneveux *et al.*, 2005; Monneveux *et al.*, 2006; Xu *et al.*, 2007; Yasir *et al.*, 2013). Present results therefore suggested that genetic differences in yield under mild UK water stress were predominantly driven by variation in water uptake rather than transpiration efficiency.

Our results showed flag-leaf $A_{max}$ and $g_s$ were positively associated with both grain yield and $\Delta^{13}C$. These findings are consistent with previous evidence in wheat genotypes of grain yield showing positive associations with $A_{max}$ and $g_s$ but negative associations with transpiration efficiency under water stress in China (Jiang *et al.*, 2000), Australia (Condon *et al.*, 2002) and France (Monneveux *et al.*, 2006). Present findings on the S × R DH lines in the field experiment indicated that higher flag-leaf $A_{max}$ was positively associated with grain under the mild drought encountered in this study (typical of UK droughts). Therefore, novel sources of high expression in flag-leaf $A_{max}$ and $g_s$ will be of value for not only for raising yield potential under optimal conditions but also for increasing yield in dry years in UK wheat breeding.

### *4.2. Genetic diversity in flag-leaf $A_{max}$ and $g_s$ in amphidiploid and ancestral wheats*

This study quantified flag-leaf activity traits over a very wide range of bread wheat, ancestral wheat and bread wheat-derived amphidiploid germplasm. In the glasshouse experiments, accessions of *Th. bessarabicum* had the highest pre-anthesis and post-anthesis flag-leaf $A_{max}$ amongst the genotype groups. The *Th. bessarabicum* accessions also had the highest flag-leaf specific weight partly associated with a smaller flag-leaf size. It can be speculated that higher FLSW was related to increased N and Rubisco per unit leaf area leading to reduced light saturation and higher photosynthetic capacity per unit leaf area (Morgan et al., 1990; Shearman et al., 2005). The associations we detected amongst the genotypes between flag-leaf chlorophyll content (SPAD) and photosynthetic rate under both irrigated and mild drought~~ed~~ conditions would also support this. There was a strong positive association between flag-leaf SPAD and flag-leaf specific weight in both irrigated and mild drought~~ed~~ treatments indicating scope for using leaf SPAD as an indirect selection tool for higher photosynthetic rate.

With regard to individual genotypes, the amphidiploid line cv. Karim × *Th. bessaribicum* had significantly higher flag-leaf $A_{max}$ and $g_s$ than the highest bread wheat S × R DH line 20 under drought. Higher flag-leaf $A_{max}$ in diploid wheat ancestors compared to hexaploid bread wheat has previously been reported in some cases to be associated with smaller flag leaf area, e.g. Austin (1982) for *T. urartu* and *Ae. speltiodes* compared to hexaploid bread wheat. Interestingly, the higher pre-anthesis $A_{max}$ for the cv. Karim x *Th. bessarabicum* amphidiploid line we observed compared to the bread wheat genotypes was related to an increased FLSW but was not associated with a significant decrease in flag-leaf area. Thus, the higher $A_{max}$ of this amphidiploid line could be a useful source of high $A_{max}$ for pre-breeding. It can be hypothesised that higher flag-leaf $A_{max}$ and FLSW was derived from genes introgressed from the *Th. bessarabicum* parent of the cv. Karim x *Th. bessaribicum* amphidiploid line and further work seems justified to test this in genetic studies. The post-anthesis $A_{max}$ was slightly higher in the field than in glasshouse conditions under mild drought, but the opposite was observed for $g_s$ under mild drought. The basis of this difference cannot be certain but may have related to N uptake being relatively more restricted in the field experiment than the glasshouse experiments under drought. It can be speculated that some soil N mineralization may have occurred in the soil columns during the glasshouse experiments increasing soil N availability.

### *4.3 Genetic diversity in TE in amphidiploid and ancestral wheats and contribution to drought tolerance*

The amphidiploid lines showed a trend for higher flag-leaf transpiration efficiency in the pre-anthesis period than the bread wheat lines under drought (4.58 vs 3.53 mol $m^{-2}$ $s^{-1}$). As discussed above, higher TE may not be a beneficial trait for yield under less severe droughts where significant amounts of rainfall occur through the season due to a trade-off with season-long water uptake. Present results showed trends for a negative relationship between flag-leaf TE and flag-leaf $g_s$ under drought for both the pre-anthesis ($P = 0.08$) and post-anthesis ($P = 0.06$) period amongst the seven amphidiploid lines. However, under more severe droughts high TE could be a breeding target to raise grain yield, e.g. positive correlations are reported between flag-leaf TE (as indicated by low grain $\Delta^{13}C$) and grain yield under severe droughts in Australia (Rebetske et al., 2002; Condon et al., 2004). Further work is required to examine whether the present *T. durum x Th. bessarabicum* amphidiploids represent higher TE than their durum wheat parents, indicating sources of high TE derived from the *Th. bessarabicum* genome with potential to introgress the relevant chromosmal segments into elite bread wheat genotypes.

Wild relatives of wheat and amphidiploids represent unadapted material, and their grain yield was not predicted to be higher than the elite bread wheat S × R DH lines and Savannah and Rialto in the present study, but they were hypothesized to have high expression of leaf stomatal aperture traits associated with higher biomass under well-watered and drought conditions. Above-ground biomass per plant was overall higher in *Ae. speltoides* accessions (54.7 g $plant^{-1}$) and the amphidiploid lines (50.8 g $plant^{-1}$) than the S x R DH bread wheat genotypes (40.9 g $plant^{-1}$) under well-watered conditions ($P< 0.001$); and there was a trend for amphidiploid lines to have higher biomass than the bread wheat genotypes under drought. Flag-leaf $g_s$ of the main shoot was strongly positively related to biomass of the main shoot in the irrigated treatment amongst the 19 genotypes; but there was no association under drought. The greater biomass in the amphidiploids than bread wheat in well-watered conditions was partly explained by their increased plant height compared to the bread wheat genotypes. Various studies have shown that taller wheat genotypes were associated with increased biomass (Fischer, 1985; Miralles and Slafer, 1997; González *et al.*, 2003; Bognár *et al.*, 2007; Toyota *et al.*, 2010). The higher biomass for *Ae. speltoides* accessions under well-watered conditions on the other hand was partly related to more shoots $plant^{-1}$ compared the bread wheat genotypes. It seems unlikely therefore that the higher biomass per plant for the *Ae. speltoides* accessions compared to the bread wheat genotypes presently observed could be maintained at commercial plant densities in the field.

In the present study, the S × R DH genotypes maintained grain yield best as a percentage of irrigated yield under drought out of the genotype groups and the *Ae. speltoides* and *Th. bessarabicum* accessions the least well. The greater relative reduction of biomass of the wild relatives compared to the elite lines or the amphidiploids under drought was mainly associated with responses for shoots per plant; the irrigation × genotype interaction for biomass per shoot was not statistically significant. Although the three *Th. bessarabicum* accessions did show better maintenance of $g_s$ under drought compared to the other genotype groups this was not associated with better maintenance of biomass.

In our experiments post-anthesis $A_{max}$ and $g_s$ measured in the field for R x S DH lines and Savannah and Rialto were strongly correlated with the corresponding glasshouse measurements for these genotypes. This demonstrated genetic variation in flag-leaf activity traits in glasshouse conditions was representative of the differences in field-grown conditions; and that flag-leaf screens for leaf stomatal traits are a valuable tool for screening for enhanced photosynthetic efficiency and stomatal conductance for improved yields under optimal and drought conditions.

### *4.4. Conclusions*

Our results showed biomass plant$^{-1}$ was higher in *Ae. speltoides* accessions and the amphidiploid lines than in the bread wheat lines under drought in the glasshouse experiments. Flag-leaf $A_{max}$ was higher in the *Th. bessarabicum* accessions and the amphidiploid lines (durum wheat × *Th. bessarabicum*) than the bread wheat lines under both irrigation and drought conditions. There was a positive association between flag-leaf specific weight and flag-leaf $A_{max}$ in both irrigated and unirrigated conditions; and there was no trade-off between flag-leaf area and flag-leaf specific weight. These results indicated that *Th. bessarabicum* accessions and amphidiploid lines offer novel variation for high expression of leaf photosynthesis rate for introgression into bread wheat genotypes to support genetic gains in biomass.

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