

Retrospective study characterizing yield and physiological changes in sorghum crop

by

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Abstract

Understanding physiological changes in response to long-term selection for yield can inform breeding decisions and hasten genetic gain. This dissertation is organized into four chapters: Chapter 1, Introduction, Chapter 2, presents yield gain achieved for US sorghum hybrids and their components (grain number and grain weight), Chapter 3 focuses on nutrient use efficiency, specifically on nitrogen (N) internal efficiency (NIE, yield to N uptake ratio), and Chapter 4, Conclusions. For Chapters 2 and 3, field trials were conducted during the 2018 and 2019 seasons in eight site-years across the states of Kansas and Texas (US) including 20 commercially available grain sorghum hybrids released by Pioneer between 1963 and 2017. Consistent with estimates using historical yield data, sorghum yield improvement was $27 \text{ kg ha}^{-1} \text{ yr}^{-1}$. Grain number increased at a rate of $100 \text{ grains m}^{-2} \text{ yr}^{-1}$, and modern hybrids had larger panicle size with greater accumulation of WSC during the vegetative period (until flowering). Additionally, greater remobilization of WSC during the reproductive period (after flowering) to grain was captured, thus, maintaining grain size on the increased grain number per unit area and harvest index (Chapter 2). The N internal efficiency (NIE, yield to N uptake ratio) increased at the expense of a reduction in grain N concentration and, in minor proportion, due to an increase in the N harvest index (NHI) at maturity for newer relative to older hybrids. Newer genotypes evidenced greater N remobilization from the stover to the grains during the reproductive period (Chapter 3). This study demonstrates the physiological foundations for yield enhancement and the N and C utilization behind yield genetic gain for sorghum hybrids. Future yield gains in sorghum can be pursued by enhancing N uptake to sustain further genetic progress.

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Dedication

*To my parents Dario and Norma,
and to my love Javier.*

Chapter 1 - Introduction

Globally, crop yield improvement represents a vital effort to the balance food supply with the demand (Potgieter et al., 2016). The crop intensification of cereals and the increase of yield potential can help maintain yield advances on existing agricultural land (Cassman et al., 2003). Over the years, potential yield gain for cereal has been reported for favorable environments, however, it is essential to continue enhancing yield potential under stress conditions (Fischer and Edmeades, 2010). Retrospective studies are relevant to document changes in the phenotype associated with the breeding process and to explore alternatives to improve yield over time.

Sorghum (*Sorghum bicolor* L. Moench) is among the five most important cereal crops produced in the world lead by United States (US) (FAO, 2019; Ciampitti and Prasad, 2020). In the major production area of the country, known as the “Sorghum Belt”, the crop is cultivated mainly under rainfed conditions. Sorghum brings several benefits to the dryland cropping system in the US due to its drought tolerance. The continuation of its adoption and use in these systems will depend on tools and methodologies that allow a sustainable increment on the realized genetic gain and subsequently make it a suitable option relative to other crops.

During the last decades, the advancement of grain sorghum yield in the US resulted from changes in genotype (G), environment (E), agronomic management (M), and their interactions (Assefa and Staggenborg, 2010; Ciampitti et al., 2019). The introduction of hybrid sorghum between 1950 and 1980 in the US agricultural systems led to yield progress of 39% (Miller and Kebede, 2015). Sorghum can increase the stability of the US dryland production system due to its tolerance to drought stress (Staggenborg et al., 2008) and its ability to survive and produce grain under hot and dry environments (Ciampitti and Prasad, 2020).

The improvement in resource use efficiency in sorghum represents a desirable trait in plant breeding programs to enhance yields and support the increasing global growth population (Ciampitti and Prasad, 2020). Therefore, retrospective studies focused on investigating changes in resource use capture and use efficiency are relevant to document the mechanisms underpinning yield improvements over time. Nitrogen (N) represents the most important nutrient related to crop yield improvement in crops, and its enhancement on use efficiency is desirable to reduce fertilizer costs (Cassman et al., 2002; Traore and Maranville, 1999). Understanding the N internal efficiency (NIE, yield to N uptake ratio) of sorghum hybrids is a useful tool for genetic improvement programs and field crop management.

This research project proposes two main research chapters, Chapter 2 to i) characterize changes in yield over time for sorghum hybrids released during the past six decades and ii) elucidate the physiological traits underpinning the yield gain of sorghum released from the 1960s until the present in the US; Chapter 3 to i) describe changes over time in NIE for US sorghum hybrids with different years of release, ii) characterize the physiological traits underlying changes on NIE, and iii) link the C (as WSC) and N dynamics during the reproductive period. This study will provide reliable information to sorghum breeders for future genotype selection aiming to increase the realized genetic gain on sorghum US breeding programs.

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Chapter 2 - Physiological changes associated with yield advance for sorghum hybrids released during the last six decades in the U.S.

Abstract

Understanding physiological changes in response to long-term selection for yield can inform breeding decisions and hasten genetic gain. The objective of this study was to characterize changes over time in yield-relevant physiological traits for hybrids with different years of release for grain sorghum (*Sorghum bicolor* L. Moench). Field trials were conducted during the 2018 and 2019 seasons in eight site-years across the states of Kansas and Texas (US) including 20 commercially-available grain sorghum hybrids released by Pioneer between 1963 and 2017. Detailed yield-related physiological traits were determined in two site-years including grain yield and its components, grain filling, plant biomass, panicle length, and water-soluble carbohydrates (WSC) during the reproductive period. Consistent with estimates using historical yield data, sorghum yield improvement was $27 \text{ kg ha}^{-1} \text{ yr}^{-1}$. For the two site-years with detailed yield-related physiological traits, no changes in final grain weight, grain filling duration and rate over time were documented. In contrast, grain number increased at a rate of $100 \text{ grains m}^{-2} \text{ yr}^{-1}$. Modern hybrids had larger panicle size and showed greater accumulation of WSC during vegetative period (as measured at start of flowering) and greater remobilization of WSC during the reproductive period (after flowering) to grain, thus, maintaining grain size on the increased grain number per unit area and harvest index. These findings suggest that WSC dynamics play a critical role on past genetic yield gain in sorghum and its potential for future improvements should be considered.

Introduction

Sorghum (*Sorghum bicolor* L. Moench) is among the five most important cereal crops produced in the world (FAO, 2019; Ciampitti and Prasad, 2020). In 2019, US growers produced 8.6 Mg from 1.8 ha distributed mainly among the states of Kansas, Texas, Colorado, Oklahoma, and South Dakota. This production region is known as the “Sorghum Belt” where sorghum is cultivated largely under rainfed conditions. While in the US, sorghum enters the value chain as feed for livestock and energy production (Rakshit et al., 2014; Ciampitti and Prasad 2020), in Asia and Africa sorghum main use is for human consumption. Yield improvement of sorghum for the semiarid global regions can increase food supply and stability of the global food system (Potgieter et al., 2016).

During the last decades, improvement of grain sorghum yield in the US resulted from changes in genotype (G), environment (E), agronomic management (M) and their interactions (Assefa and Staggenborg, 2010; Ciampitti et al., 2019; 2020). The introduction of hybrid sorghum between 1950 and 1980 in the US agricultural systems led to yield improvement of 39% (Miller and Kebede, 2015). In addition, improved crop management tailored to hybrids further increased yields over a short period of time (Monk et al., 2015). Sorghum can increase the stability of US dryland production system because of its tolerance to drought stress (Staggenborg et al., 2008) and its ability to survive and produce grain under hot and dry conditions (Ciampitti and Prasad, 2020). The continuation of sorghum adoption and use in the dryland systems will depend on tools and methodologies that allow sustainable and continued increments on the realized genetic gain for grain yield to make it a suitable crop option relative to other crops.

Molecular breeding methods augmented by physiological knowledge proved effective to hasten genetic gain in maize (Cooper et al., 2014; Messina et al., 2018). The understanding of the physiological changes caused by long-term selection for yield can inform the design of breeding programs and phenotyping strategies that fully leverage biological knowledge (Cooper et al., 2014; Messina et al., 2018). Prospective analysis of genetic gain is an effective approach to inform selection as demonstrated in the development of drought tolerant maize (*Zea mays* L.) (Cooper et al., 2014; Gaffney et al., 2015). However, to fully implement this approach, it is necessary to understand the genetic variation for adaptive traits, the accessible data on key physiological traits, genetic information, and the architecture of breeding programs and characterization of the target population of environments (Messina et al., 2011; Reynolds et al., 2021). Recent studies revealed that grain yield has increased from 1960 to 2010's in commercial sorghum hybrids and in inbreds lines (Pfeiffer et al., 2019). Furthermore, understanding the physiological determinants of yield stability can improve decision making at advanced stages of selection (Gizzi and Gambin, 2016; Messina et al., 2020).

Understanding physiological changes of US sorghum hybrids released from the 1960s until the present decade can provide reliable information to guide the selection process and increase the realized genetic gain in sorghum. Messina et al., (2020) established that long-term genetic gain research in maize under water limited environments, was determined by improvements in reproductive resilience but not water capture. Plant-to-plant variation was a key yield determinant of maize yields under stress conditions (Otegui et al., 1995). Similar to maize, sorghum kernel (grain) number was highly correlated with variation in growth during a critical window (van Oosterom and Hammer, 2008; Messina et al., 2019; Gambín et al., 2008).

However, changes in root architecture and water capture have been implicated in the improved drought tolerance in sorghum (Singh et al., 2010).

In this study, a causal framework was used to formulate three hypotheses to explain the realized genetic gain for yield in Pioneer sorghum hybrids over the last 60 years:

$$\begin{aligned} Y &= GN \times GW \\ Y &= W \times HI \\ GW &= GFR \times GFD \end{aligned}$$

where Y is yield, GN is grain number per square meter, GW: grain weight, W: total plant mass per square meter, HI: harvest index, GFR: grain filling rate, and GFD: grain filling duration. The hypotheses are: i) yield increased due to increased GN (and HI) as the result of increased allocation of mass to the panicle, ii) GN increased without compensating reduction in GW due to increased water-soluble carbohydrates (WSC) utilization, and iii) yield improvement due to increased W via enhanced resource capture/conversion efficiency. The overall objective was to characterize changes over time in yield-relevant physiological traits for hybrids with different years of release for sorghum.

Materials and Methods

Experimental conditions

Eight field studies (site-year combinations) were conducted at Corteva Agriscience research stations throughout Kansas (KS) and Texas (TX), USA, during the 2018 and 2019 growing seasons (Table 1). Across all sites, the experimental design was a randomized complete block design, with 20 sorghum hybrids and three replications. Sorghum hybrids, all from Corteva Agriscience, spanned six decades of genetic selection (from 1963 until 2017). Hybrids were selected based on their commercial and breeding relevance for each decade. For the 2018

growing season, the plot size was 2-row plots with 5.3 m long and 0.76 m row spacing in all locations. For the 2019 season, the plot size was 4-row plots with 5.3 m long and 0.76 m row spacing in all locations, except Riley (KS) where 8-row plots were used. Yield data was collected from all locations during 2018 and 2019. Detailed physiological descriptors of grain yield formation were obtained from one location (Riley, KS) in both seasons (2018 and 2019). Due to the high plant density tested in these studies (heads number = plant number), the contribution of tillers to yield was not negligible. Specific details of planting date, harvest date, plant density, and other management information from each site/year combination are presented in Table 1.

Phenotypic measurements and laboratory analysis

Aboveground plant biomass was measured at flowering (Vanderlip and Reeves, 1972) and at harvest time during the 2018 season by collecting four consecutive plants per plot due to the small plot size. For the 2019 season, biomass samples were collected at three times during the vegetative phase (5 fully expanded leaves, 8-9 fully expanded leaves, and 15 days before flowering) and three times during the reproductive phase (flowering, 15 days after flowering, maturity) in a 0.7 m² harvest area. During the vegetative stages, plants were partitioned into leaves (leaf blades) and stem (stem + leaf sheaths). During reproductive stages, plants were partitioned into leaves, stem, and panicle (plus grain). Dry weight was obtained after drying plant fractions in an air-forced oven at 65°C until constant weight.

Phenology was recorded daily for tagged individual plants before flowering and during the reproductive period. During the grain-filling period, one plant per plot was collected in intervals of 4-to-5 days to characterize seasonal dynamics of grain dry matter and moisture

content. The panicle was separated from the rest of the plant (leaf + stem) by cutting 1 cm below the first branch and placed in plastic bags to conserve grain moisture. At the laboratory, 40 kernels per main panicle were sampled inside of a humidity chamber by collecting 10 kernels from each of four visually determined sections of the head (Fig. 1A). Individual kernels were weighted, and fresh weight was collected prior to drying in an air-forced oven at 65°C until constant weight to record dry weight. Lastly, the rest of the plant was partitioned into stem and leaves, and dry biomass weights were collected after drying as described for other samples. Harvest index was estimated as the ratio of grain dry weight to total above ground biomass.

Grain yield was obtained from a plot combine, harvesting two rows per plot (approximately 8.17 m²) and adjusted to a standard 150 g kg⁻¹ moisture. Grain number per unit of land area was calculated as the ratio between total grain yield and individual grain weight.

In the 2019 season, images of ten consecutive panicles per plot with metric reference were collected at physiological maturity to determine panicle uniformity using the panicle length as a proxy measurement (Fig. 1). A total of 30 panicle measurements per hybrid [10 panicles per plot in each of the three replications (3)] were conducted for a total of 600 images for all hybrids. Panicle length was defined as the length in centimeters of the panicle from the first branch to the top of the panicle (Fig. 1B).

For laboratory analyses, stem samples were ground through a 0.25-mm sieve. Water-soluble carbohydrates (WSC) were analyzed in the stem fraction using the anthrone reagent method, water extraction followed by a colorimetric reaction (Galicía-Flores et al., 2009). Biomass samplings at the beginning of flowering and during the reproductive phase until physiological maturity were used for WSC analyses. The total WSC content in the stem was

determined by multiplying the stem dry mass by the WSC concentration. WSC remobilization was calculated as the difference of WSC at flowering minus at physiological maturity.

Statistical analysis

Mixed-effects models were fitted with nlme (Pinheiro et al., 2018) and lme4 (Bates et al., 2015) packages in RStudio (RStudio, 2015) for grain yield and its components (grain number and grain weight). For seasonal dynamics of biomass, and WSC generalized additive models (GAMs) were fit to the data using the mgcv package (Wood, 2017). Within models, the effect of the relative maturity of the genotypes, the year of the experiment, and the location (nested within year) were included as random components. Best Linear Unbiased Estimators (BLUEs) for each genotype and trait measured were calculated. For each trait, BLUEs were regressed against released year of the hybrids.

Grain dry weight was modeled on a day-time basis from flowering to harvest maturity. Similar to method used by Prasad et al., (2006) grain filling rate (GFR) and grain filling duration (GFD) were estimated fitting a bi-linear model [equations (1) and (2)]:

$$\begin{array}{ll} \text{Grain weight (mg grain-1)} = a + b * x & \text{for } x < c \quad [1] \\ \text{Grain weight (mg grain-1)} = a + b * c & \text{for } x > c \quad [2] \end{array}$$

where x are the days after flowering, a is the y-intercept (mg grain-1), b is the GFR (mg grain⁻¹ d⁻¹), and c is the total GFD (in days). The bi-linear function was fit to the data using non-linear mixed effects models with the nlme package (Pinheiro and Bates, 2018). An adjustment for different variances per sampling date was used to correct for heterogeneity of residuals due to the increased sample variance over time.

Results

Yield gain over time

Grain yield linearly increased over the year of release period (1963-2017) at an average rate of $27 (\pm 8.4, \text{SE}) \text{ kg ha}^{-1} \text{ yr}^{-1}$ (0.42% relative to the mean) for the evaluated US sorghum hybrids across all eight site-years (Fig. 2). For Riley (KS) site, where detailed physiological descriptors were assessed, a similar yield increase trend of $31 (\pm 17) \text{ kg ha}^{-1} \text{ yr}^{-1}$ was documented ($Y = 31 (\pm 17)x + 5247(\pm 588); r^2 = 0.1; p < 0.1$). The lowest estimated yield across hybrids was 5064 kg ha^{-1} for the hybrid released in 1963, while it was 6486 kg ha^{-1} for the hybrid released in 2017 at Riley (KS) site.

Yield components

All yield components and yield-related physiological traits were investigated only at Riley (KS) site during the 2018 and 2019 growing seasons. Individual grain weight did not change ($p > 0.1$) with year of release (Fig. 3A) with mean of $29(\pm 0.08) \text{ mg}$. However, considerable variation was observed for this trait across the decades with values ranging from 18.7 to 37.9 mg per grain. Similarly, neither the grain-filling rate nor the grain fill duration changed with year of release. The grain-filling rate ranged from 0.55 to $1.34 \text{ mg grain}^{-1} \text{ day}^{-1}$, while the grain filling duration ranged from 30 to 40 days (Fig. 3B, C).

Kernel (grain) number per unit area was the main component accounting for the grain yield increase reported over time. An increase of $96(\pm 45) \text{ kernels m}^{-2} \text{ yr}^{-1}$, represented an overall improvement of 0.43% relative to the mean yield obtained across decades in this study (Fig. 4).

Physiological traits associated with grain yield gain

Changes in panicle dry biomass at flowering time were not statistically significant ($p > 0.1$) across decades (Fig. 5A). Consequently, kernel number per unit of biomass at flowering increased over time [Fig. 4 and 5A; $p < 0.05$, $y = 1.05(\pm 0.4)x - 1928(\pm 854)$], which reflects the grain set efficiency during the initial stages of the reproductive period. Furthermore, there is a significant positive trend for panicle length across decades (Fig. 5B), supporting the overall increase in kernel number per panicle for modern hybrids. In contrast, the panicle-to-panicle variability, estimated from the variability of panicle length of consecutive plants in a row and expressed as a coefficient of variation (CV, %) did not change significantly with year of release ($p > 0.1$ data not shown). However, it should be noted that the oldest hybrid showed larger variability (4.1%, 1963-year of release) relative to the newest hybrid (3.4%, 2017-year of release). Lastly, while HI at physiological maturity increased with years of release (Fig. 5C) the overall mass at maturity did not (Supplementary Table S1). Consequently, dry matter allocation to the grain (grain/g) has changed in parallel with increases in panicle length and grain number.

The difference between stem WSC concentration at flowering and maturity (Table S1) was used to estimate the remobilization of WSC reserves during the reproductive period. Reduction in WSC during grain-filling increased with the year of release of the hybrids (Fig. 6). This scenario portrays greater remobilization of WSC for modern hybrids relative to older sorghum hybrids. Dry biomass suggests no trend over time as well as the relationship between dry biomass and HI ($p > 0.1$). These results provide evidence that modern hybrids presented an increase in WSC remobilization with superior grain number and harvest index when compared to early released hybrids. Despite all these changes, final biomass at maturity was not affected suggesting a source/sink remodeling favoring overall yield estimates in newer hybrids.

Discussion

Globally, yield gains reported for sorghum range from 8 to 44 kg ha⁻¹ yr⁻¹ (0.2-0.7% yr⁻¹) (Pfeiffer et al., 2019; Gizzi and Gambin, 2016; Mason et al., 2008) for similar historical periods to this study. Here we show that genetic improvement in elite US sorghum hybrids has been mainly associated with improvements in the reproductive efficiency (grain number per unit biomass) and harvest index of the crop. An increase in kernel number due to an increase in panicle size and reproductive dry matter allocation underpinned the rate of genetic gain in this study. However, genetic gain in yield for sorghum is still inferior relative to other hybrid crops such as maize (Mason et al., 2008), which has yield gains ranging from 37 to 109 kg ha⁻¹ yr⁻¹ (0.4-1.8% yr⁻¹) (Duvick, 2005; Mason et al., 2008; Cooper et al., 2014b; Assefa et al., 2018). This positive trend on yield gains in sorghum is not close to a plateau (Fig. 2), which suggests there are further opportunities for crop improvement in the US rainfed environments. It is important to note that crop improvement opportunities in sorghum are ample; improving sink set through reproductive resilience and allocation, and improving growth by leveraging genetic variation in water capture and transpiration efficiency (Messina et al., 2021, Hammer et al., 2014; 2020).

Experimental results support the hypothesis that sorghum yield gain was mainly supported by changes in grain number per unit area (96 grains m⁻² yr⁻¹), similar to those reported for US commercial (Mason et al., 2008) and public hybrids (Pfeiffer et al., 2019). Gizzi and Gambin (2016) observed similar trends on a set of 43 commercial hybrids released over 30 years (1984 to 2014) in Argentina. Strong positive relationships were observed between grain number and grain yield in grain sorghum even under stress conditions such as high temperatures (Prasad et al., 2015) and drought (Mutava et al., 2011). Furthermore, similar responses on yield gain

related to changes in grain number across decades have been reported for other field crops such as wheat (*Triticum aestivum* L.) (Donmez et al., 2001; Sadras and Lawson, 2011; Xiao et al., 2012), soybean (*Glycine max* (L.) Merr.) (Tamagno et al., 2020), and maize (Mason et al., 2008; Duvick, 2005; Cooper et al., 2020). Increases in the number of grains per unit area are consistent with the increment in panicle size over time. Pfeiffer et al. (2019) demonstrated this relationship when studying plant yield potential as a result of an increase in grain number and panicle size.

Our results indicate stability in individual grain weight with increases in number over time and an increase in grain number per unit of biomass. The stability of individual grain weight over time is relevant for yield improvement in crops (Sadras, 2007; Uribelarrea et al, 2008). Grain weight stability reported here is in agreement with similar studies conducted with Argentinian sorghum genotypes released between 1984 and 2014 (Gizzi and Gambin, 2016). Lack of variation in the duration of grain filling was implicated in the stability of grain weight (Gizzi and Gambin., 2016). However, it is important to note that newest hybrids, with more grains to fill, retained comparable grain growth rates to older genotypes. This demonstrates that there has not been a tradeoff between grain number and individual grain weight with selection in sorghum, in agreement with what has been reported for maize (Otegui et al., 2015).

This study shows that the increase in grain set, and dry matter allocation or partitioning could be related to effects on WSC. Sugar concentration has been implicated in signaling (Koch, 2004), and previous research has confirmed the relationship between greater sugar levels and grain set by influencing floral abortion in grain sorghum (Jain et al., 2007), maize (Boyer and McLaughlin, 2006) and wheat (Dreccer et al., 2014). The dominance of grain number over individual grain weight for yield formation has been shown to rely on the early determination of the grain number component around flowering (van Oosterom and Hammer, 2008). In addition,

greater storage of carbohydrate reserves during stem elongation is recognized to provide support to the grain filling, especially under drought (Rathey et al., 2009; Liu et al., 2020), heat (Tahir and Nakata, 2005), and nitrogen stresses (Gebbing et al., 1999; Fernandez et al., 2020).

Modifications in WSC levels are known to emerge as a result of genotypic differences in lodging and tillering in crops (Foulkes et al., 2011; Sadras and Lawson, 2011; Dreccer et al., 2013).

Thus, a higher WSC accumulation may also play a key role behind adjustments in tillering and lodging traits across historical sorghum hybrids (Monk et al., 2015).

Taken together, our results suggest that carbohydrate concentrations could underpin the increased kernel set, and thus contribute to set an increasingly higher potential harvest index.

Panicle size has been recently reported to be related to the remobilization of carbohydrates from the stem to the panicle during grain filling for sorghum (Tovignan et al., 2020). Pfeiffer et al.

(2018) found in new hybrids less sucrose concentration in the biomass at maturity when compared to older hybrids, corroborating the higher remobilization rate on newer genetics.

Although increases over time in panicle size represent a larger sink potential for stored WSC in the stem, further enhancement on the panicle-to-panicle variability (and in plant-to-plant variation) should be one of the focus in future breeding programs. In addition, WSC content and remobilization are genetically influenced and as such could be considered within any breeding selection strategy, either directly or indirectly through other biomass components (Murray et al., 2009, Brenton et al., 2020). Here, we show that an improved reproductive efficiency (kernel set and grain number) was associated with greater WSC at flowering, and that greater grain yield and associated higher harvest index were achieved through greater WSC remobilization. These findings suggest that WSC dynamics play a critical role in yield formation and its potential to be used for direct or indirect selection in future sorghum-breeding programs should be investigated.

Conclusions

Yield gain across time was mainly related to increments on grain number per area rather than grain weight. Lack of significance for grain filling duration and grain filling rate explained grain weight stability. Moreover, greater grain numbers per area (grain set) in newer hybrids are associated with increments in panicle size as well as harvest index. The increase in WSC accumulation in stems during the vegetative period seem to contribute to set a higher yield potential through higher grain numbers, and during grain-filling to realize higher grain yield and higher harvest index via enhanced WSC remobilization from stems to grains.

Future research on plant growth rate and WSC dynamics around the critical stages of grain setting or grain growth could elucidate the mechanisms for the observed increases in grain number over time and carbohydrate partitioning. Exploring these physiological aspects are valuable not only to understand the reasons of genetic gain into yield-component traits but also as a knowledge to guide selection strategies in current breeding programs.

Figures

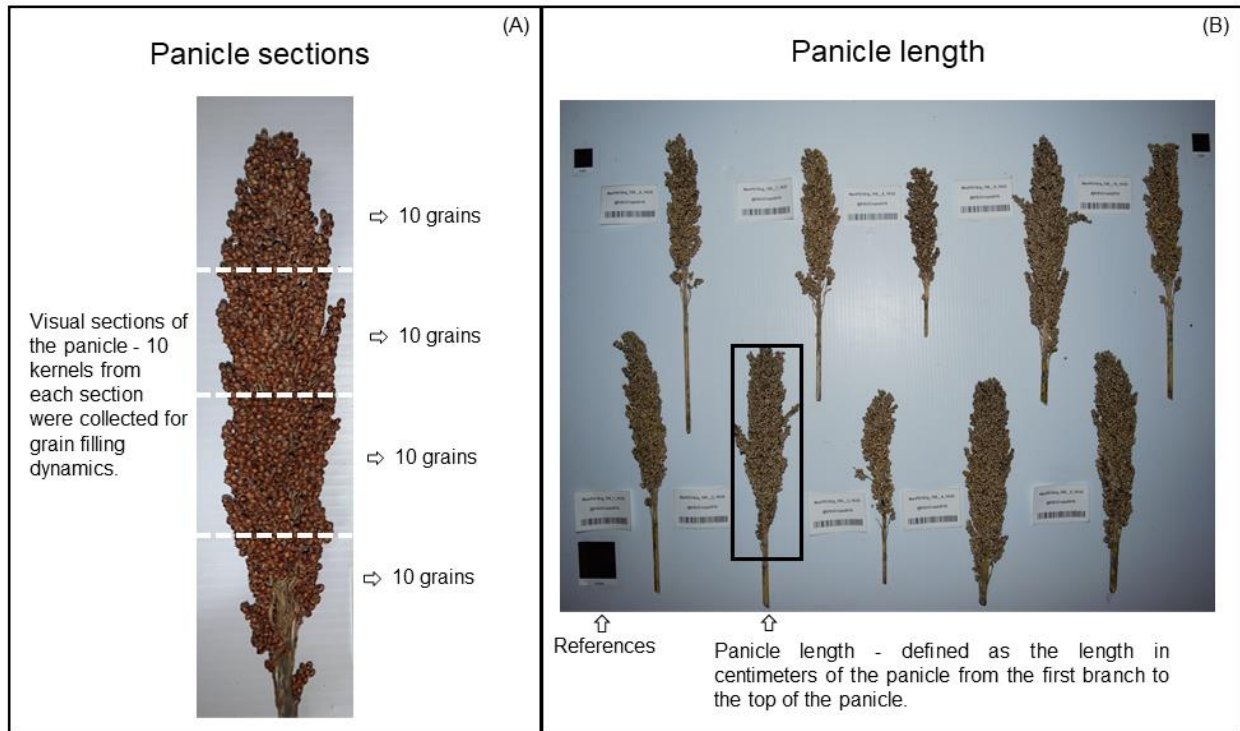


Figure 2.1. Representation of sorghum panicle measurements performed in Riley (KS). Visual panicle sections for seed filling dynamics (A). Panicle length of 10 consecutive plants (B).

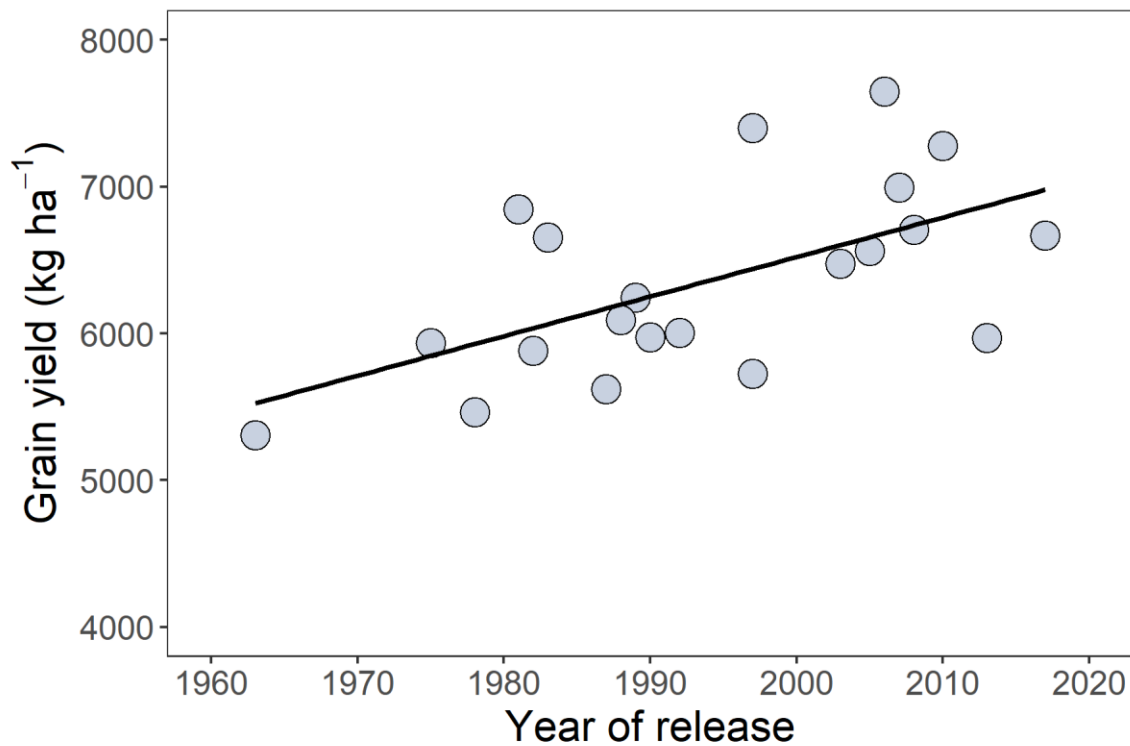


Figure 2.2. Relationship between best linear unbiased estimators (BLUEs) for grain yield (kg ha⁻¹) for all site-years $Y = 27 (\pm 8)x + 5524(\pm 285)$; $r^2 = 0.3$; $p < 0.005$ relative to the year of release of the hybrids.

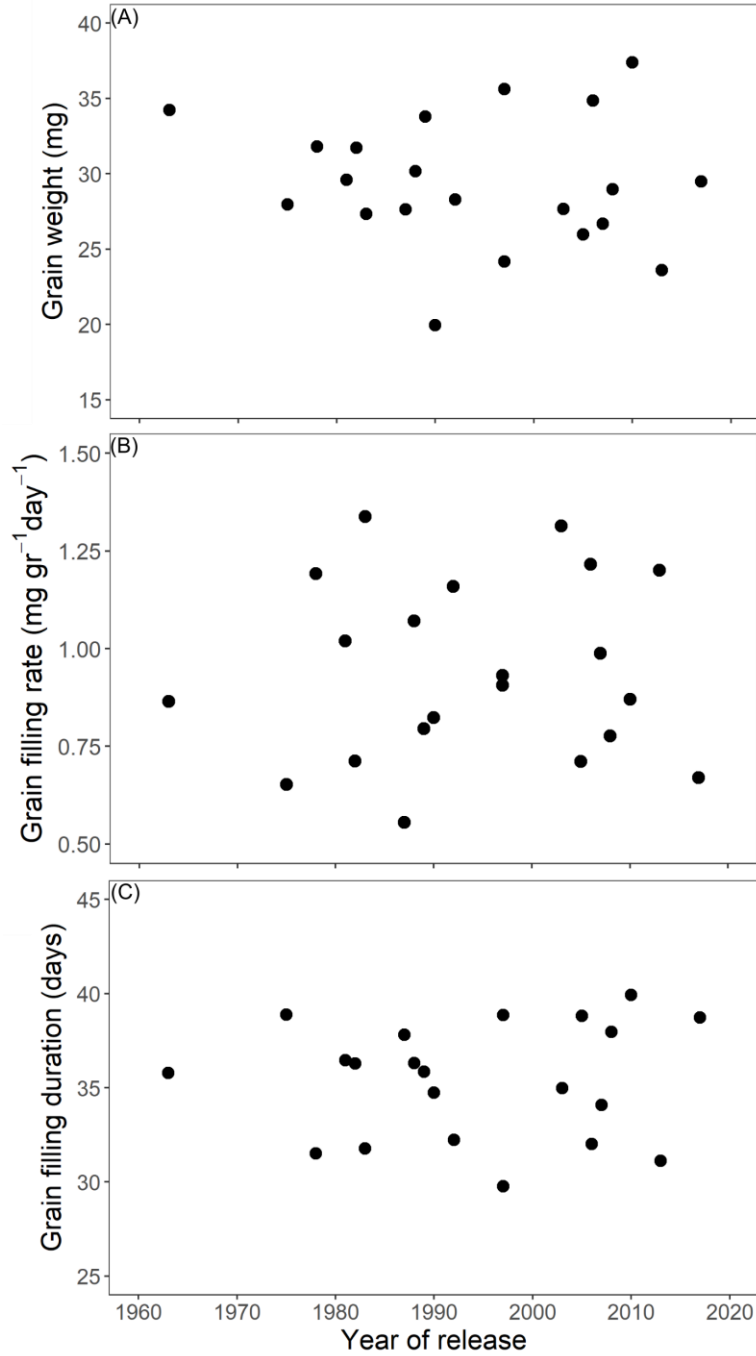


Figure 2.3. Grain weight (mg kernel⁻¹) (A), grain filling rate (mg gr⁻¹ year⁻¹) (B), and grain filling duration in days (C) all relative to the year of release of the sorghum hybrids at Riley (KS, US) during the 2018 and 2019 growing seasons. Each observation represents best linear unbiased estimators of three replications per hybrid and two site-years.

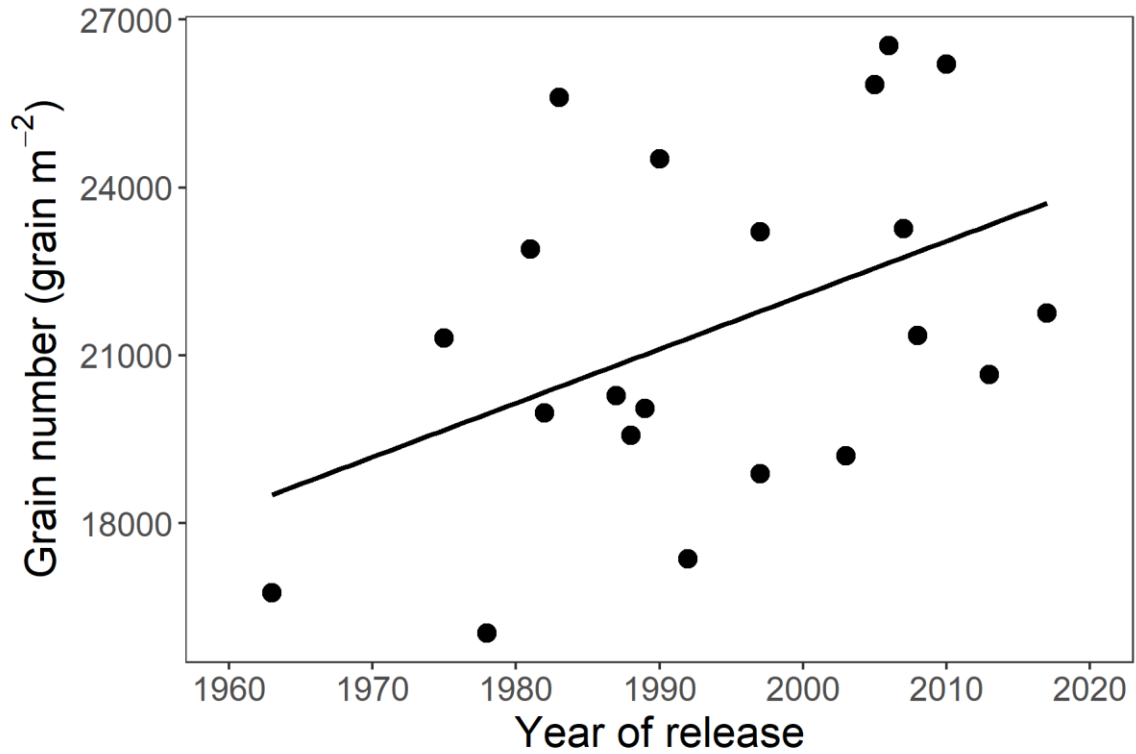


Figure 2.4. Relationship between kernel (grain) number (number m⁻²) and the year of release of the sorghum hybrids at Riley (KS, US) during the 2018 and 2019 growing seasons $Y = 96 (\pm 45)x + 18509(\pm 1523)$; $r^2 = 0.2$; $p < 0.05$. Each observation represents best linear unbiased estimators of three replications per hybrid and two site-years.

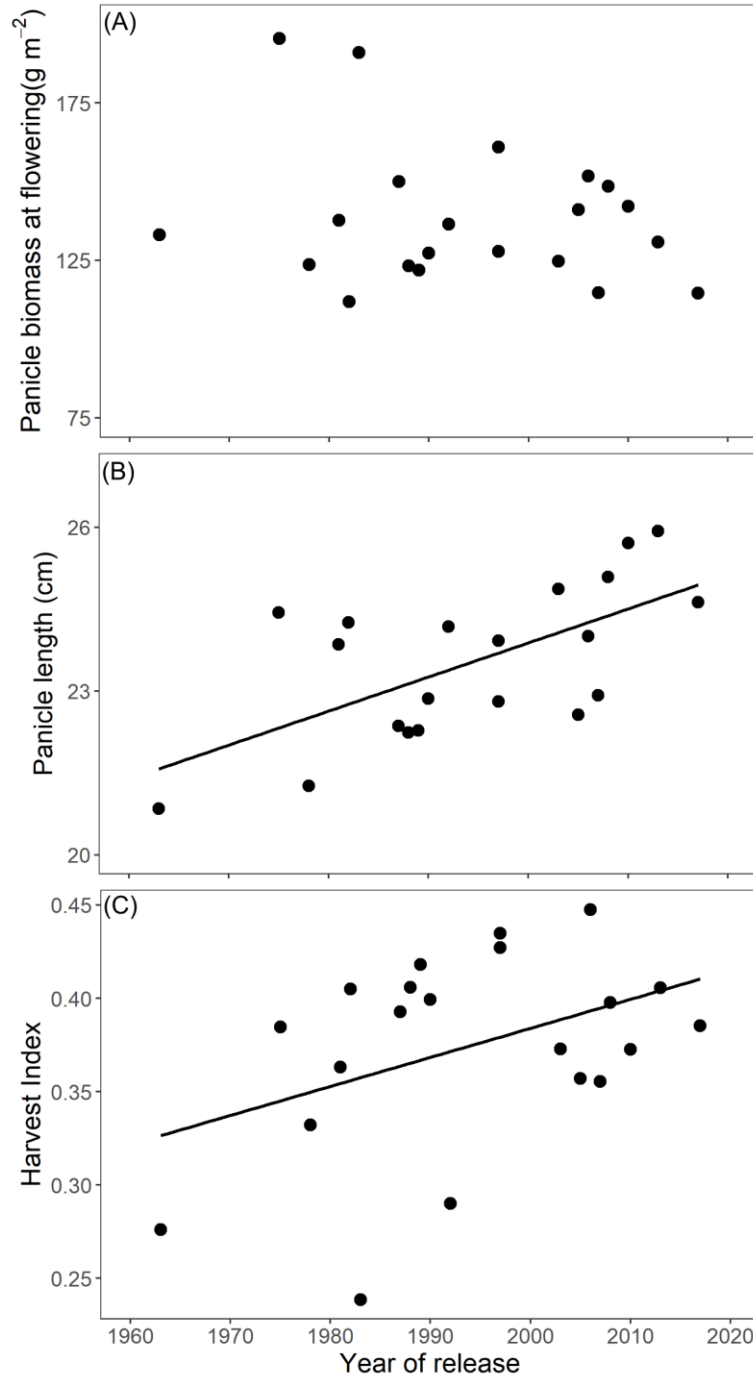


Figure 2.5. Panicle dry biomass (g m⁻²) at flowering (A), panicle length at maturity (cm) $Y = 0.1(\pm 0.003)x + 22(\pm 0.1)$; $r^2 = 0.4$; $p < 0.001$ (B), and harvest Index $Y = 0.0015 (\pm 0.0008)x + 0.3(\pm 0.3)$; $r^2 = 0.13$; $p < 0.1$ (C) all related to the year of sorghum hybrid release for experiments at Riley (KS, US). Each observation represents best linear unbiased estimators of three replications per hybrid and two site-years. Best linear unbiased estimators for total dry biomass (g m⁻²), stem water soluble carbohydrates (WSC) concentration (mg g⁻¹), and stem WSC content (g m⁻²) relative to the years of release of the hybrids are reported in Supplemental Table S1.

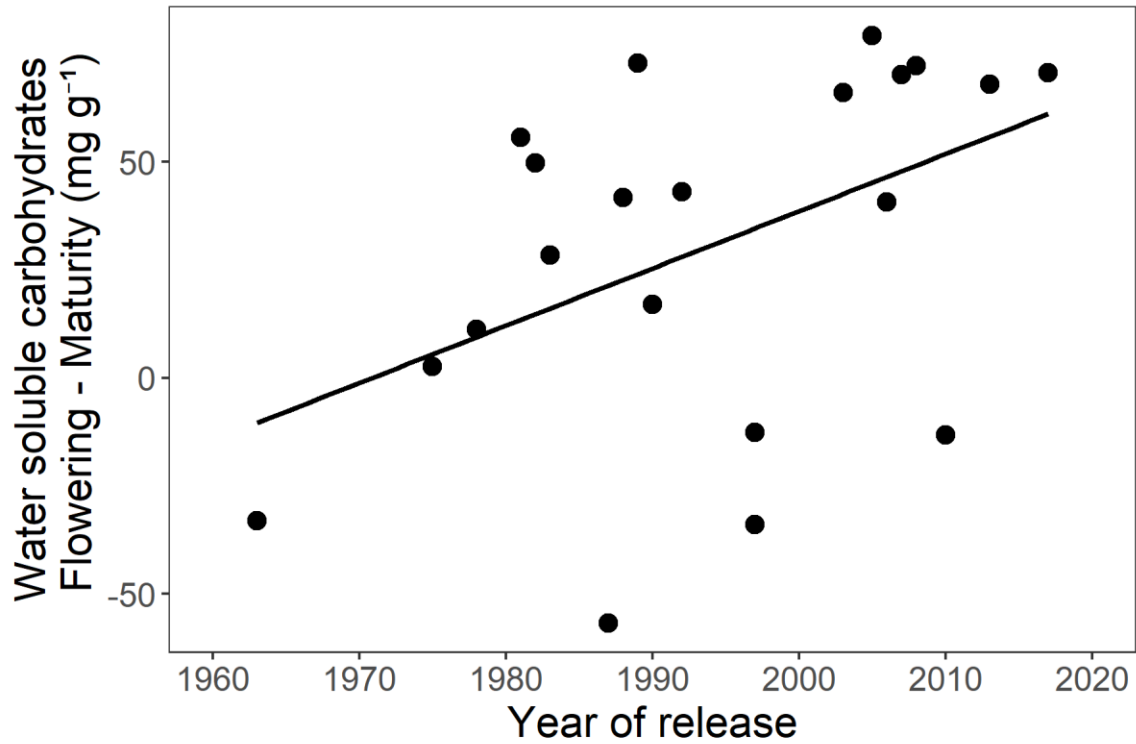


Figure 2.6. Water Soluble Carbohydrates (WSC) remobilization in concentration (mg g^{-1}) (WSC at flowering - WCS at maturity) related to years of the release of the hybrids at Riley (KS, US) during the 2018 and 2019 seasons $Y = 1.3(\pm 0.6)x - 11(\pm 20)$; $r^2 = 0.2$; $p < 0.05$. Each observation represents the best linear unbiased estimators of three replications per hybrid and two site-years.

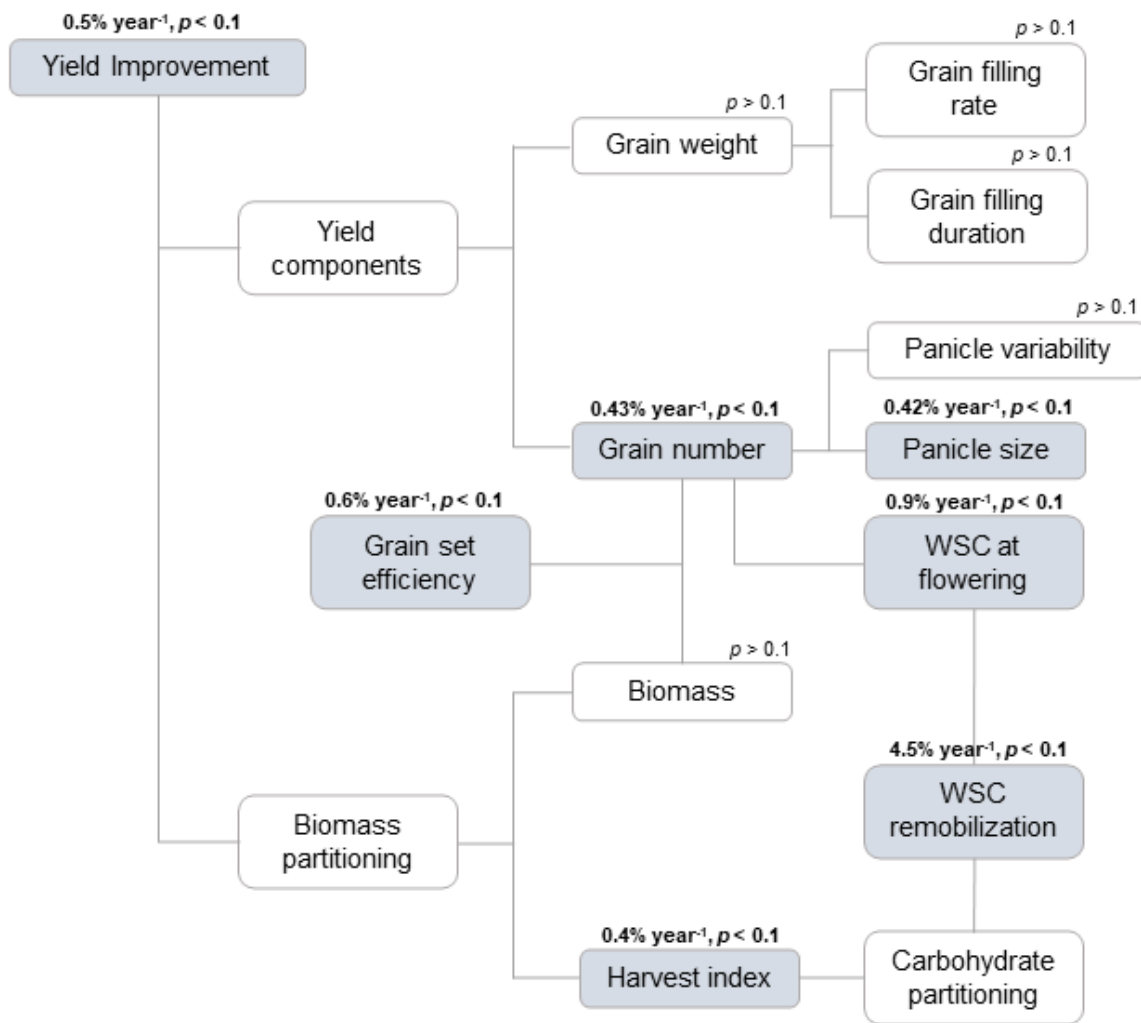


Figure 2.7. Graphical abstract: Historical yield improvement in sorghum crop, portraying significant ($p < 0.1$) relative gains (%) in key plant traits over the last six decades.

Tables

Table 2.1. General information for the 2018 and 2019 growing season for field trial locations.

Location	Year	Sowing date	Harvest date	Row Length (m)	Row Width (m)	Rows Per Plot	Harvest rows	Irrigation	Irrigation level	Plant density (pl/ha)	Previous crop
Cloud, KS	2018	6/6/2018	11/16/2018	5.3	0.76	2	2	Dryland	None	173,000	Soybean
Finney, KS	2018	6/10/2018	10/29/2018	5.3	0.76	2	2	Irrigated	Full	148,000	Wheat/Fallow
Moore, TX	2018	6/29/2018	11/20/2018	5.3	0.76	2	2	Irrigated	Limited	94,000	Corn
Riley, KS	2018	6/7/2018	11/21/2018	5.3	0.76	2	2	Irrigated	Limited	173,000	Corn
Cloud, KS	2019	7/1/2019	11/6/2019	5.3	0.76	4	2	Dryland	None	173,000	Soy
Dallam, TX	2019	6/14/2019	10/28/2019	5.3	0.76	4	2	Irrigated	Limited	89,000	Fallow
Hale, TX	2019	5/31/2019	10/9/2019	5.3	0.76	4	2	Irrigated	Limited	148,000	Sorghum
Riley, KS	2019	6/8/2019	11/8/2019	5.3	0.76	8	2	Dryland	None	173,000	Wheat

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Chapter 3 - Nitrogen internal efficiency improved over the past six decades in US sorghum hybrids

Abstract

Retrospective studies are critical to investigate changes in physiological traits related to nitrogen (N) uptake and yield formation. For sorghum (*Sorghum bicolor* L. Moench), N is a key yield-limiting nutrient, and improving its N internal efficiency (NIE, yield to N uptake ratio) should be a research priority. In addition, study changes in N efficiency across time linked to carbon (C) economy can provide insights for future breeding targets. This study aims to characterize N and C dynamics (via studying changes in water-soluble carbohydrates, WSC) of twenty US sorghum hybrids released from 1963 to 2017 period. Field trials were conducted during the 2018 and 2019 growing seasons in Riley, Kansas (US). Seasonal changes in biomass accumulation, N uptake and WSC were characterized during both vegetative and reproductive periods. Modern hybrids presented greater NIE compared to those from the 1960's. Overall, beyond the yield gain documented for modern sorghum hybrids, the NIE increased at the expense of a reduction in grain N concentration and, in minor proportion, due to an increase in the N harvest index (NHI) at maturity for newer relative to older hybrids. Newer genotypes evidenced greater N remobilization from the stover to the grains during the reproductive period. This study demonstrates the physiological foundations for an increase in N and C utilization behind yield genetic gain for sorghum hybrids. Future yield gain in sorghum could be pursued by enhancing N uptake to sustain further genetic progress.

Introduction

The improvement in resource use efficiency in cereal crops represents a desirable trait in plant breeding programs to enhance yields and support the increasing global growth population (Ciampitti and Prasad, 2020). Therefore, retrospective studies focused on investigating changes in resource use capture and use efficiency are relevant to document the mechanisms underpinning yield improvements over time. Sorghum (*Sorghum bicolor* L.) is one of the five most important cereal crops in agriculture, its capacity to better tolerate stress conditions (e.g., heat, drought) represents a valuable alternative in crop rotations (Stone et al., 2006). For US sorghum hybrids, grain yield increased about 27 kg ha⁻¹ yr⁻¹ during the last decades (Chapter 2). Overall, yield gain was strongly associated with the increase in grain number, harvest index (in minor proportion), and carbon (C) remobilization during the reproductive period (Pfeiffer et al., 2019; Chapter 2). Nitrogen (N) represents the most important nutrient related to crop yield improvement in crops, and its enhancement on use efficiency is desirable to reduce fertilizer costs (Cassman et al., 2002; Traore and Maranville, 1999). Understanding the N internal efficiency (NIE, yield to N uptake ratio) of sorghum hybrids is a useful tool for genetic improvement programs and field crop management.

From N use efficiency perspective, progress on this trait can be driven by increases in plant N uptake, reduction on grain N concentration as yield improves, and/or by improvement on the overall reproductive N remobilization (Bollam et al., 2021; Muchow, 1998). Previous studies indicate the effect of the genotypes in NIE for sorghum and other cereals (Bollam et al., 2021; Ciampitti and Vyn, 2012). More specifically for sorghum, Ciampitti and Prasad (2016) documented increases in NIE with more than half of the variation on this trait accounted for reductions in grain N concentration, but with no clear trends on NIE over time. However, a more

detailed retrospective characterization study is vital to better understand potential changes in NIE over time and to dissect the main physiological factors underlying the changes on this trait for US sorghum.

Nitrogen represents the most important nutrient influencing the growth and development of sorghum plants; therefore, it is strongly associated with crop yields (Ciampitti and Prasad, 2016; van Oosterom et al., 2010). Physiologically, in plants, N plays a key role in C fixation, biomass accumulation, and grain yield (Dechorgnat et al., 2010). Nitrogen is absorbed by the plant during the vegetative and reproductive period. During the vegetative period N is allocated to the leaf to be utilized in photosynthesis and to the stems acting as reservoir organs for the plant (Ciampitti et al., 2013; Ciampitti and Prasad, 2016). During the grain filling period, reserves of the plants are remobilized to fill the grains (van Oosterom et al., 2010). At this moment C and N are utilized for cell division at the lag phase of seed filling and cell growth during the exponential growth phase (Lemcoff and Loomis, 1994; Singletary and Below, 1989). Nitrogen remobilization have been described for sorghum and corn as one of the mechanisms related to yield gain over time (Ciampitti and Vyn, 2012; Bender et al., 2013; Ciampitti and Prasad, 2016; Mueller et al., 2019).

Sorghum hybrids in the US have increased yield over the past decades by increasing the number of grains per unit area (Chapter 2). Biomass accumulation was described as a trait with small or null changes over time for sorghum (Gizzi and Gambin, 2016; Chapter 2). However, the plant physiological mechanisms underpinning yield gains over time indicate a better utilization of resources in modern genotypes (Ciampitti and Prasad, 2016; Pfeiffer et al., 2019). For sorghum, increments in NIE have been reported due to an increase in N remobilization, manifested in a low N content in stover at physiological maturity in high yielding hybrids (van

Oosterom et al., 2010). In parallel to maize (*Zea mays* L.) yield improvement (Ciampitti and Vyn, 2012), additional increases in yield resulted in unintentional reductions in grain N concentration (Ciampitti and Prasad, 2016). The understanding of these physiological mechanisms for sorghum is helpful to continuing enhancing yields for future generations.

This study aims to i) describe changes over time in NIE for US sorghum hybrids with different years of release, ii) characterize the physiological traits underlying changes on NIE, and iii) link the C (as WSC) and N dynamics during the reproductive period. Overall, our hypothesis states that direct selection for yield in US sorghum has indirectly resulted in N efficient hybrids via reductions in grain N concentration and greater N remobilization, without modifying plant biomass accumulation and N uptake.

Materials and methods

Field study

This study was conducted during the 2018 and 2019 growing seasons at Corteva Agriscience research stations at Riley County (Kansas), US. Detailed information about the research study is presented in Chapter 2. The experimental design was a randomized complete block design (RCBD), with 20 sorghum hybrids and three replications. Sorghum hybrids, (Corteva Agriscience, Johnston, IA), were selected based on their commercial and breeding relevance spanning a total of six decades (from 1963 until 2017). For the 2018 growing season, the plot size was 2-row plots and for the 2019 season, the plot size was 8-row for Riley County, KS. In the study due to the high plant density (heads number = plant number), the contribution of tillers to yield was not negligible. Lastly, the planting date for 2018 was 6/7/2018, and for 2019 was 6/8/2019. Plant population for both years was about 173,000 pl ha⁻¹, and the N requirements

for the crops were supplied before planting time following standard agronomic practices for the area.

Measurements and laboratory analysis

Aboveground plant biomass was measured at flowering and at harvest time during the 2018 season. In 2019 season aboveground biomass was measured three during vegetative and reproductive periods. During the vegetative period, plants were partitioned into leaves (leaf blades) and stem (stem + leaf sheaths). During the reproductive period, plants were partitioned into leaves, stems, and panicles (plus grain). Dry weight was obtained after drying plant fractions in an air-forced oven at 65°C until constant weight. Phenology was recorded daily from the tagged individual plants during the entire growing season. During the grain-filling period, one plant per plot was collected in intervals of 4-to-5 days to characterize the dynamics of grain dry matter and moisture content. Grain mass (g m^{-2}) during the entire grain filling was calculated by multiplying the grain mass for each grain filling sampling date by the final grain number. Grain yield was obtained from a plot combine, harvesting two rows per plot (approximately 8.17 m^{-2}), and adjusted to a standard 150 g kg^{-1} moisture.

Biomass samples (and plant fractions) were used to determine WSC and N concentrations. For the WSC laboratory analyses, stem samples were ground through a 0.25-mm sieve and analyzed using the anthrone reagent method, water extraction followed by a colorimetric reaction (Galicía-Flores et al., 2009). The total stem WSC content was determined by multiplying the stem dry mass by the WSC concentration. For N analyses, all plant fractions (stem, leaves, and panicle) were analyzed by near-infrared analysis (NIR, model DA 7250 NIR analyzer). A subset of 194 samples (out of the 1540 samples) were selected for analysis by dry

combustion and used to calibrate Partial Least Squares (PLS) models for each fraction to estimate N concentration. The validation results of the models are included in the supplementary material (Fig. S1). The N content in all the fractions was determined by multiplying the dry mass by the N concentration.

Nitrogen related traits

Nitrogen internal efficiency (NIE) is defined as the ratio between grain yield on a dry basis (in kg ha⁻¹) over the N uptake at physiological maturity (Moll et al., 1982). The NHI is defined as the relationship between the grain N content over the total N uptake (Ciampitti and Vyn, 2012). The N remobilization efficiency was calculated as the ratio between N remobilization from flowering to maturity (g m⁻²) divided by the total N uptake (g m⁻²) at flowering time.

Statistical analysis

For analysis purposes, all twenty hybrids were grouped according to years of release and the number of hybrids in each category (Table1). Mixed-effects models were fitted using nlme (Pinheiro et al., 2018) and lme4 (Bates et al., 2015) packages in RStudio (RStudio, 2015) for grain yield and NIE. For seasonal dynamics of biomass, N, and WSC generalized additive models (GAMs) were fit to the data using the mgcv package (Wood, 2017). Within models, the effect of the relative maturity of the hybrids, the year of the experiment, and the location (nested within the year) were included as random components.

Grain dry weight was modeled on a day-time basis from flowering to harvest maturity. Similar to the method used by Prasad et al., (2006) grain filling rate (GFR) and grain filling duration (GFD) was estimated fitting a bi-linear model [equations (1) and (2)]:

$$\begin{aligned} \text{Grain weight (mg grain-1)} &= a + b * x && \text{for } x < c \quad [1] \\ \text{Grain weight (mg grain-1)} &= a + b * c && \text{for } x > c \quad [2] \end{aligned}$$

where x is the days after flowering, a is the y-intercept (mg grain-1), b is the GFR (mg grain⁻¹ d⁻¹), and c is the total GFD (in days). The bi-linear function was fit to the data using non-linear mixed-effects models with the nlme package (Pinheiro and Bates, 2018). An adjustment for different variances per sampling date was used to correct for heterogeneity of residuals due to the increased sample variance over time.

The rate of grain mass accumulation, changes in grain dry weight, in g m⁻² d⁻¹, stover nitrogen uptake (g m⁻² d⁻¹), and stem WSC content (g m⁻² d⁻¹) were calculated using the first derivative of the GAMs model for each variable. All rates were estimated for each group of hybrids on a given day after flowering.

Results

A significant yield gain of 0.43% yr⁻¹ was found for US sorghum hybrids. The modern hybrids, last decade (2006-2017), presented significantly greater yield relative to the oldest hybrids, first two decades (1963-1983) (Fig. 1A). The historical changes in NIE are similar to the ones documented for the genetic yield gain. The modern sorghum hybrids presented an overall increase of 0.37% yr⁻¹ relative to the oldest group, from the 1960's (Fig. 1B).

Similar trends over time were observed for total biomass, stem, and leaf biomass (Fig. 2A, B, C). Likewise, for total N uptake (and stem N uptake) a lack of changes over time has been

recorded across sorghum groups (Fig. 2D, E). However, a greater N allocation to the leaves for modern hybrids (Fig. 2F) could be an indication of the greater WSC content in the stem fraction at flowering for the newest relative to the oldest sorghum hybrids (Fig. 2G).

The grain N concentration decreased over time, with the modern sorghum hybrids presenting a lower grain N concentration (0.177 mg g^{-1}) relative to the older group (0.230 mg g^{-1}) (Fig. 3A, Table 2). In addition, the progression of NHI during the grain filling period reflected an improved partitioning for modern sorghum hybrids (2006-2017) relative to the rest of the groups (Fig 3B, Table 2). Clearly, the two older groups (1963-1983, and 1984-1994) presented a lower final NHI (0.36) relative to the two modern sorghum groups (0.43) (1995-2005, and 2006-2017).

Over the past decades, the N remobilization efficiency has increased roughly $2.4\% \text{ yr}^{-1}$, for US sorghum hybrids (Fig. 4A). In accordance, the stover N content (denoted as the sum of stem N plus leaf N content) at maturity was significantly reduced by roughly $-0.43\% \text{ yr}^{-1}$ for the modern group of hybrids relative to the older ones (Fig. 4B).

Dynamics on rates for grain mass, stover N uptake, and stem WSC reflect a substantial change between the oldest and newest sorghum groups (Fig. 5). Our results indicate that modern hybrids presented a greater grain mass rate, earlier and faster stover N depletion, and greater WSC remobilization during the reproductive period, all relative to the oldest sorghum hybrids (Fig. 5A, B, C). These results support the concept of much faster remobilization of N from stover and WSC from the stem organ to accompany the greater increase in grain mass documented for modern sorghum hybrids. Modern sorghum hybrids presented a greater initial grain mass rate, sustained close to 26 days after flowering and with a greater peak ($17.5 \text{ g m}^{-2} \text{ d}^{-1}$) relative to the older sorghum group ($15 \text{ g m}^{-2} \text{ d}^{-1}$) (Fig. 5A). For stover N depletion, a similar initial N reservoir

has been recorded for both groups but with a faster N remobilization from these plant fractions (leaf+stem organs) and presenting a dual-phase, rapid until 10 days after flowering and less than proportional N decrease until 20 days after flowering, plateauing right after until the end of grain filling (Fig. 2B). On the other hand, the older sorghum hybrids presented a more steady but slower N remobilization until the end of grain filling. Lastly, for the stem WSC both decay rates were similar, but the modern hybrids display a sharper decrease until 15 days after flowering ($-1.8 \text{ g m}^{-2} \text{ d}^{-1}$) and then increasing, while the older group showcases a smooth decline, following by an increase in WSC at a similar time (Fig. 2C).

Discussion

Nitrogen internal efficiency increased over the past six decades for US sorghum hybrids. The overall increase in NIE is mainly linked to the yield gain, reduction in grain N concentration, increase in NHI (minor proportion), and due to greater remobilization of N and C (WSC) from the reservoir plant fractions. These results are consistent with other cereals such as maize for the increase in NIE over time (Ciampitti and Vyn, 2012), but the overall changes are slightly different. For maize, the increase in NIE over time was related to improvement on N uptake for both pre-and post-flowering periods and driven not only by better N remobilization but also by increases in overall plant N uptake (Ciampitti and Vyn, 2012). In sorghum during the past decades, no changes in total plant biomass and plant N uptake were recorded in this research study. Therefore, the changes on NIE are mainly linked to yield gains and improve remobilization/partitioning.

Previous studies in cereal crops indicate that increments in yield are unintentionally related to reductions in grain N concentration over time (Ciampitti and Vyn, 2012; de Oliveira

Silva et al., 2020; Muchow, 1998; Yang et al., 2016). Likewise, our study documented a dilution of N in the grain for new sorghum hybrids, most likely this outcome is derived by the increment in grain number and maintaining similar individual kernel weight over decades (Chapter 2). These results are consistent with previously reported increments on NIE at expenses of grain N dilution for sorghum (Ciampitti and Prasad, 2016). In addition, the same synthesis paper documented a positive relationship between NIE and NHI (and the latter with grain HI). That relationship explains the overall outcome reported on this retrospective study with improvements on NIE accounted for greater NHI of modern hybrids during the grain filling period (Ciampitti and Prasad, 2016). These traits related to NIE indicate an improvement over time in nutrient utilization efficiency.

In crops, changes in NIE can be driven by greater plant N uptake, or by improvement in N remobilization during the reproductive period (Bollam et al., 2021; Ciampitti and Vyn, 2013; Muchow, 1998). Ciampitti and Vyn (2013) illustrated for maize that the proportion of remobilized N represents a large contribution to the final grain N content for modern hybrids. In this study, we provided scientific evidence that grain N demand was highly dependent on N remobilization from the stover fraction for modern sorghum hybrids. Additionally, lower stover N content at physiological maturity in new genotypes supports the notion that N remobilization is the main N source underpinning the enhancement of NIE for modern hybrids over time. Future breeding programs for sorghum should focus on improvements on plant N uptake before flowering, increasing overall N reservoir, and after flowering (as did occur for maize) to sustain greater plant growth, grain N demand, and superior yields (Borrell et al., 2000).

The remobilization of N and WSC during grain filling seems to play an important role in yield gain for sorghum and maize (Fernandez et al., 2021). Over the past decades, the

remobilization of WSC from flowering to maturity contribute to support an increase in grain number via enhancement of better harvest index for sorghum hybrids (Chapter 2). Fernandez et al. (2021) demonstrated a close relationship of N and WSC remobilization during the reproductive period for modern maize hybrids, and the importance of this process to sustain higher grain growth rates and yields. In wheat (*Triticum aestivum* L.), stem reserves storage and utilization represent an effective mechanism to sustain grain filling under stress (Blum et al., 1998). Our study also demonstrates that the early depletion rates for both stover N content and stem WSC are relevant to sustain the higher grain mass accumulation rate during grain filling for newer sorghum hybrids.

Conclusion

This study reports for the first time for US sorghum hybrids an overall improvement in NIE by $0.37\% \text{ yr}^{-1}$ in the past six decades. This NIE improvement was mainly linked to yield gain and dilution in grain N concentration in modern hybrids. Lastly, N remobilization plays a key role in the historical enhancement of NIE in sorghum, with this point reflected with a sustained reduction in stover N content at maturity to sustain the greater N demand in modern sorghum hybrids.

Future studies should investigate the main factors driving the C and N demand and investigate in more detail potential changes linked to improved photosynthesis efficiency (and RUE) at the canopy level to better understand the overall C economy in sorghum plants.

Figures

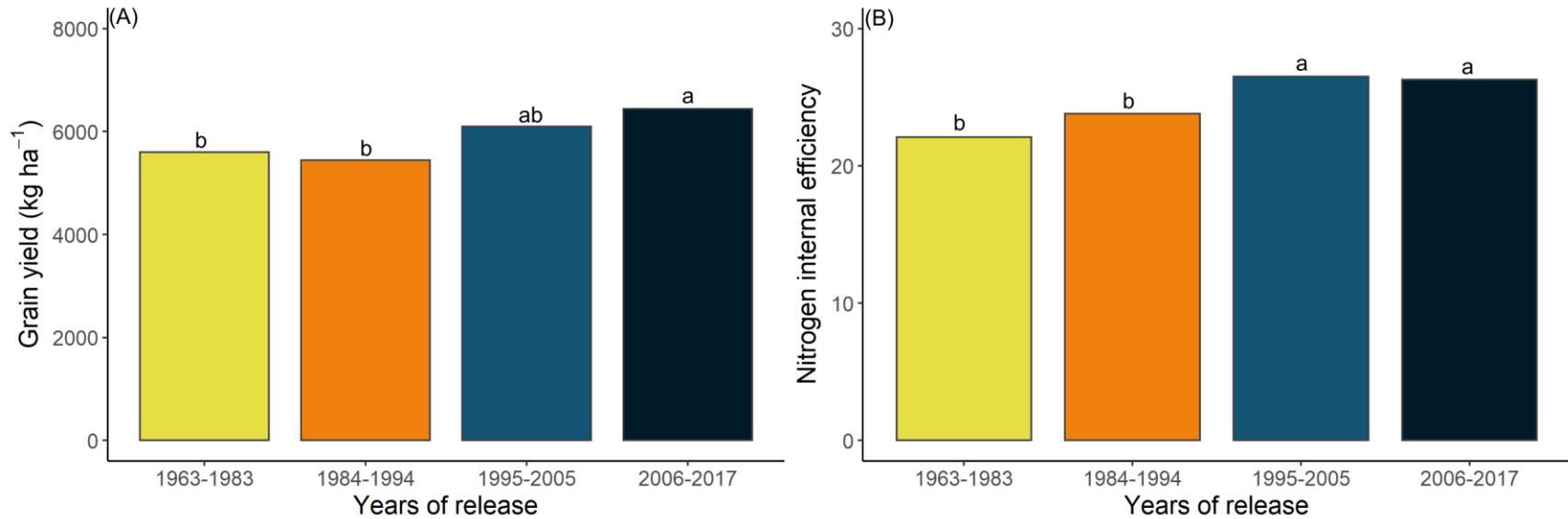


Figure 3.1. Grain yield by groups of hybrids for Riley, KS during 2018 and 2019 growing seasons (kg ha^{-1}) (A), nitrogen internal efficiency (NIE) in $\text{kg ha}^{-1} / \text{kg ha}^{-1}$ (B). Within each group of hybrids, different letters indicate significant differences at a p-value < 0.1 . Colors represent the group of hybrids according to years of release ● 1963-1983, ● 1984-1994, ● 1995-2005, and ● 2006-2017.

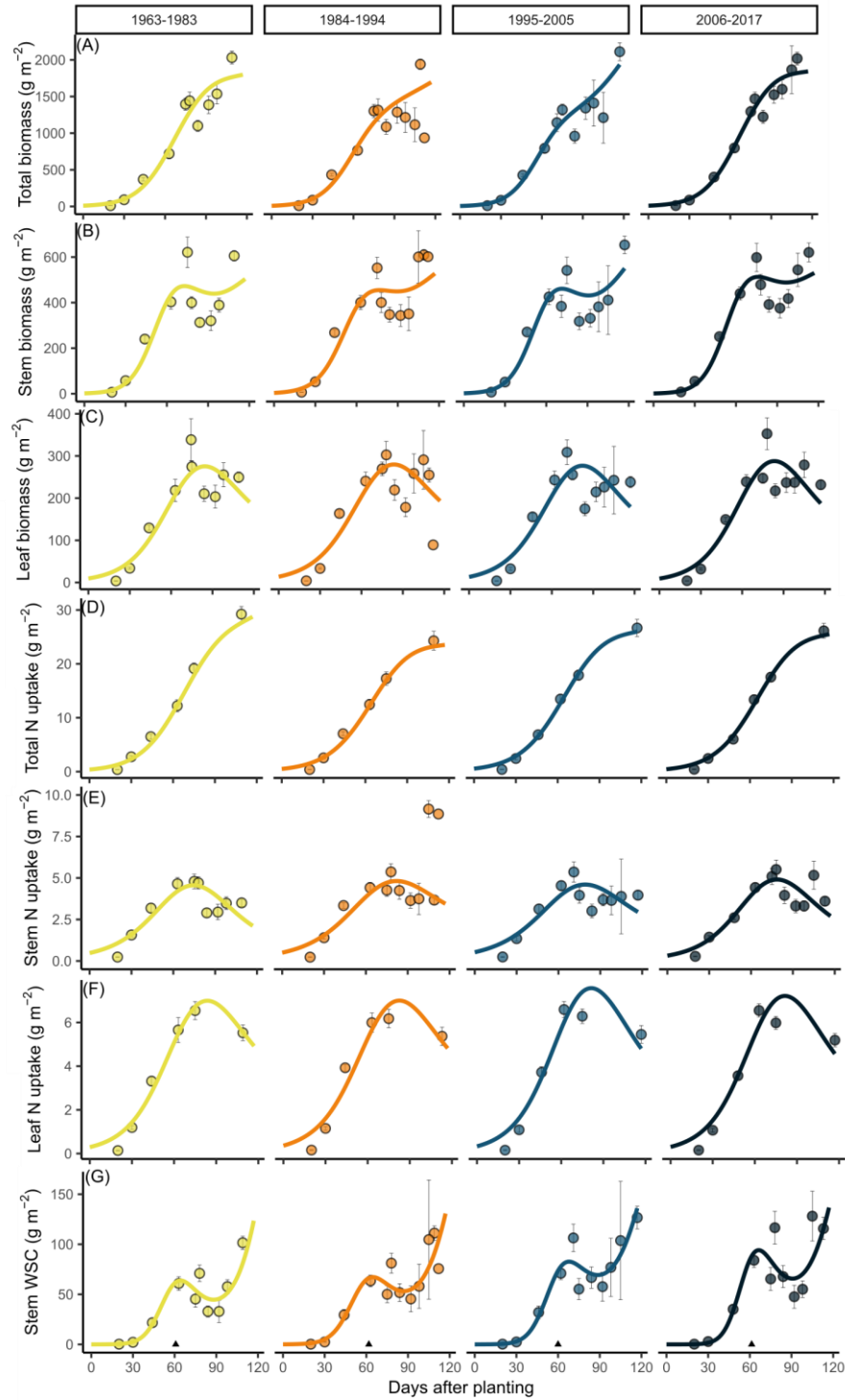


Figure 3.2. Total dry biomass (A), stem biomass (B), leaf biomass (C), total nitrogen uptake (D), stem nitrogen uptake (E), leaf nitrogen uptake (F), and stem water-soluble carbohydrates (G) all over the growing season. The symbols $\blacktriangle$ indicate the flowering time for each group of hybrids. Vertical bars denote the standard error of the mean. Colors represent the group of hybrids according to years of release $\bullet$ 1963-1983, $\bullet$ 1984-1994, $\bullet$ 1995-2005, and $\bullet$ 2006-2017.

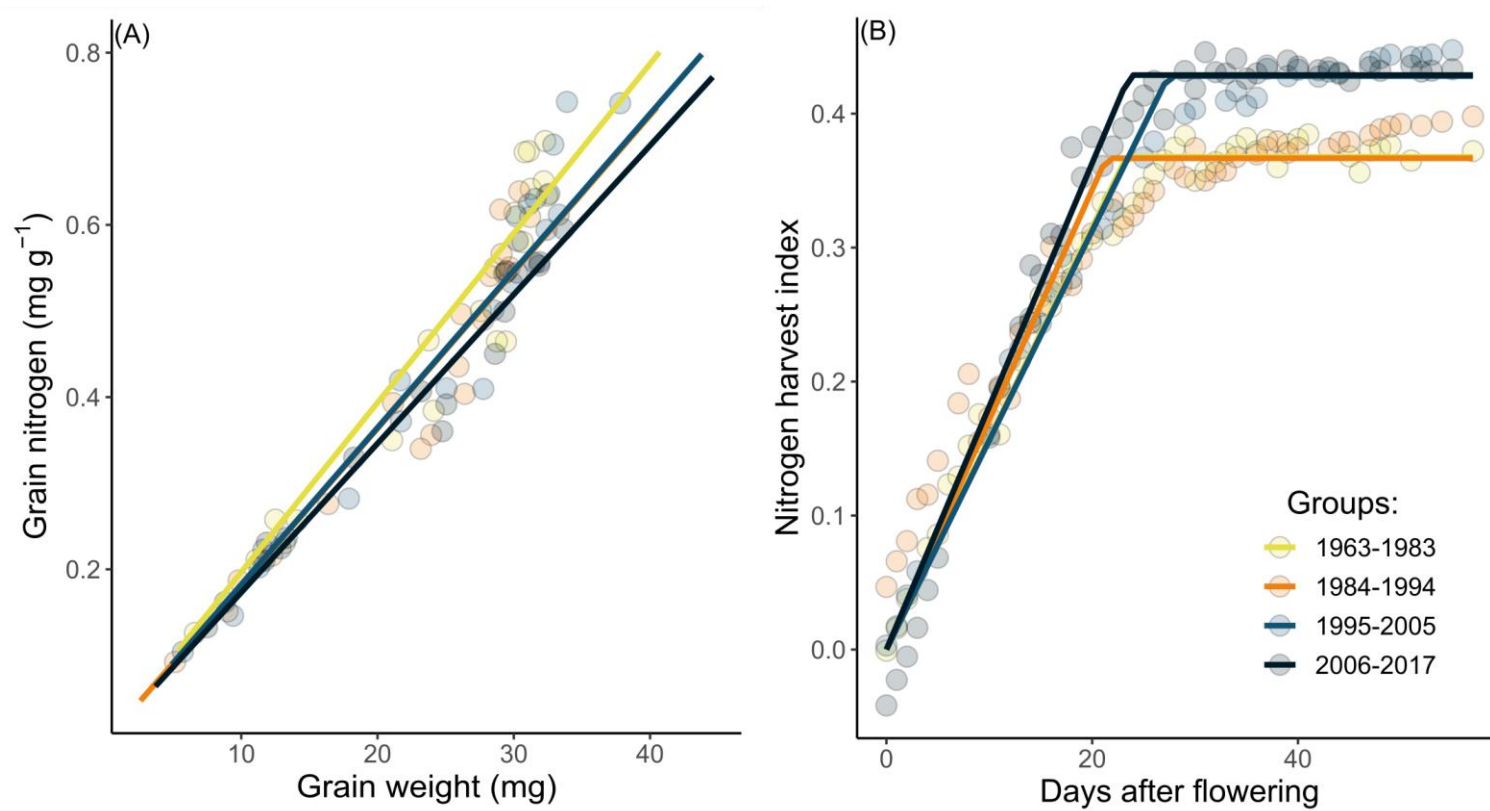


Figure 3.3. Relationship between grain nitrogen and grain weight (A), and nitrogen harvest index over the grain filling period (B). Colors represent the group of hybrids according to years of release ●1963-1983, ●1984-1994, ●1995-2005, and ●2006-2017.

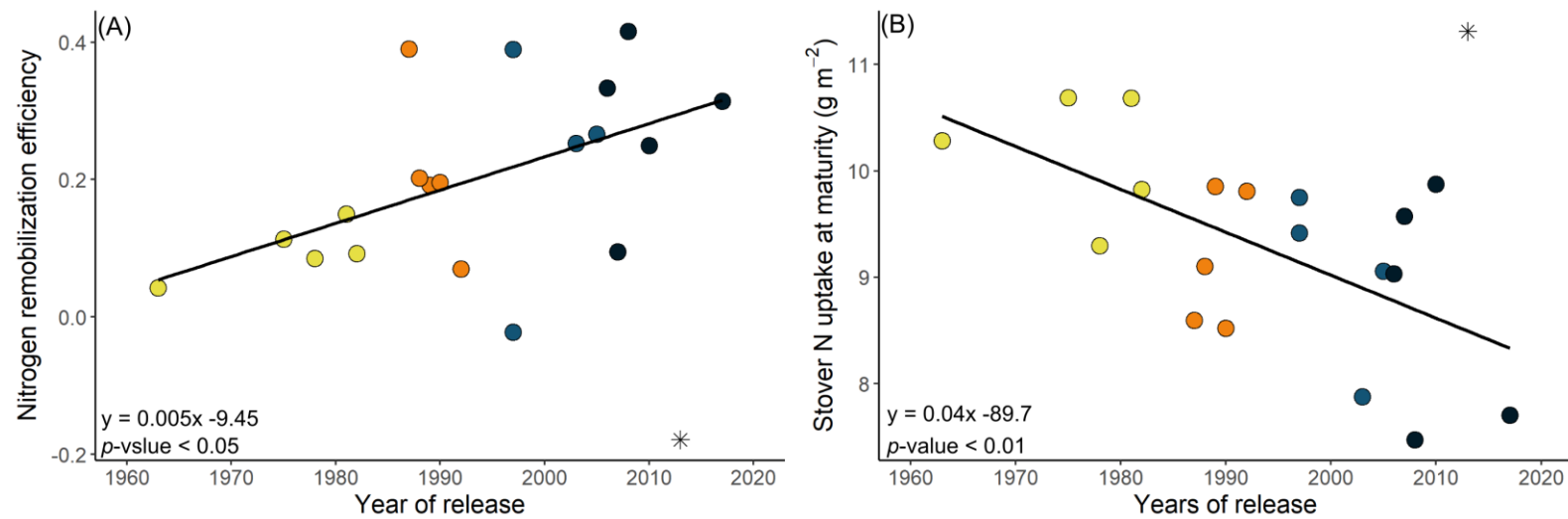


Figure 3.4. Nitrogen remobilization efficiency from flowering to maturity over years of release (A), and stover (sum of stem plus leaves N) nitrogen uptake at physiological maturity over years of the release of the hybrids (B). Colors represent the group of hybrids according to years of release ●1963-1983, ●1984-1994, ●1995-2005, and ●2006-2017.

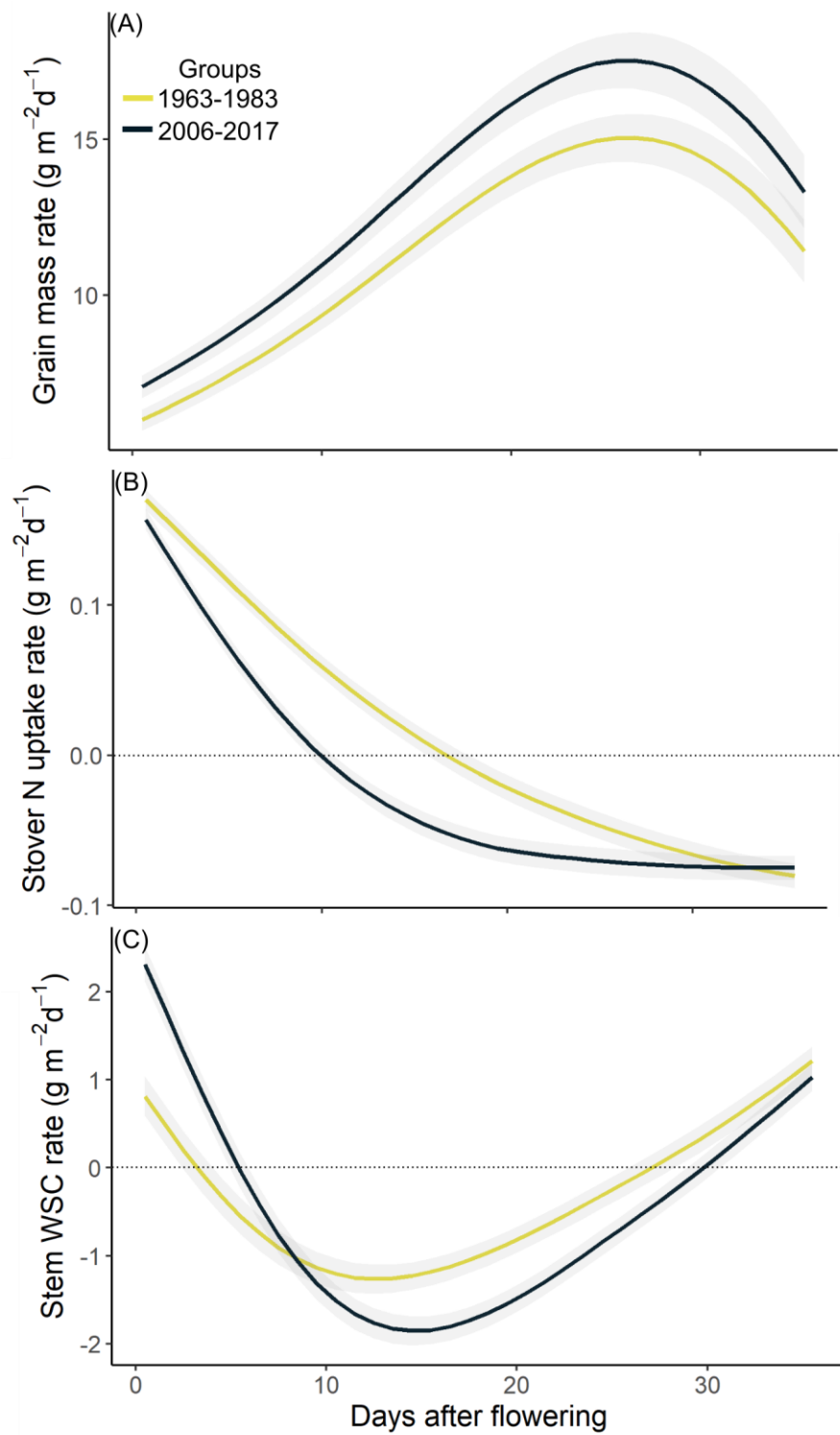


Figure 3.5. Rate of grain mass accumulation in $\text{g m}^{-2} \text{d}^{-1}$ (A), stover nitrogen uptake ($\text{g m}^{-2} \text{d}^{-1}$) (B), and stem WSC ($\text{g m}^{-2} \text{d}^{-1}$) (C), all during days after flowering. Colors represent the group of hybrids according to years of release.

Tables

Table 3.1. Group of sorghum hybrids according to the year of release, clustered in four different decades for sorghum yield improvement. Groups represent de denomination for each group.

Groups	Maturity	RM	Year of release
1963-1983	Mid-Late	71	1963
	Mid	69	1975
	Mid	68	1978
	Mid	68	1981
	Mid-Late	70	1982
1984-1994	Mid-Late	73	1987
	Mid	68	1988
	Mid-Late	70	1989
	Mid-Early	65	1990
	Mid	68	1992
1995-2005	Mid-Late	72	1997 A
	Mid-Early	66	1997 B
	Mid-Early	65	2003
	Mid	68	2005
2006-2017	Mid-Late	70	2006
	Mid-Early	65	2007
	Mid	69	2008
	Mid-Late	71	2010
	Mid-Early	64	2013
	Mid	68	2017

Table 3.2. Rate of Grain N concentration, rate, and duration of the nitrogen harvest index (NHI) during the grain filling period for all groups of sorghum hybrids.

Rate				Rate			Duration			
Group	Grain N	Grain N confidence		NHI	NHI Confidence		Days after flowering (DAF)	DAF Confidence		
	(mg g ⁻¹)	interval (95%)			interval (95%)			interval (95%)		
1963-1983	0.023	0.0217	0.0244	a	0.0158	0.015 - 0.016	a	23	22 - 24	a
1984-1994	0.0203	0.0190	0.0215	b	0.0172	0.016 - 0.018	b	21	20 - 23	b
1995-2005	0.0197	0.0184	0.0210	bc	0.0156	0.015 - 0.016	c	27	26 - 28	c
2006-2017	0.0177	0.0165	0.0189	c	0.0181	0.017 - 0.019	d	24	23 - 25	d

Within each group of hybrids, different letters indicate significant differences at p-value < 0.1.

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Chapter 4 - Final remarks

The present study provides valuable information about the physiological changes underpinning yield gain for US sorghum hybrids during the last six decades. The understanding of these physiological mechanisms for sorghum is helpful to continuing enhancing yields for future generations. Overall, yield gain across time was mainly related to increments in grain number per area rather than grain weight. Although grain filling duration and grain filling rate did not change, it supports grain weight stability over time. Additionally, greater grain number per area (grain set) in modern hybrids were associated with increments in panicle size as well as with harvest index. The WSC accumulation increased in stems during the vegetative period, and this mechanism seems to contribute to set a higher yield potential through higher grain number. The greater WSC content at flowering in new hybrids contributes to the realization of yield gain and higher harvest index via increased WSC remobilization from stems to grains.

Nitrogen (N) represents the most important nutrient influencing the growth and development of sorghum plants; therefore, it is strongly associated with crop yields (Ciampitti and Prasad, 2016; van Oosterom et al., 2010). Our results report for the first time improvements in N internal efficiency (NIE, yield to N uptake) in US sorghum hybrids during the last six decades of breeding efforts. The NIE improvement was related to yield gain and to a dilution in grain N concentration for modern hybrids. Furthermore, N remobilization plays a key role in the historical enhancement of NIE in sorghum, with this point reflected with a sustained reduction in stover N content at maturity to sustain the greater N demand in modern sorghum hybrids.

Future research should focus on understanding the mechanisms driving the enhancement on grain number for sorghum, this study can reveal key physiological triggers for yield improvement in sorghum. Additionally, the investigation of the main mechanisms for the

partitioning of carbohydrates via source-sink manipulations can reveal substantial evidence on the contribution of the plant reserves to the grain number enhancement while grain weight remains stable over time. Furthermore, studies should investigate the main factors driving the C and N demand and links to improve photosynthesis efficiency (and RUE) at the canopy level. To better understand the overall C economy in sorghum plants, studies targeting the dynamics of photosynthesis/carbon fixation/respiration can help to understand mechanism of C economy. Exploring these physiological aspects is important to guide selection strategies in current and future breeding programs.

Appendix A - Figures and Tables Chapter 2

Table A.1. Best linear unbiased estimators (BLUEs) for total dry biomass in g m^{-2} , stem water soluble carbohydrate (WSC) concentration (mg g^{-1}), and stem WSC content (g m^{-2}) relative to the years of release of the hybrids at flowering and physiological maturity.

Year of Release	Flowering			Physiological maturity		
	Total dry biomass (g m^{-2})	Stem WSC concentration (mg g^{-1})	Stem WSC content (g m^{-2})	Total dry biomass (g m^{-2})	WSC concentration (mg g^{-1})	WSC content (g m^{-2})
1963	766.2	97.3	41.3	1806.5	130.3	31.8
1975	722.9	124.5	47.6	1898.9	121.8	72.6
1978	557.5	174.1	76.8	1376.0	163.0	59.9
1981	686.4	154.1	61.9	1767.0	98.4	51.4
1982	716.1	137.2	35.6	1626.4	87.4	46.4
1983†	1001.2	146.6	72.3	1842.7	118.2	64.4
1987	1179.6	124.6	60.5	1629.1	181.4	73.5
1988	729.5	172.9	65.3	1388.6	131.1	52.4
1989	883.9	176.8	71.3	1455.0	103.9	59.0
1990	678.9	169.1	63.7	1501.8	152.0	61.6
1992	676.9	186.2	67.9	1509.8	143.2	73.8
1997 A	1078.2	140.7	74.1	1729.6	153.3	77.7
1997 B	625.5	180.2	59.3	1181.1	214.1	100.3
2003	624.7	191.5	66.4	1599.7	125.4	53.6
2005	878.7	229.3	114.0	1491.9	150.1	55.0
2006	805.6	191.9	78.4	1918.2	151.2	47.4
2007	649.4	217.0	67.5	1605.8	153.9	82.8
2008	702.2	196.7	125.1	1515.6	124.4	44.7
2010	1011.4	144.4	77.0	1649.2	157.6	67.4
2013	489.5	190.3	48.7	1401.9	122.4	56.3
2017‡	655.3	226.3	94.4	1804.2	155.7	83.2
Year of release	ns	***	**	ns	ns	ns
b (yr^{-1})	-	1.86	0.84	-	-	-

+Significant at $p < 0.1$, *Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.001$, ns: not significant $p > 0.1$.

†Hybrid included only in 2018 growing season.

‡Hybrid included only in 2019 growing season.

Appendix B - Figures and Tables Chapter 3

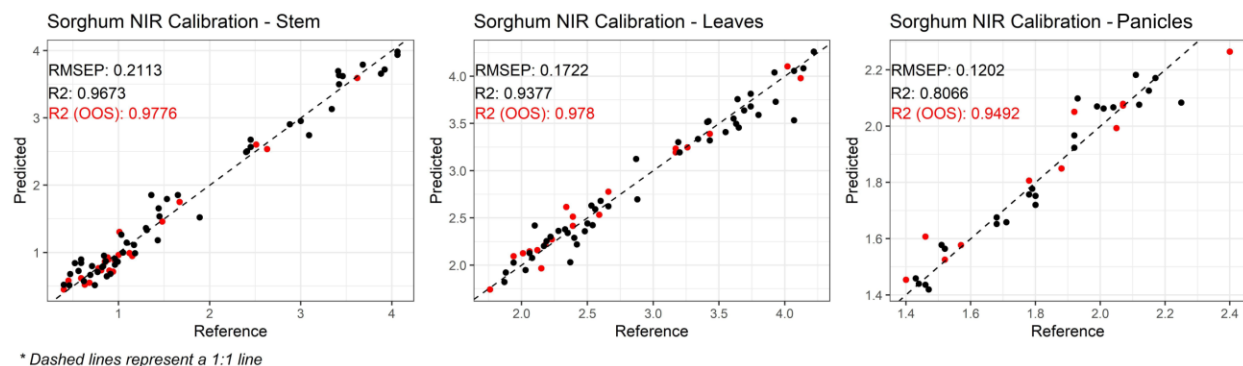


Figure B.1. Validation results for N determination using lab method versus NIR-derived N concentration in stems (A), leaves (B), and panicles ($\text{g m}^{-2} \text{d}^{-1}$) (C) for all samples collected during the entire growing season for sorghum crop.