



# Wheat grain number: Identification of favourable physiological traits in an elite doubled-haploid population



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## ABSTRACT

A detailed and accurate phenotyping of mapping populations is an important “bottleneck” for the understanding of the phenotype–genotype relationships. Grain number per unit area (GN), the main wheat yield component, can be analyzed through physiological components as spike dry weight at flowering, determined by crop growth rate and biomass partitioning to spike during stem elongation phase, and fruiting efficiency. The phenotypic variability of these physiological traits responsible for variation in GN and the relationships among them was analyzed in an elite wheat doubled-haploid (DH) population grown in two different environments. Positive transgressive segregation (i.e. DH lines that exceed parental phenotypic values) was observed for all GN determining traits, suggesting that increases in GN could be achieved through their improvement. However, focusing on top DH lines, which represent a feasible genetic improvement, fruiting efficiency was the most relevant physiological trait for consistently improving GN, and thereby grain yield, in both environments.

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## 1. Introduction

Wheat (*Triticum aestivum* L.) is a key component of food security (it provides ca. 20% of the calories to the world's population) and the crop most widely grown worldwide, being adapted to a broad range of environments (Reynolds et al., 2012). As wheat global demand is projected to increase during coming decades, it is broadly agreed that improvements in yield potential must be accelerated to achieve a sustainable production increment.

**Abbreviations:** GN, grain number per m<sup>2</sup>; SDW, spike dry weight at flowering; FE, fruiting efficiency; SEP, stem elongation phase; CGR, crop growth rate; BPS, biomass partitioning to spike; RUE, radiation use efficiency; DH, doubled-haploid; BA, Buenos Aires; CO, Ciudad Obregón; PAR, photosynthetically active radiation; Ri, incident solar radiation; Rt, transmitted solar radiation; RIE<sub>midday</sub>, radiation interception efficiency at midday; RIE<sub>day</sub>, daily radiation interception efficiency; BLUE, best linear unbiased estimation; B, Bacanora; W, Weevil.

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Current rates of wheat genetic gains in many countries (largely inferior to 1% per year) are lower than those required to meet the projected cereal demand (Reynolds et al., 2012; Hall and Richards, 2013). It has been proposed that indirect selection based on physiological traits would help achieving the required rates of genetic gains. Consequently, many efforts have been made to understand the association between grain yield improvements and changes in physiological traits. Currently, there are several proposals that support the feasibility of using this physiological assistance to improve yield potential (Slafer, 2003).

Recent advances in molecular biology have developed the potential to identify and map genes or quantitative trait loci related to any trait, and phenotyping has become the “bottleneck” delaying progress in breeding for complex traits (Furbank and Tester, 2011; Fiorani and Schurr, 2013). In order to use physiological criteria in a breeding program, it is first required to identify traits that determine grain yield and then to detect genetic factors controlling them in order to make their manipulation easier and/or to predict their selection response (Slafer, 2003). The usefulness of physiological information obtained by accurate phenotyping is unquestionable, not only for a better understanding of the relationships between

grain yield and its determining traits, but also to improve, in combination with molecular tools, the current knowledge about the genotype–phenotype relationship (Slafer, 2003; Edmeades et al., 2004). On the other hand, phenotyping, an “elite” population, has an evident practical application, as breeding programs routinely make progress with elite lines and are interested in improved combinations among them (Ratley et al., 2009).

Grain number per unit area (GN) is the main yield component in wheat as in the majority of grain crops (Fischer, 2008). Improvement of GN is critical to achieve genetic gains in wheat yield, particularly if large increases are required (Slafer et al., 2014), as it was the main component associated with yield progress through breeding and it is still the main component limiting yield in many regions of the world (e.g. Peltonen-Sainio et al., 2007). A common approach to understand GN (and yield) determination is to dissect it into their numerical components, i.e. spike number per unit area and grain number per spike (Slafer et al., 2014 and references quoted therein). Even though this approach is useful to analyze grain yield strategies (e.g. differences between cultivars) or a management practice effect (e.g. different sowing rates), from a physiological perspective, it is inefficient for predicting the impact of manipulating a single trait on GN determination. This limitation arises because a trade-off between spike number and grain number per spike almost inevitably appears, implying that improvements in one of these components is negatively counterbalanced by reductions in the other one, failing to deliver more grains (Fischer, 1983; Slafer, 2003), particularly for modest to moderate yield improvements (Slafer et al., 2014). Therefore, GN determination would be better analyzed in terms of crop dry matter economy during the critical period (Fischer, 2008).

The critical period for GN determination in wheat occurs when the spikes are actively growing during the stem elongation phase, from a few weeks before to immediately after anthesis (Fischer, 1985). During this critical period, GN determination is strongly source-limited and the degree of such limitation is reflected in the proportion of both floret primordia able to reach the stage of fertile florets, avoiding floret death (Kirby, 1988), and tillers surviving to become fertile spikes (Thorne and Wood, 1987). Thus, different evidence shows a strong positive association between GN and spike dry weight at flowering (Fischer, 2011). However, GN may vary among genotypes beyond differences in spike dry weight due to differences in fruiting efficiency, i.e. the number of grains set per unit of spike weight (Abbate et al., 1998; González et al., 2011). Therefore, GN can be analyzed in terms of spike dry weight at flowering determinants [i.e. stem elongation phase (SEP), crop growth rate (CGR) and biomass partitioning to spike (BPS) both during SEP] and fruiting efficiency (FE; Eq. (1)) (Fischer, 1983, 2008).

$$GN = SEP * CGR * BPS * FE \quad (1)$$

This assimilate-based approach considers that if: (i) more solar radiation is intercepted, and/or (ii) this radiation is more efficiently converted into biomass, and/or (iii) this biomass is more favorably partitioned to the juvenile spikes, and/or (iv) spike biomass is more efficiently used to set grains, an increment in GN could be achieved. This approach represents a useful guide to carry out a detailed and accurate phenotyping of elite mapping populations.

As stated above, spike dry weight at flowering is a good indicator of wheat GN (Fischer, 2011) and most of the efforts to improve GN are focused in its linked traits. The idea of manipulating the stem elongation phase through photoperiod sensitivity in order to increase spike dry weight has been suggested (Slafer et al., 2001). The positive impact of longer stem elongation phase on spike dry weight at flowering and GN has been experimentally tested (Miralles et al., 2000; González et al., 2003; Serrago et al., 2008), however this trait could have an important genotype by environment interaction which affects its early selection (García

et al., 2011). The improvement of crop growth rate through higher pre-flowering radiation use efficiency (RUE) has contributed to yield progress of UK cultivars released between 1970s to 1990s (Shearman et al., 2005), and today several works aim to improve RUE for increasing GN and yield (Reynolds et al., 2012). The biomass partitioning to spike, a key trait during the Green Revolution, must also be improved without affecting the root system functionality or increasing the lodging risk (Berry et al., 2007). On the other hand, although the importance of the fruiting efficiency is recognized, its relationship with the spike dry weight has been scarcely studied. Works that evaluated the genetic progress or the performance of modern cultivars have reported a positive association between fruiting efficiency and GN, in both bread (Abbate et al., 1998; Acreche et al., 2008; González et al., 2011) and durum wheat (Ferrante et al., 2012). These results, together with evidence of cultivar's variability (Shearman et al., 2005; Serrago et al., 2008; González et al., 2011) and likely responsiveness to selection (Abbate et al., 2013), position fruiting efficiency as a relevant trait to improve GN, although keeping in mind that a trade-off between fruiting efficiency and spike dry weight may occur (Dreccer et al., 2009; Ferrante et al., 2012; Lázaro and Abbate, 2012). Therefore, when testing the opportunities to raise GN through improved fruiting efficiency, it is necessary to evaluate whether the improved in this trait does bring about a trade-off in spike dry weight.

The two main objectives of this work were: (i) to analyze the phenotypic variability in physiological traits responsible for genotypic variation in GN (Eq. (1)) and (ii) to identify possible trade-offs among those traits, in an elite wheat doubled-haploid (DH) population grown in two contrasting environments. The relevance of the present work lies both in (i) the phenotyping of GN determination based on the physiological approach proposed by Fischer (1983), rarely reported for large populations, and (ii) the use of a population derived from high-yielding CIMMYT cultivars contrasting in grain yield components. Crop-physiological studies based in this sort of population are far less common than those based on populations derived from wide crosses. The latter normally offer wide ranges of variation exacerbating the breadth of expression of physiological traits, but using populations derived from two high-yielding cultivars provides more meaningful conclusions for breeding programs, as when breeders aim to increase grain yield potential normally cross elite-by-elite materials and search for transgressive segregations. Furthermore, this population is being genotyped at the John Innes Centre (UK) and therefore it might be possible to count in the near future with genetic markers associated to the traits identified in the present work, which may become tools for empirical application of these conclusions in the selection process.

## 2. Material and methods

### 2.1. Plant material and environments

The wheat population used in this work consisted of 105 DH lines derived from a cross between two well-adapted and high-yielding cultivars of spring wheat, Bacanora and Weebil, with similar phenology but consistently differing in yield components: Bacanora normally has more grains than Weebil, while the latter has heavier grains than Bacanora (García et al., 2013). The cross was carried out in CIMMYT (Mexico) and the DH lines, derived from F<sub>1</sub> using the maize cross method (Laurie and Bennett, 1988), were developed by the John Innes Centre (UK). Both parents are modern cultivars within the elite CIMMYT germplasm. The population was phenotyped under field conditions in two environments: (i) the experimental field of Facultad de Agronomía - Universidad de Buenos Aires (34°35'S, 58°29'W, 26 masl) in Buenos Aires (Argentina) during the 2009 growing season (BA), and (ii) the

Norman E. Borlaug experimental station (27°25'N, 109°54'W, 38 masl) near Ciudad Obregón (Mexico) during the 2009/2010 growing season (CO). BA has a silty clay loam soil, classified as Vertic Argiudoll, and CO has a coarse sandy clay soil mixed with montmorillonitic clay, classified as Typic Calciorthid. The entire population was evaluated in CO, while in BA 85 DH lines were phenotyped, due to lack of enough seeds for the rest of the lines.

Field trial in BA was arranged in a completely randomized design with three replicates, sown in flat plots, 2.1 m long and 0.9 m wide with five rows (0.175 m between rows). Field experiment in CO had an alpha lattice design with two replicates sown in a 2.5 m long and 0.8 m wide plots, each one consisting of one raised bed with two rows per bed (0.25 m between rows). These cropping systems are representative of those most commonly used by farmers from the Pampas and the Yaqui Valley, respectively. The population was sown on 5 July 2009 in BA and on 2 December 2009 in CO, which correspond to sowing dates recommended to maximize productivity at each of these sites. Sowing densities were that required to obtain 250 and 180 plants m<sup>-2</sup> in BA and CO, respectively. The actual density of seeds sown per m<sup>2</sup> was ca. 10–20% higher, depending on the vigor index of each DH line analyzed before sowing. Since in BA, the harvest and processing had to be done manually, restricting severely the sample size to be processed for such a large number of plots (261), an extremely careful and secure system allowing a small sample to be representative was used. Soon after emergence (seedlings mostly with two expanded leaves), sectors in which plant density was exactly the one expected (and identical for all the plots) and seedlings were on the same stage were labelled, both in the row to be sampled and in the borders of that sampling area. Then samples were exclusively taken from these pre-labelled areas. Both field trials were irrigated (irrigation by sprinklers in BA and furrow irrigation in CO) and fertilized adequately (i.e. fertilization was adequate to obtain more than 20 ppm of phosphorus per hectare at sowing and 180 kg nitrogen ha<sup>-1</sup> at tillering in both sites). Plant pathogens, pests and weeds were prevented or controlled with recommended chemical treatments.

## 2.2. Phenotyping

In BA and CO, GN, phenology and biomass accumulation and partitioning at flowering were measured in the whole population. Approximately a week after physiological maturity, each plot was harvested. All spikes of a sample hand-harvested in 0.3 m of two central rows of each plot in BA were threshed in a small stationary thresher, while the whole plot was mechanically harvested in CO. Grain yield per unit area (corrected to 0% of moisture content) and average grain weight (through two samples of 100 grains) were determined, and GN was calculated as the quotient between grain yield and grain weight. Timing of emergence (GS11; Zadoks et al., 1974), onset of stem elongation (GS31; Zadoks et al., 1974) and anthesis (GS65; Zadoks et al., 1974) were recorded. GS11 stage was registered when 50% of the seedling in the plot have emerged, GS31 stage was recorded when 50% of 10 main shoots chosen randomly had their first node detectable above the soil surface, and GS65 was established when 50% of the plants in the plot reached that stage. To estimate biomass accumulation and partitioning, above-ground dry matter cuts were carried out at flowering. In BA, biomass in 0.5 m of the central row was harvested at GS65, while in CO, 0.5 m of the two rows of a raised bed were harvested 7 days after GS65. In BA, the entire sample was separated into spikes and culms (i.e. stem plus leaves), oven-dried (60 °C, 72 h) and weighed. In CO, a sub-sample of the fresh weight of the entire sample (ca. 30%) was obtained, and then, it was separated into spikes and culms, oven-dried and weighed. Plant height from the soil to the base of the spike was recorded in five plants per plot around mid-grain filling.

Spike dry weight-defining traits and fruiting efficiency were determined for the whole population in both environments. The stem elongation phase was considered as the thermal time (base temperature: 0 °C, Kirby et al., 1985) between GS31 and GS65 (BA) or GS65 + 7 days (CO). Temperatures used to calculate the thermal time were registered in weather stations located at the experiment (BA) or at less than 1500 m (CO). The pre-flowering crop growth rate was calculated as the quotient between above-ground dry matter per unit area and flowering time, considered as the thermal time between emergence and GS65 (BA) or GS65 + 7 days (CO). The biomass partitioning to spike (i.e. proportion of above dry matter in spikes at flowering) was determined as the ratio between spike dry weight and above-ground dry matter at flowering. Finally, the fruiting efficiency was calculated as the quotient between GN and spike dry weight at flowering.<sup>1</sup>

The crop growth rate depends on the crop capacity to intercept and accumulate photosynthetically active radiation (PAR) and to convert it into biomass (i.e. RUE) (Fischer, 1983). Both traits were determined during pre-flowering for the whole population in BA. Solar radiation interception was periodically (twice weekly) measured from emergence to flowering with a linear ceptometer (BAR-RAD 100, Cavadevices.com, Buenos Aires, Argentina) on clear days at noon, for 12 and 14 h. Four measurements were recorded per plot each time. For the first one, the ceptometer was placed above the canopy, while for the other three measurements the ceptometer was positioned below the canopy between two central rows and parallel to them (on the left, on the right and in the middle). The first record corresponds to the incident radiation (R<sub>i</sub>), while the average of the other three records represents the transmitted radiation (R<sub>t</sub>). The radiation interception efficiency at midday (RIE<sub>midday</sub>) was calculated as (R<sub>i</sub> - R<sub>t</sub>)/R<sub>i</sub>, and the daily radiation interception efficiency (RIE<sub>day</sub>) was estimated according to the methodology (Eq. (2)) proposed by Charles-Edwards and Lawn (1984):

$$\text{RIE}_{\text{day}} = 2 * \text{RIE}_{\text{midday}} / (1 + \text{RIE}_{\text{midday}}) \quad (2)$$

Afterwards, the evolution of RIE<sub>day</sub> during pre-flowering was estimated using an optimization model that fitted the recorded data iteratively (determination coefficients were higher than 0.90 in all cases) through a curve-fitting software (Radushev et al., 2007) to the following logistic model:

$$\text{RIE}_{\text{day}} = \frac{A}{1 + e^{-B(t-C)}} \quad (3)$$

where *A* is the maximum RIE<sub>day</sub>, *B* is the relative rate of change in RIE<sub>day</sub>, *C* is the time at which the inflection point occurred and *t* is the time (i.e. days from emergence). The parameters *A*, *B* and *C* were estimated by the model from recorded data. Solar radiation daily intercepted and accumulated was calculated as the product between the modelled RIE<sub>day</sub> for this particular day and the daily solar radiation registered in the weather station close to the trial (50% of this solar radiation was considered PAR). Finally, the pre-flowering RUE was calculated as the quotient between biomass accumulated at flowering and accumulated intercepted PAR from emergence to flowering.

## 2.3. Statistical analyses

Firstly, each environment was analyzed separately according to its experimental design. The mean and the best linear unbiased estimation (BLUE) for each DH line (or parent) and trait phenotyped

<sup>1</sup> Note that fruiting efficiency in CO was much lower than in BA because spike dry weight was assessed in CO 7 days after flowering and thus included the weight of the very young grains that had commenced growth at that time.

was estimated. These analyses were performed with two software: InfoStat (Di Rienzo et al., 2010) for the completely randomized design of BA, and the MIXED procedure from SAS (SAS, 1999) for the alpha lattice design of CO. Secondly, population means, standard deviations, quartiles, and variability ranges for the different traits evaluated (InfoStat) using means(BA) or BLUEs (CO) were used to provide descriptive statistics for population distributions. In third place, correlation coefficients (Pearson) between two random variables were estimated (InfoStat) to determine statistical relationships between GN and its main physiological components and to study possible trade-offs between them.

Finally, the performance of top DH lines with respect to the parental mean was evaluated. DH lines that ranked in the top quartile of GN in both environments were selected and the ratio between each one of them and the parental mean was calculated for stem elongation phase, crop pre-flowering growth rate, biomass partitioning to spike, fruiting efficiency, flowering time (i.e. time between GS11 and GS65) and plant height.

### 3. Results

#### 3.1. Variability in physiological traits determining grain number

During the critical period, the DH population explored different photothermal conditions in each of the two environments (i.e. BA and CO) where phenotyping was carried out. Focusing on the stem elongation phase, the DH population was exposed to different mean photoperiod (13.2 and 12.1 h in BA and CO, respectively), mean temperature (13.8 and 15.6 °C in BA and CO, respectively) and average incident radiation (8.2 and 9.9 MJ m<sup>-2</sup> day<sup>-1</sup> of incident PAR, in BA and CO, respectively). These environmental differences in climatic factors linked with crop development and growth process during the critical period were reflected in GN establishment, which averaged higher values in BA than in CO (21,700 and 14,900 grains m<sup>2</sup>, respectively).

A detailed description and discussion of the phenotypic variability of this population in grain yield strategies across contrasting environments, including BA and CO, has been reported in García et al. (2013). From this work, it is important to highlight some characteristics of the population performance which were similar in both environments. Firstly, although variability in flowering time and plant height was observed, 50% of the population reached the GS65 stage in a range of 5 or 6 days in BA or CO, respectively, and both parental lines as and most of DH lines showed a plant height within the range considered optimal for grain yield (between 0.7 and 1 m; Richards, 1992). Secondly, as expected, Bacanora established a greater GN than Weebil in agreement with the basis for the initial choice as parents and, interestingly, several DH lines set more grains than Bacanora (13 and 50% of the population in BA and CO, respectively).

All the physiological traits responsible for GN determination (Eq. (1)) showed transgressive segregation (Fig. 1). Consequently, some DH lines exhibited improvements in these traits compared to the parental lines. Parental performance was consistent across the two environments, though the differences magnitude was generally larger in BA than in CO. Bacanora had a higher fruiting efficiency than Weebil, while Weebil had heavier spikes, despite having slightly less biomass partitioning to spike, due to its slightly longer stem elongation phase and higher crop growth rate than Bacanora (Fig. 1). The magnitude of transgression depended on the particular trait and environment. Thus, 24% (BA) and 47% (CO) of the DH lines had heavier spikes than Weebil, and 20% (BA) and 34% (CO) of the population had higher fruiting efficiency than Bacanora. Additionally, 39% (BA) and 67% (CO) of the DH lines had longer stem elongation phase than Weebil. Regarding pre-flowering crop

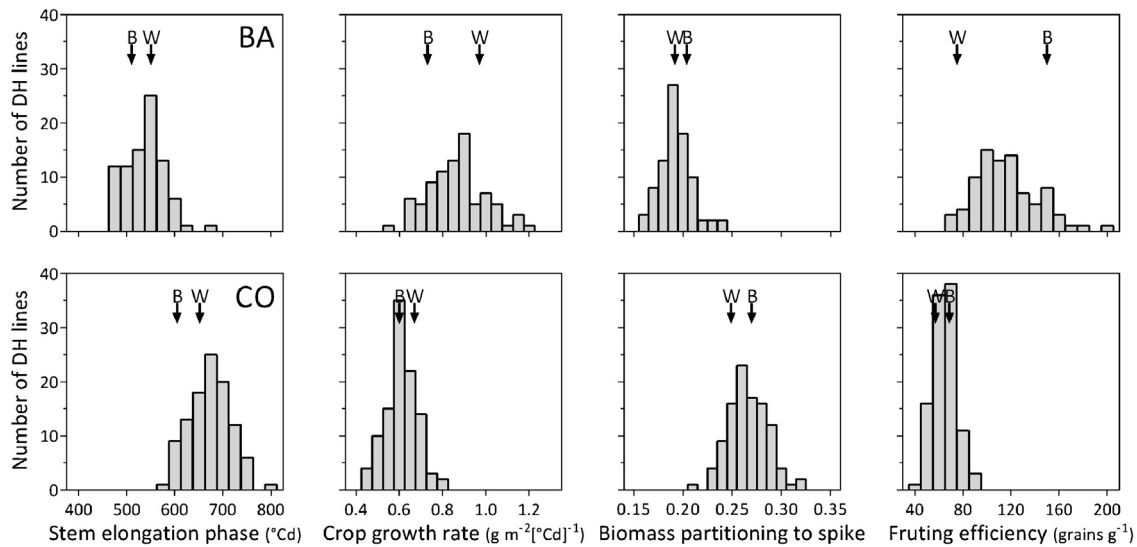
growth rate, Weebil was outperformed by 20% of the population in both environments. Finally, 20% (BA) and 41% (CO) of the DH lines had higher biomass partitioning to spike than Bacanora. Most of the DH line distributions were approximately normal for the different physiological traits in both environments (Fig. 1). Only the distributions for stem elongation phase and fruiting efficiency in BA were slightly skewed towards smaller values (and in the latter, the skewness was generated by only one datum).

#### 3.2. Contribution of physiological traits to grain number determination and possible trade-offs

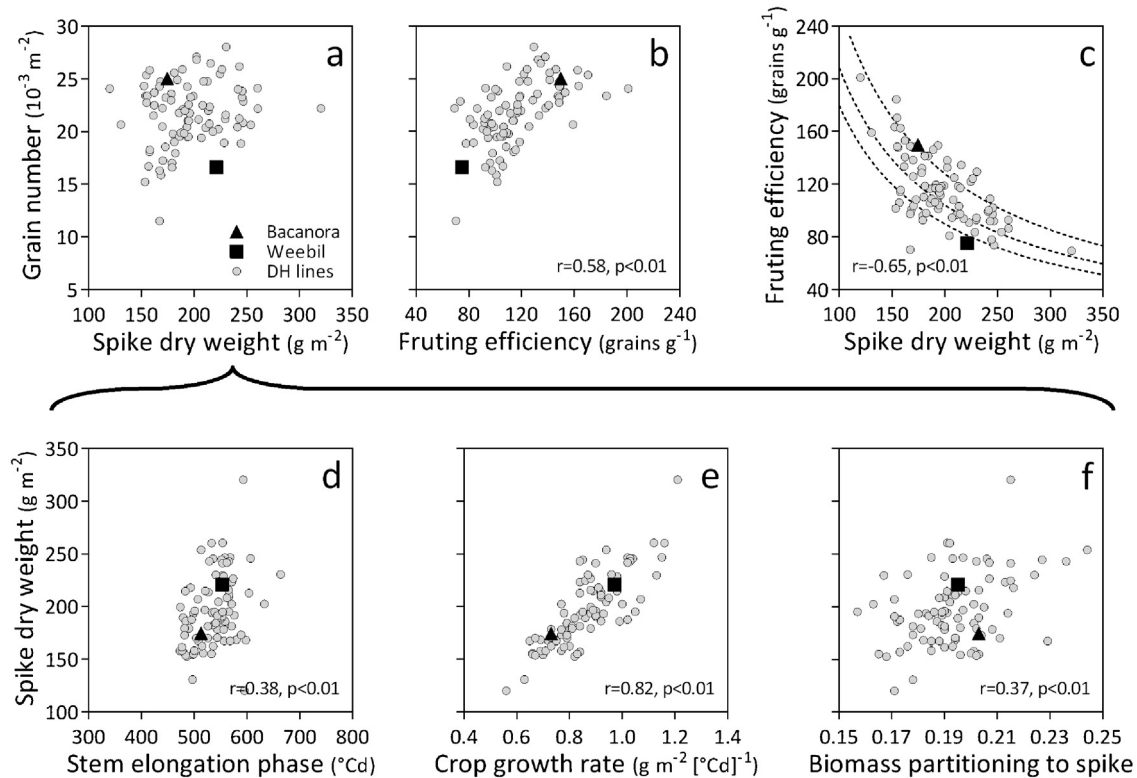
At the whole population level, GN tended to be positively associated with spike dry weight at flowering and fruiting efficiency, but the relative relevance of these variables depended on the environment. Variations in GN were more associated with differences in fruiting efficiency than in spike dry weight in BA (Fig. 2a and b), while the opposite occurred in CO (Fig. 3a and b). Consequently, physiological traits determining spike dry weight at flowering were positively correlated to GN in CO ( $r=0.33$ ,  $p<0.01$  for stem elongation phase;  $r=0.38$ ,  $p<0.01$  for crop growth rate; and  $r=0.34$ ,  $p<0.01$  for biomass partitioning to spike), but in BA, with the exception of stem elongation phase ( $r=0.32$ ,  $p<0.01$ ), the correlations were weak. Spike dry weight at flowering and fruiting efficiency were negatively associated; however, there was a large degree of variability indicating that combinations of high values for both traits might be possible (Figs. 2c and 3c). For example, fruiting efficiency ranged from 93 to 141 grains g<sup>-1</sup> for a spike dry weight of ca. 180 g m<sup>-2</sup> and this last trait ranged from 154 to 246 g m<sup>-2</sup> for a fruiting efficiency of ca. 100 grains g<sup>-1</sup> in BA (Fig. 2c). In CO, fruiting efficiency ranged from 47 to 72 grains g<sup>-1</sup> for a spike dry weight of 243 g m<sup>-2</sup>, while spikes weighted between 173 and 289 g m<sup>-2</sup> for a fruiting efficiency of 65 grains g<sup>-1</sup> (Fig. 3c).

In general, the variations in spike dry weight at flowering were more associated with changes in growth than in development or partitioning. Although the association between spike dry weight and pre-flowering crop growth rate was the strongest, stem elongation phase and biomass partitioning to spike were also positively correlated to spike dry weight in both environments (Figs. 2d–f and 3d–f). In line with the negative relationship found between spike dry weight at flowering and fruiting efficiency, all physiological components of the former trait, but stem elongation phase in BA, were negatively correlated with fruiting efficiency in both environments ( $r=-0.26$ ,  $p=0.01$  for stem elongation phase in CO;  $r=-0.59$ ,  $p<0.01$ ; and  $r=-0.41$ ,  $p<0.01$  for crop growth rate in BA and CO, respectively; and  $r=-0.29$ ,  $p=0.01$  and  $r=-0.36$ ,  $p<0.01$  for biomass partitioning to spike in BA and CO, respectively). No negative association was observed among spike dry weight determining traits. Only a positive correlation between stem elongation phase and biomass partition to spike in CO ( $r=0.40$ ,  $p<0.01$ ) was registered.

Pre-flowering crop growth rate showed an important range of variation within the population (from 0.6 to 1.2 g m<sup>-2</sup> [°Cd]<sup>-1</sup>) which was associated with differences in RUE more than with intercepted PAR (Fig. 4). Although the differences in crop growth rate between parental lines were associated with changes in both traits, the range of variation observed in the population for pre-flowering RUE was higher than for intercepted PAR (69 vs. 29% of the parental mean, respectively) and similar to the range of variation in crop growth rate (76%). This result was due to the fact that 50% of the population showed values of RIE<sub>day</sub> between 0.89 and 0.93 at GS39 (i.e. flag leaf appearance) and between 0.95 and 0.98 at GS65 (inset in Fig. 4a). This tendency was expected, as the population derived from the cross of two high-yielding cultivars producing DH lines that could be considered elite germplasm in terms of adaptation and grain yield. On the other hand, and similar



**Fig. 1.** Distribution of doubled-haploid (DH) line values for grain number determining traits (stem elongation phase, pre-flowering crop growth rate, biomass partitioning to spike and fruting efficiency), in Buenos Aires (BA, top panel) and Ciudad Obregon (CO, lower panel). The analysis comprised 85 and 105 DH lines in BA and CO, respectively. Parental line values (B, Bacanora; W, Weebil) are indicated with arrows.



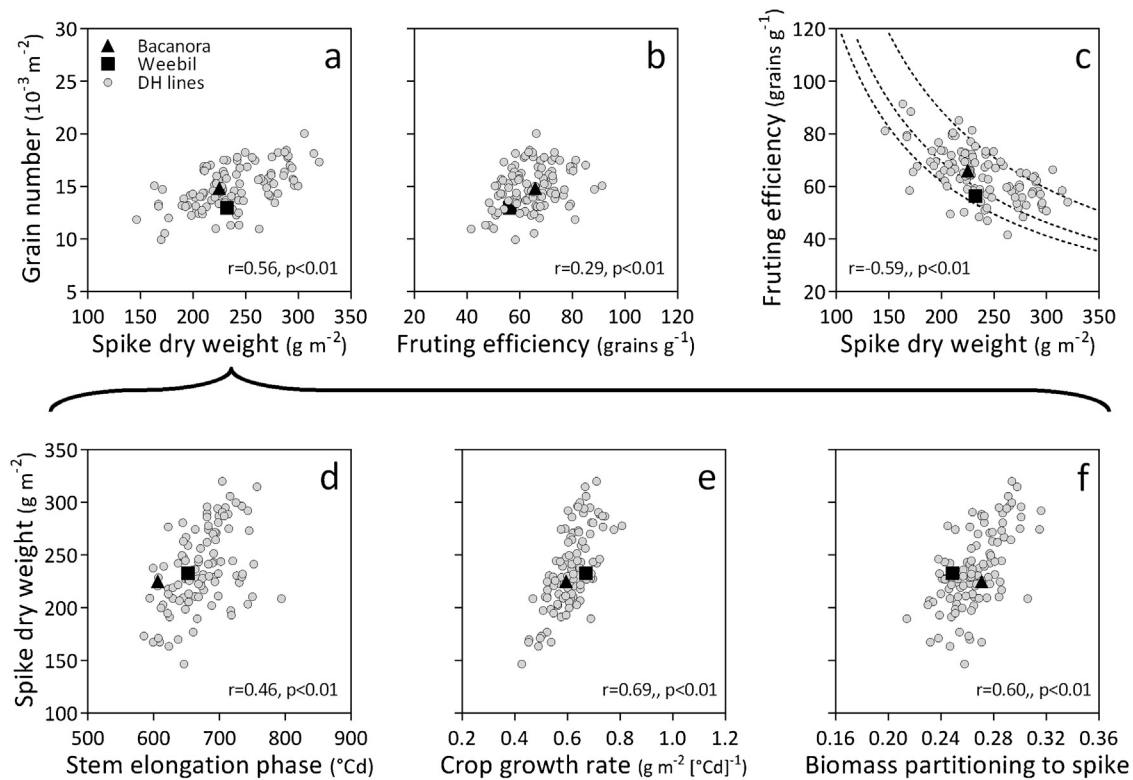
**Fig. 2.** Relationships between grain number and its main determining traits (spike dry weight at flowering and fruting efficiency, top panel) and between spike dry weight and its determining traits (stem elongation phase, pre-flowering crop growth rate and biomass partition to spike, lower panel) for doubled-haploid (DH) and parental lines (Bacanora and Weebil) in Buenos Aires. The analysis comprised 85 and 105 DH lines in BA and CO, respectively. Correlation coefficients (Pearson) and probabilities are included only when  $p$ -value is lower than 0.01. In (c), dotted lines represent iso-grain number curves corresponding to the 90th percentile (upper), the parental mean and the 10th percentile (lower).

to others traits, significant transgressive segregation was observed in pre-flowering RUE (Fig. 4b), with 47% of the DH lines more efficient than Weebil (the parental line with higher RUE). Although  $\text{RIE}_{\text{day}}$  was only periodically measured in BA, measurements of  $\text{RIE}_{\text{day}}$  around flowering in CO showed that the value of this trait during the critical period in CO was higher than 80% in most DH lines (data not shown). Therefore, it is expected that the variability

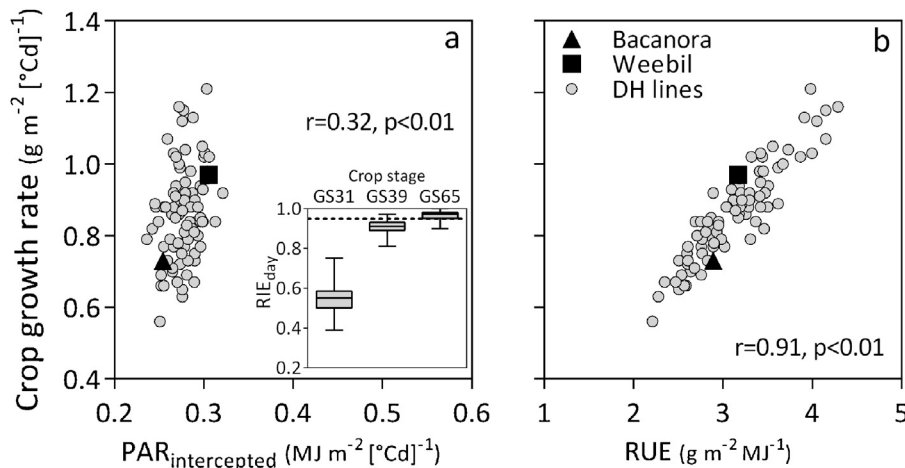
in pre-flowering crop growth rate observed in CO was also more related to differences in pre-flowering RUE than in intercepted PAR.

### 3.3. Top DH lines performance

We further analyzed the performance of DH lines included in the top quartile of GN in both environments to determine which of



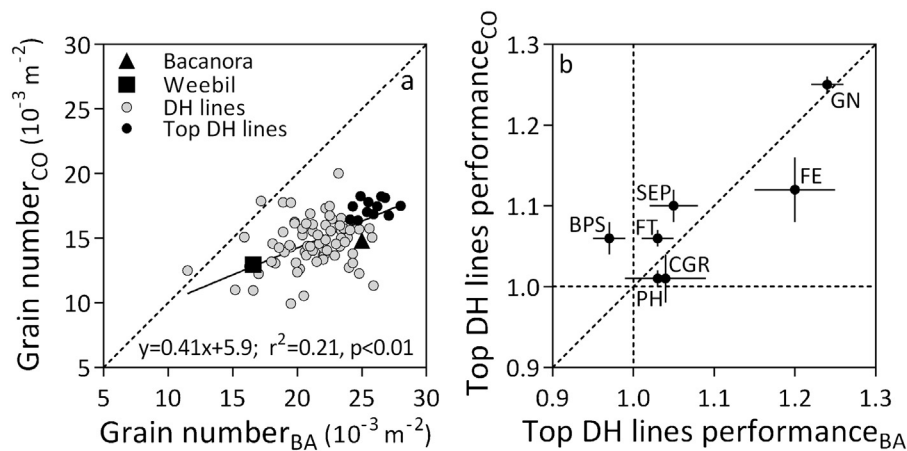
**Fig. 3.** Relationships between grain number and its main determining traits (spike dry weight at flowering and fruiting efficiency, top panel) and between spike dry weight and its determining traits (stem elongation phase, pre-flowering crop growth rate and biomass partition to spike, lower panel) for doubled-haploid (DH) and parental lines (Bacanora and Weebil) in Ciudad Obregon. The analysis comprised 85 and 105 DH lines in BA and CO, respectively. Correlation coefficients (Pearson) and probabilities are included only when  $p$ -value is lower than 0.01. In (c), dotted lines represent iso-grain number curves corresponding to the 90th percentile (upper), the parental mean and the 10th percentile (lower).



**Fig. 4.** Relationship between pre-flowering crop growth rate and (a) photosynthetically active radiation (PAR) intercepted per thermal time unit during pre-flowering and (b) pre-flowering radiation use efficiency (RUE) for 85 doubled-haploid (DH) and parental lines (Bacanora and Weebil) in Buenos Aires. Correlation coefficients (Pearson) and probabilities are included. Box and Whiskers of DH line means for daily radiation interception efficiency ( $RIE_{day}$ ) at different crop stages are included (inset), indicating the population minimum, first quartile, median, third quartile and maximum. Dotted line represents a  $RIE_{day}$  of 0.95.

the physiological attributes determining GN were most strongly responsible for their consistent improved performance (Fig. 5). Although average GN was higher in BA than in CO, there was a positive association between environments (Fig. 5a), and top performing DH lines represented an improvement in GN over the parental lines of very similar magnitude (Fig. 5b). Within this sub-population, which does represent the lines which would have been selected in a breeding program aimed to improve GN, fruiting efficiency was the most relevant physiological component of GN

responsible for the improved performance (Fig. 5b). On average, top DH lines set 25% more grains than the parental mean with a fruiting efficiency 20 and 12% higher in BA and CO, respectively. With the exception of stem elongation phase, none of the other physiological components was consistently relevant for the improved performance of the top lines representing consistent increases in GN, even though some may have been significantly related to GN for the whole population. For instance, improved biomass partitioning to spike had a contribution to improved GN in CO but not in



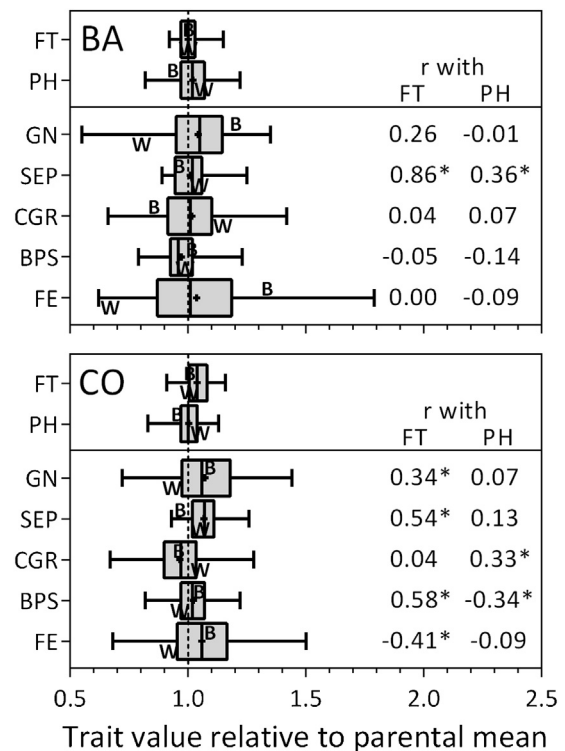
**Fig. 5.** Top doubled-haploid (DH) lines performance in both environments. (a) Contrast between grain number per unit area (GN) in Buenos Aires (BA) and in Ciudad Obregon (CO), for DH and parental lines (Bacanora and Weebil). The analysis comprised 85 and 105 DH lines in BA and CO, respectively. Slope, intercept, regression coefficient (model II) and probabilities are included. Top DH lines, considered as the lines which are in the top quartile in both environments, are indicated with closed circles. (b) Contrast between the top DH lines average relative to the parental mean (standard errors are indicated) of GN, stem elongation phase (SEP), pre-flowering crop growth rate (CGR), biomass partitioning to spike (BPS), fruiting efficiency (FE), flowering time (FT) and plant height (PH) in CO and BA. Horizontal and vertical dotted lines indicate parental mean in CO and BA, respectively. Crosswise dotted lines represent the 1:1 relationship in both sub-figures.

BA and crop growth rate of the top performing lines did not seem significantly higher than that of the parental lines in any of the two environments. Longer stem elongation phase showed an improvement of GN respect to the parental lines and it was consistent in both environments, but its magnitude is comparable with that of fruiting efficiency only in CO. On the other hand, agronomic important traits such as flowering time and plant height were not relevant for GN improvement in this population (Fig. 5b). Although DH top lines tended to have later flowering time than the parental mean, mainly in CO, the magnitude of this difference was smaller than the one for fruiting efficiency.

#### 4. Discussion

A detailed and accurate phenotyping of mapping populations is an important “bottleneck” not only for identifying simple traits functionally linked to GN (and yield), but also to understand their genetic bases (Furbank and Tester, 2011; Fiorani and Schurr, 2013). As the parental lines are high-yielding cultivars, the DH population phenotyped in this work represents the germplasm routinely used in a breeding program. In a previous study with this population, García et al. (2013) highlighted that GN was the main determinant of grain yield and that it did not show association with flowering time or plant height across four contrasting environments. Similarly, in the present experiment, GN and its determining traits did not show a consistent association with flowering time or plant height in BA and CO separately as those relationships were not of the same sign (positive or negative) and significant across the two contrasting environments (Fig. 6). Only the length of stem elongation phase was consistently and positively associated with flowering time, as has also been reported for other wheat populations (García et al., 2011; Borràs-Gelonch et al., 2012). In addition, if we consider only the top DH lines, neither flowering time nor plant height was a relevant physiological component for their improved GN. Two interpretations arose from this phenotyping, according to the analysis carried out: (i) at the whole population level, the phenotyping was useful to describe the variability in physiological traits determining GN; while (ii) focusing on top DH lines, which represent a feasible genetic improvement, the phenotyping allowed for the identification of fruiting efficiency as a key physiological trait for improving GN, and thereby grain yield, at least in the population derived from this cross.

Positive transgressive segregation (i.e. DH lines with better performance than the parental lines) was observed for all physiological traits phenotyped. Depending on the trait and environment, between 20 and 50% of the DH lines outperformed the parental



**Fig. 6.** Doubled-haploid (DH) population variability and influence of flowering time (FT) and plant height (PH) on grain number per unit area (GN) and its determining traits in Buenos Aires (BA, top panel) and Ciudad Obregon (CO, lower panel). The analysis comprised 85 and 105 DH lines in BA and CO, respectively. On the left, Box and Whiskers of DH line values relative to the parental mean are represented for FT, PH, GN, stem elongation phase (SEP), pre-flowering crop growth rate (CGR), biomass partitioning to spike (BPS) and fruiting efficiency (FE), indicating the population minimum, first quartile, mean (cross), median, third quartile and maximum, and the parental lines values (B, Bacanora; W, Weebil). On the right, a table with correlation coefficients (Pearson) between FT or PH and GN, SEP, CGR, BPS and FE is included. Asterisks indicate significant correlation ( $p < 0.01$ ).

lines. Positive transgressive segregation has been found in other elite populations, for instance, in traits related to phenology and tillering dynamics in wheat (Borràs-Gelonch et al., 2012) and barley (Borràs et al., 2009), or in drought-adaptive traits in wheat (Olivares-Villegas et al., 2007; Rattey et al., 2009). Nevertheless, as far as we are aware, this is the first evidence of positive transgressive segregation in physiological traits determining wheat grain number.

At the whole population level, both fruiting efficiency and pre-flowering RUE appeared as key traits for GN improvements, but the former was clearly the one determining the improved performance of top DH lines. Regarding parental lines, Bacanora set more grains than Weebil due to a higher fruiting efficiency. However, several DH lines showed a better combination than Bacanora for both spike dry weight at flowering and fruiting efficiency, resulting in higher GN. This suggests that crossing parents with different GN determination mechanisms could be a valuable breeding strategy to put together a better combination of physiological traits in the same genotype to improve GN (Bustos et al., 2013). In this case, population variability in spike dry weight at flowering depended more on growth than development and/or partitioning of assimilates during the critical period, and pre-flowering crop growth rate was strongly related to differences in RUE, in agreement with the idea of increasing spike dry weight through photosynthetic capability at the crop level (Reynolds et al., 2012). Nevertheless, when the analysis was limited to top DH lines, fruiting efficiency was consistently the most relevant physiological determinant of GN, responsible for the improved performance of these top lines compared to the parents under both environments. This result is in agreement with Bustos et al. (2013) who evaluated the same population in the high-yielding environments of South Chile, comparing the performance of top DH lines with highest-yielding Chilean cultivars. In that work, the top yielding DH lines out-yielded the Chilean checks by setting a greater GN associated with higher fruiting efficiency.

Although several works have concluded that spike dry weight at flowering was the main physiological component of GN (Slafer and Andrade, 1993; Abbate et al., 1997; Demotes-Mainard et al., 1999; González et al., 2003), the apparent conflict with our results may be related to differences between those studies and the present one. In general terms, when a given cultivar explores different growth and/or development conditions through different radiation levels (Thorne and Wood, 1987; Abbate et al., 1997), variations in nitrogen availability (Demotes-Mainard et al., 1999; Ferrante et al., 2012), changes in photoperiod and vernalization (González et al., 2003) or the combination of some of these factors (Abbate et al., 1995; Demotes-Mainard and Jeuffroy, 2004), the spike dry weight at flowering is the main trait explaining environmental effects on GN. Similarly, the variations in GN were well explained by differences in spike dry weight when cultivars released before and after the introgression of major dwarfing genes was compared (Siddique et al., 1989; Slafer and Andrade, 1993; Miralles et al., 1998). However, when comparing performance of modern cultivars (all of them exhibiting optimized plant height), fruiting efficiency appears as the main determinant of GN differences (Abbate et al., 1998; González et al., 2011). Therefore, taking into account that the present work evaluated materials with a similar genetic background (i.e. DH lines from a cross between modern cultivars of the same breeding program with similar plant height), growing under similar growth and development conditions in each site (i.e. narrow flowering time range in most of the population), a higher contribution of fruiting efficiency rather than spike dry weight is not surprising, especially when comparing top DH lines with the parents. On the other hand, at the whole population, the main GN determinants (i.e. spike dry weight and fruiting efficiency) tended to be negatively associated, suggesting a possible trade-off. However, when the analysis was carried out within top DH lines, the

evident benefit of higher fruiting efficiency was not compensated by reductions in spike dry weight at flowering. In fact, all the determinants of this last trait (excepting biomass partitioning to spike in BA) were similar or improved with respect to parental mean.

Although more detailed studies are needed, improvements in fruiting efficiency could be associated with better intra-spike partitioning with a concomitant increase in biomass being delivered to developing florets instead of structural components of the spike (Slafer and Andrade, 1993), or to florets of smaller size (Dreccer et al., 2009). If the former is the main mechanism, potential grain weight would not be affected (Acreche et al., 2008); but in the second case, the achieved increase in GN would bring about compensations in grain weight; as the smaller the size of the fertile florets, the smaller the potential weight of the grains (Calderini et al., 2001). In the present study, average grain weight tended to be negatively associated with fruiting efficiency in both environments at the whole population level ( $r = -0.38$ ,  $p < 0.01$  in BA and  $r = -0.28$ ,  $p < 0.01$  in CO), but this relation did not affect grain yield which was driven by GN (García et al., 2013). In the same way, top DH lines showed a lower average grain weight than the parental lines (ca. 16%), but this reduction was not able to compensate the GN improvement (ca. 25%). Thus, the reduction in average grain weight might be simply the reflection of an increased proportion of distal grains (Miralles and Slafer, 1995), representing a non-competitive partial compensation between fruiting efficiency and final grain weight. A recent study with a large set of Argentinean high-yielding cultivars differing in fruiting efficiency did not report a negative relationship between this trait and potential grain weight, reinforcing the idea that the fruiting efficiency could be increased to improve GN in elite wheat cultivars (González et al., 2014).

Summarizing, from the positive transgressive segregation observed in this elite wheat population, fruiting efficiency appears as key trait to improve GN. As a complex trait that includes processes like the assimilate partitioning inside the spike (or spike structure), floret development and survival and grain setting (Fischer, 1983), more detailed studies about physiological and genetic bases of fruiting efficiency could help to understand how this trait drives GN, and thereby grain yield improvements.

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